USER GUIDE

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by life technologies™

GeneArt® High-Order Genetic Assembly System

For efficient, simultaneous, seamless assembly of up to 10 DNA fragments and a vector, totaling up to 110 kb in length

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Contents

Kit Contents and Storage

Types of Kits

This manual is supplied with the following products.

Product	Amount	Cat. no.
GeneArt® High-Order Genetic Assembly System	10 reactions	A13285
GeneArt® High-Order Genetic Assembly System *(with Yeast Growth Media)*	10 reactions	A13286

Kit Components

The GeneArt® High-Order Genetic Assembly System contains modules with the following components.

Component	Size	Quantity
–80°C Module		
MaV203 Competent Yeast Cells	100 µL/tube	11 tubes
pMAB37 DNA (250 ng/µL)	10 µL/tube	1 tube
PEG/Lithium Acetate (LiAc) solution	1.5 mL/tube	5 tubes
One Shot® TOP10 Electrocomp™ *E. coli*	50 µL/tube	11 tubes
pUC19 DNA (10 pg/µL)	50 µL/tube	1 tube
Control insert (50 ng/µL)	5 µL/tube	1 tube
GeneArt® pYES1L Vector with Sapphire™ Technology (50 ng/µL)	8 µL/tube	1 tube
Room Temperature Module		
Lysis buffer	1 mL/tube	1 tube
Glass beads	1 mg/tube	1 tube
S.O.C. media	6 mL/bottle	1 bottle

CSM Media for MaV203 Yeast Cells

CSM Media for MaV203 Yeast Cells, included in the GeneArt® High-Order Genetic Assembly System *(with Yeast Growth Media)* (Cat. no. A13286), is also available separately from Life Technologies (Cat. no. A13292). It includes the following prepackaged media for your convenience. **Keep the media dry and store at room temperature.**

Media	Size	Quantity
CSM-Trp Agar Yeast Media minus Glucose	32.4 g/pouch	2 pouches
20% glucose	100 mL/bottle	2 bottles

Continued on next page

Kit Contents and Storage, continued

Shipping and Storage

Components of the GeneArt® High-Order Genetic Assembly System are shipped as described below. Upon receipt, store each component as detailed.

Item	Shipping	Storage
MaV203 Competent Yeast Cells	Dry ice	–80°C
pMAB37 DNA	Dry ice	–80°C
PEG/Lithium Acetate (LiAc) solution	Dry ice	–80°C
One Shot® TOP10 Electrocomp™ *E. coli*	Dry ice	–80°C
pUC19 DNA	Dry ice	–80°C
Control insert	Dry ice	–80°C
GeneArt® pYES1L Vector with Sapphire™ Technology	Dry ice	–80°C
Lysis buffer	Room temperature	Room temperature
Glass beads	Room temperature	Room temperature
S.O.C. media	Room temperature	Room temperature
CSM-Trp Agar Yeast Media minus Glucose	Room temperature	Room temperature
20% glucose	Room temperature	Room temperature

MaV203 Competent Yeast Cells

The MaV203 Competent Yeast Cells have been developed for the routine introduction of plasmid DNA into yeast cells. The high transformation efficiency of these cells (≥ 1×10^6 transformants per µg DNA) makes them ideally suited for use with the GeneArt® High-Order Genetic Assembly System, which relies on transformation-associated recombination.

Genotype of MaV203 Cells

MAT α; leu2-3,112; trp1-901; his3Δ200; ade2-101; cyh2^R; can1^R; gal4Δ; gal80Δ; GAL1::lacZ; HIS3$_{UASGAL1}$::HIS3@LYS2; SPAL10 $_{UASGAL1}$::URA3.

One Shot® TOP10 Chemically Competent *E. coli*

One Shot® TOP10 Chemically Competent *E. coli,* included in the GeneArt® High-Order Genetic Assembly System, have a transformation efficiency of ≥ 1×10^8 transformants per µg of plasmid.

Genotype of TOP10 Cells

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$^-$

Description of the System

GeneArt® High-Order Genetic Assembly System

GeneArt® High-Order Genetic Assembly Technology

The GeneArt® High-Order Genetic Assembly System is a highly efficient system for the simultaneous and seamless assembly of up to 10 DNA fragments and a vector, totaling up to 110 kb in length (including the vector). The system relies on yeast's ability to take up and recombine DNA fragments with high-efficiency. This process, termed transformation-associated recombination (Larionov et al., 1996), greatly reduces *in vitro* handling of DNA and eliminates the need for enzymatic treatments of DNA such as restriction and ligation, while allowing precise fusions of DNA sequences.

Components of the GeneArt® High-Order Genetic Assembly System

The GeneArt® High-Order Genetic Assembly System is offered in two configurations, the core system for 10 high-order DNA assemblies (Cat. no. A13285) and the expanded system that include yeast growth media for your convenience (Cat. no A13286). The system contains the following components:

- MaV203 Competent Yeast Cells for use in the transformation-associated recombinational assembly of up to 10 DNA fragments and a vector. The high transformation efficiency of these cells (≥ 1×10^6 transformants per µg DNA) makes them ideally suited for co-transformation with your DNA fragments.
- pMAB37 DNA as a control vector for assessing the transformation efficiency of MaV203 Competent Yeast Cells.
- GeneArt® pYES1L Vector with Sapphire™ Technology, a 9.4 kb linearized YAC-BAC shuttle plasmid, as a control vector for the assembly of DNA fragments in MaV203 cells and for the transfer of the assembled recombinant DNA molecule into *E. coli*. You can also use the pYES1L vector as a cloning vector, if desired.
- Control insert, a 702 bp DNA fragment sharing a 30-bp homology with the linear pYES1L at both ends, as a control for the high-order DNA assembly in MaV203 cells.
- PEG/Lithium Acetate (LiAc) solution for transforming the linear pYES1L vector and the DNA fragments of interest into MaV203 Competent Yeast Cells.
- Glass beads and lysis buffer to extract the assembled recombinant DNA molecule from positive yeast colonies.
- One Shot® TOP10 Electrocomp™ *E. coli* and S.O.C. medium for large-scale plasmid preparations of the assembled DNA construct for downstream applications.
- pUC19 control DNA for assessing the transformation efficiency of the One Shot® TOP10 Electrocomp™ *E. coli.*
- Yeast growth medium and 20% glucose (with Cat. no. A13286 only) for the growth and maintenance of transformed MaV203 cells.

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How GeneArt® High-Order Genetic Assembly System Works

In the yeast *Saccharomyces cerevisae*, homologous recombination is the preferred pathway for DNA double-strand break repair (Orr-Weaver et al., 1981). Moreover, this pathway mediates highly efficient intermolecular recombination between homologous DNA sequences when they are co-transformed into yeast cells (Ma et al., 1987; Gibson et al., 2008; Raymond et al., 2002).

The GeneArt® High-Order Genetic Assembly System takes advantage of transformation-associated recombination in yeast to join pre-existing DNA fragments or chemically synthesized oligonucleotides into a single recombinant molecule (Larionov et al., 1994; Larionov et al., 1996). Because the system relies on homologous recombination, the adjacent DNA fragments must share end-terminal homology (Ma et al., 1987; Gibson, 2009) or they must be "stitched" together by means of recombination linkers (Raymond et al., 1999; Raymond et al., 2002). Recombination linkers are synthetic oligonucleotides that provide sequence overlaps with adjacent sequences that are to be joined, thus promoting recombinational joining of unrelated DNA fragments (DeMarini et al., 2001; Raymond et al., 2002).

To seamlessly assemble up to 10 DNA fragments into a single recombinant DNA molecule using the GeneArt® High-Order Genetic Assembly System:

1. Combine the linear cloning vector (pYES1L Vector with Sapphire™ Technology or your own yeast-adapted vector) and the DNA fragments to assemble in a centrifuge tube. If you are assembling non-homologous DNA fragments, also include the stitching oligonucleotides in the same tube.

   Note: We recommend that you use our web-based GeneArt® Primer and Construct Design Tool, available at **http://bioinfo.invitrogen.com/oligoDesigner**, to confirm end-terminal homology between your adjacent DNA fragments, check for potential homologous regions in the fragments that could lead to undesired recombination, and/or to design PCR primers and stitching oligonucleotides.
2. Co-transform the linear cloning vector and the DNA fragments into MaV203 Competent Yeast Cells supplied with the system. Plate the transformed yeast cells onto CSM-Trp agar plates and incubate for three days.
3. Perform yeast colony PCR to screen positive yeast colonies containing your assembled recombinant DNA molecule. Isolate one or more of the positive colonies to transfer the assembled DNA molecule into *E. coli*.
4. Lyse the positive yeast colony and use the lysate to transform the assembled DNA molecule into One Shot® TOP10 Electrocomp™ *E. coli* for large scale plasmid prep for downstream applications.

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GeneArt® High-Order Genetic Assembly System, continued

Oligonucleotide Stitching

In the oligonucleotide stitching feature of the GeneArt® High-Order Genetic Assembly System, short synthetic linkers (i.e., recombination linkers) bridge the ends of adjacent DNA fragments. This feature enables the precise assembly of unrelated DNA fragments that do not share end-terminal homology. In addition, oligonucleotide stitching allows editing of the fragment junctions to generate deletions (i.e., deletion editing), insertions (i.e., insertion editing), and other desired imperfections. This feature also allows the reuse of existing DNA fragments or elements obtained by restriction enzyme digestion, without the need for reamplifying them.

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 31.

CSM Media for MaV203 Yeast Cells

CSM Media for MaV203 Yeast Cells, optimized to provide the maximum cloning efficiency, is used to prepare CSM-Trp agar plates. Because MaV203 yeast cells are auxotrophic for tryptophan (*trp1-901*), only cells that have been successfully transformed with pYES1L (or your own yeast-compatible cloning vector prepared using the GeneArt® Vector Conversion Cassette with Sapphire™ Technology) grow on CSM-Trp agar plates.

For your convenience, the CSM Media for MaV203 Yeast Cells is included prepackaged in the GeneArt® High-Order Genetic Assembly System (*with Yeast Growth Media*). These cells are also available separately from Life Technologies (Cat. no. A13292).

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GeneArt® High-Order Genetic Assembly System, continued

Advantages of the GeneArt® High-Order Genetic Assembly System

- **Speed** – Facilitates the assembly of up to 10 DNA fragments and the vector, totaling up to 110 kb in length, simultaneously.
- **Simplicity** – Greatly reduces *in vitro* handling of DNA and eliminates the need for enzymatic treatments of DNA such as restriction, ligation, and even PCR (if you use the oligonucleotide stitching feature of the system).
- **Precision** – Enables seamless assembly of DNA fragments in a precise and pre-determined order without extra sequences.
- **Flexibility** – Allows the assembly of multiple DNA fragments from any source using any yeast-adapted multifunctional cloning vector for protein expression in mammalian cells, other yeast species, *E. coli*, and other expression systems. The oligonucleotides stitching feature of the system allows end-editing and reuse of pre-existing DNA fragments with the need for re-amplification.
- **Efficiency** – Generates up to 90% positive clones depending on the number and length of DNA fragments assembled.

Possible Applications

The recombinant DNA molecule assembled using the GeneArt® High-Order Genetic Assembly System can be used in a variety of downstream applications. These may include:

- Rapid construction of large, genome-size DNA molecules from synthetic or naturally occurring DNA fragments
- Creation of genetic pathways, including combinatorial libraries where each member of the library has a different combination of gene variants
- Construction of synthetic genetic pathways that include genes, transcriptional promoters and terminators, and signals for protein synthesis and processing
- Assembly of hard-to-clone DNA fragments
- Manipulation of medically or industrially important microbial genomes from hard-to-culture organisms
- Construction of modular expression vectors with interchangeable parts
- Creation of knock-out constructs

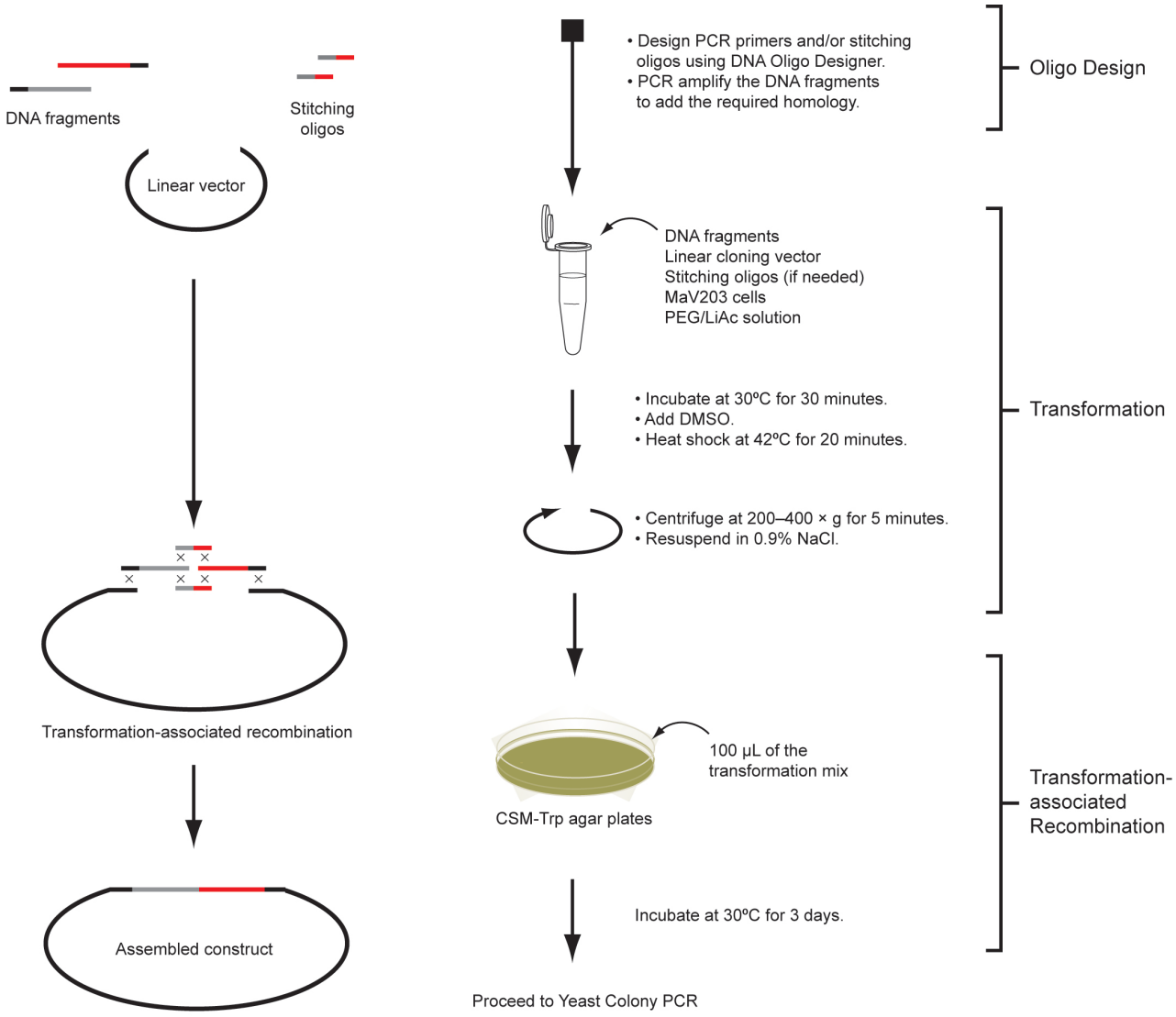
Some of the many applications that will benefit from the GeneArt® High-Order Genetic Assembly System are the following:

- Synthetic biology-related applications
- Drug discovery
- Biofuels
- Bioremediation
- Plant Biotechnology
- Synthetic vaccines
- Bioproduction of chemicals and drugs
- Metabolic Engineering

GeneArt® High-Order Genetic Assembly Workflow

Workflow for High-Order Genetic Assembly

The figure below summarizes the workflow for the high-order genetic assembly using the GeneArt® High-Order Genetic Assembly System. In this example, the system is used to assemble two DNA fragments plus the vector. The fragments share end-terminal homology with the linear cloning vector, but not with each other; therefore, the fragments are joined together using a pair of stitching oligonucleotides (i.e., sense and anti-sense oligonucleotides).



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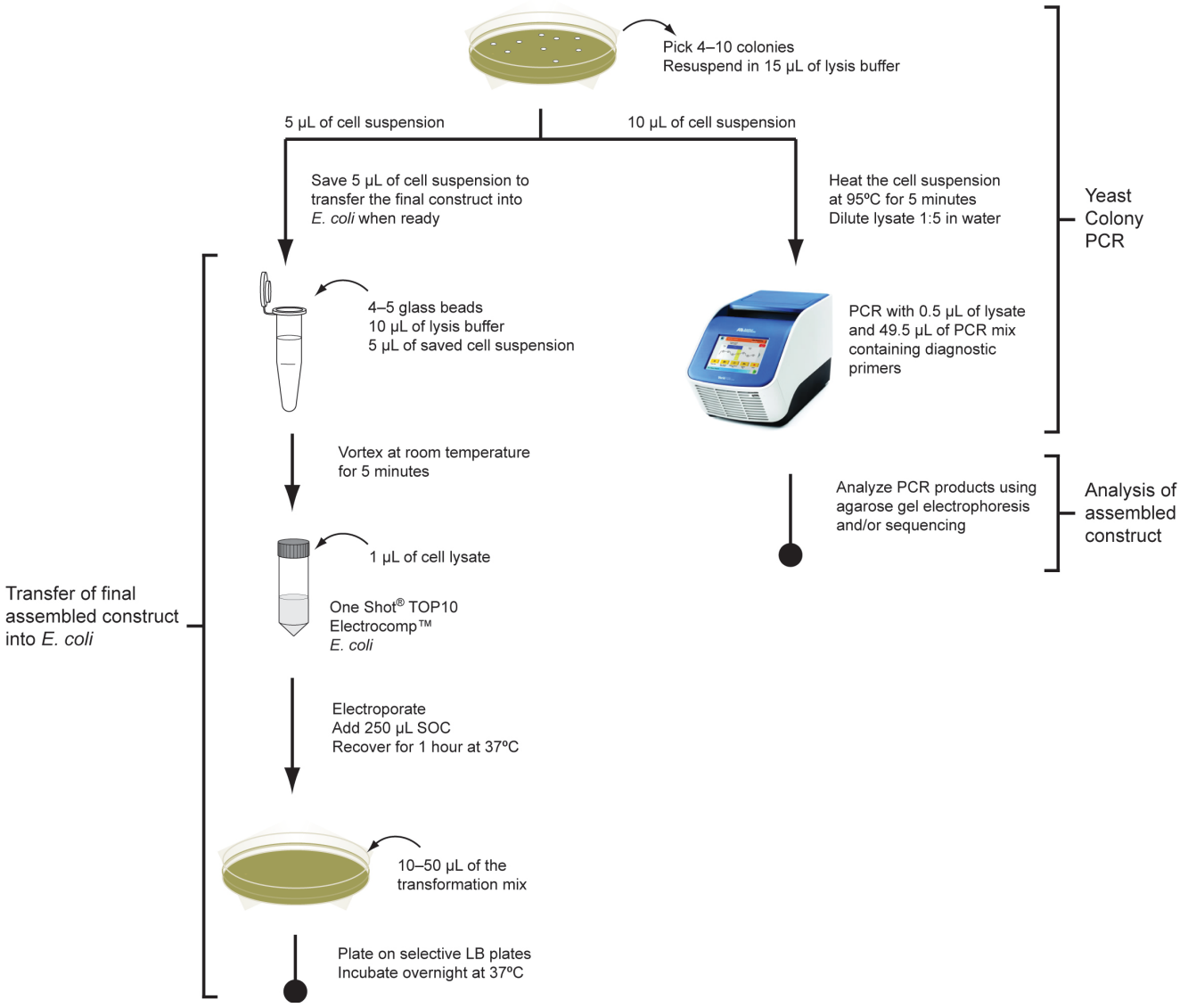
GeneArt® High-Order Genetic Assembly Workflow, continued

Workflow for Analysis of Assembled Construct

The figure below summarizes the workflow for the analysis of the final construct after it was assembled using the GeneArt® High-Order Genetic Assembly System. After you verify your "positive" colonies containing the final assembled construct, transfer the construct into *E. coli* for large scale plasmid preps for downstream applications.



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GeneArt® High-Order Genetic Assembly Workflow, continued

Experimental Outline

The table below describes the major steps required to assemble your recombinant DNA molecule using the GeneArt® High-Order Genetic Assembly System. 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, design your PCR primers and stitching oligonucleotides, and perform *in silico* cloning to verify your strategy	31–32
2	Synthesize your DNA fragments and/or stitching oligonucleotides (if needed)	11–20
3	Co-transform GeneArt® pYES1L Vector with Sapphire™ Technology (or your own linear yeast-compatible cloning vector), your DNA fragments, and stitching oligonucleotides (if needed) into MaV203 competent yeast cells and incubate	21–23
4	Perform yeast colony PCR to screen for positive colonies and to verify the correct assembly of your DNA fragments	26–27
5	Transform One Shot® TOP10 Electrocomp™ *E. coli* using the lysate from the "positive" yeast colonies	28

Methods

Requirements for DNA Oligonucleotides and Fragments

Introduction

The GeneArt® High-Order Genetic Assembly technology relies on transformation-associated recombination in yeast (TAR). Therefore, the DNA fragments used for assembly must share end-terminal homology (Ma et al., 1987; Gibson, 2009) or, if they do not share any homology, they must be "stitched" together using recombination linkers (Raymond et al., 1999; DeMarini et al., 2001; Raymond et al., 2002).

The extent of end-terminal homology shared by adjacent DNA fragments and the design of the DNA oligonucleotides used for "stitching" are critical for their successful assembly in yeast. You can achieve successful assembly by one of three methods:

- Using pre-existing fragments that already contain the required homologous sequences for recombination
- Generating the fragments to assemble by PCR amplification using PCR primers with 5′ overhangs that provide the required homology with the adjacent fragments
- Using stitching oligonucleotides to link DNA fragments that do **not** share the required end-terminal homology

We highly recommend that you use our web-based GeneArt® Primer and Construct Design Tool, available at **http://bioinfo.invitrogen.com/oligoDesigner**, to design your DNA oligonucleotides and to verify your assembly strategy. The GeneArt® Primer and Construct Design Tool minimizes the planning time required for designing your DNA fragments, PCR primers, and stitching oligonucleotides (i.e., recombination linkers), identifies potential pitfalls linked to your specific sequences, and performs *in silico* cloning using your sequences. For more information on GeneArt® Primer and Construct Design Tool, see pages 31–32.

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Requirements for DNA Oligonucleotides and Fragments, continued

Homology Requirements

- DNA fragments used for constructs up to 60 kb must have a total overlap (i.e., end-terminal homology) of 30 nucleotides with adjacent fragments.
- DNA fragments used for constructs larger than 60 kb must have total overlap of 50 nucleotides with adjacent fragments.

  Note: The 30-bp or 50-bp overlap for constructs ≤ 60 kb and > 60 kb, respectively (see points above), is required between pre-existing DNA fragments, and **not** between a stitching oligonucleotide and a fragment.
- You can generate end-terminal homology by PCR amplifying the DNA fragments to assemble using PCR primer pairs with 5′ overhangs that provide the required homology. For more information, see **Preparing DNA Fragments by PCR**, pages 13–15.
- If the DNA fragments used for assembly do **not** share any end-terminal homology, they must be stitched together using recombination linkers (i.e., stitching oligonucleotides). For more information, see **Using Stitching Oligonucleotides for Assembly**, page 16.

Purity and Concentration of Oligonucleotide Stocks

- For general assembly applications, you can use standard purity oligonucleotides (i.e., desalted or cartridge-purified). However, higher purity oligonucleotides (i.e., PAGE- or HPLC-purified) may result in slightly increased cloning efficiencies.

  Note: For custom DNA oligonucleotide synthesis options available from Life Technologies, visit **www.lifetechnologies.com/oligos** or contact Technical Support (see page 41).
- Prepare oligonucleotide stocks and DNA fragments at a final concentration of 100 µM in 1X TE buffer, pH 8 (10 mM Tris-HCl, 1 mM EDTA, pH 8). Before use, dilute the stock solution 1:10 to 1:50 in DNase- and RNase-free water for easier pipetting.
- Store oligonucleotides and DNA fragments at –20°C.

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Preparing DNA Fragments by PCR

Before You Begin

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. For more information on GeneArt® Primer and Construct Design Tool, see pages 31–32.

Guidelines for PCR Primers

The GeneArt® High-Order Genetic Assembly reaction requires that each DNA fragment share a 30 to 50 bp end-terminal homology with the adjacent fragment (including the cloning vector). Therefore, PCR primers used for generating your fragments must have 5′ overhangs to provide this homology with the adjacent fragments.

Follow the guidelines below when designing your PCR primers and refer to the PCR primer examples on pages 14–15.

- Design your PCR primers such that each DNA fragment to be assembled is between 100 bp and 5 kb in length.

  Note: Large fragments (>5 kb) are more susceptible to damage in a gel extraction procedure. Therefore, when using DNA fragments generated by PCR, 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 the sequence that is homologous to the 30–50 bases at one end of the adjacent DNA fragment (i.e., the vector or another insert).

  Note: The 30-bp or 50-bp overlap is for constructs of ≤ 60 kb and > 60 kb.

- If you are attaching the insert to the linearized cloning vector, all nucleotides providing the requisite homology must be on the 5' end of the primer. If you are connecting two adjacent inserts, you may split the 30- to 50-bp homology between the fragments (e.g., 15 bp on the reverse primer of fragment 1 and 15 bp on the forward primer of fragment 2 for a 30-bp homology, or 25 bp on the reverse primer of fragment 1 and 25 bp on the forward primer of fragment 2 for a 50-bp homology).

  Note: You can split the homology between adjacent fragments in any combination (e.g., 15+15 as in the example above or 20+10, 25+5 etc. for a 30-bp homology).

- The 3′ end of each primer must be specific to your DNA element.
- PCR primers should be up to 75 nucleotides in length (30–50 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 end-terminal homology between adjacent fragments, one PCR primer may have as few as 18 nucleotides and no tail whatsoever. For example, in a 0+30 homology split, the reverse primer of fragment 1 would not contribute to the homology but would have 18–25 gene-specific nucleotides, while the forward primer of fragment 2 would contribute the entire 30 bp for the homology and have 18–25 gene-specific nucleotides (for a total length of 48–55 nucleotides).

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Preparing DNA Fragments by PCR, continued

Examples for PCR Primers

The 30- to 50-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 30- to 50-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 figures below shows examples of PCR primers used for preparing the DNA fragments for assembly in linear vectors with 5′ overhangs, blunt ends, and 3′ overhangs. The primer sequences providing the 30-bp homology required for constructs of < 60 kb are shown in bold; the remaining sequences of each primer (i.e., the 3′ end) are specific to the DNA element you want to assemble.

Vector with 5′ Overhangs

Vector with Blunt Ends

Vector with 3′ Overhangs

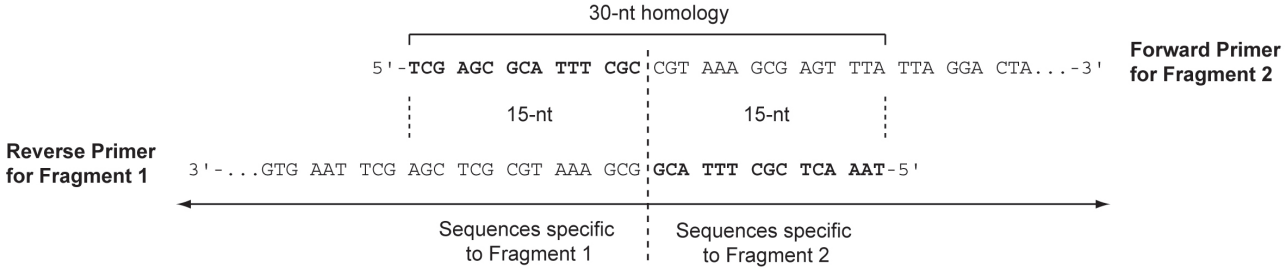
Continued on next page

Preparing DNA Fragments by PCR, continued

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 30- to 50-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 each provide 15-bp of the 30-bp homology required for recombination when assembling a construct of <60 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 you want to assemble.



Guidelines for PCR

- Prepare oligonucleotide stocks for PCR at a final concentration of 100 µM in DNase- and RNase-free water.
- We recommend using Platinum® PCR SuperMix High Fidelity (Cat. no. 12532-016) for amplifying DNA fragments up to 5 kb for general assembly applications. If you require higher PCR fidelity, make sure to use a processive and proof-reading polymerase.
- 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, available separately from Life Technologies (Cat. no. K3100-01).
- After generating your DNA fragments by PCR, verify the PCR products by gel electrophoresis. If you obtain multiple bands, you must gel purify your DNA fragments. In this case, 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 solubilization might be required to obtain the best results.

Using Stitching Oligonucleotides for Assembly

Before You Begin

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 stitching oligonucleotides to assemble DNA fragments that do **not** share the required homology for transformation-associated. For more information on GeneArt® Primer and Construct Design Tool, see pages 31–32.

Guidelines for Oligonucleotide Stitching

- If the DNA fragments used for assembly do **not** share any end-terminal homology, they must be stitched together using recombination linkers (i.e., stitching oligonucleotides).
- Each junction between adjacent fragments requires two oligonucleotides for oligonucleotide stitching, a sense and an anti-sense oligonucleotide. The sense and anti-sense oligonucleotides do not have to be annealed prior to transformation in yeast.
- We recommend that you do **not** assemble more than 5 fragments using 3 pairs of sense and anti-sense stitching oligonucleotides (a total of 6 single-stranded oligonucleotides).
- You may assemble up to 5 fragments plus a vector using stitching oligonucleotides, provided that at most three junctions are formed by the stitching oligonucleotides and the remaining junctions are produced by shared end-terminal homology (see the previous guideline).
- Oligonucleotides used for oligonucleotide stitching of up to 3 non-homologous fragments of < 5 kb must be 80-mers (i.e., they must have a 40-bp overlap with each adjacent fragment).
- Prepare stitching oligonucleotide stocks at a final concentration of 100 µM in 1X TE buffer, pH 8 (10 mM Tris-HCl, 1 mM EDTA, pH 8).

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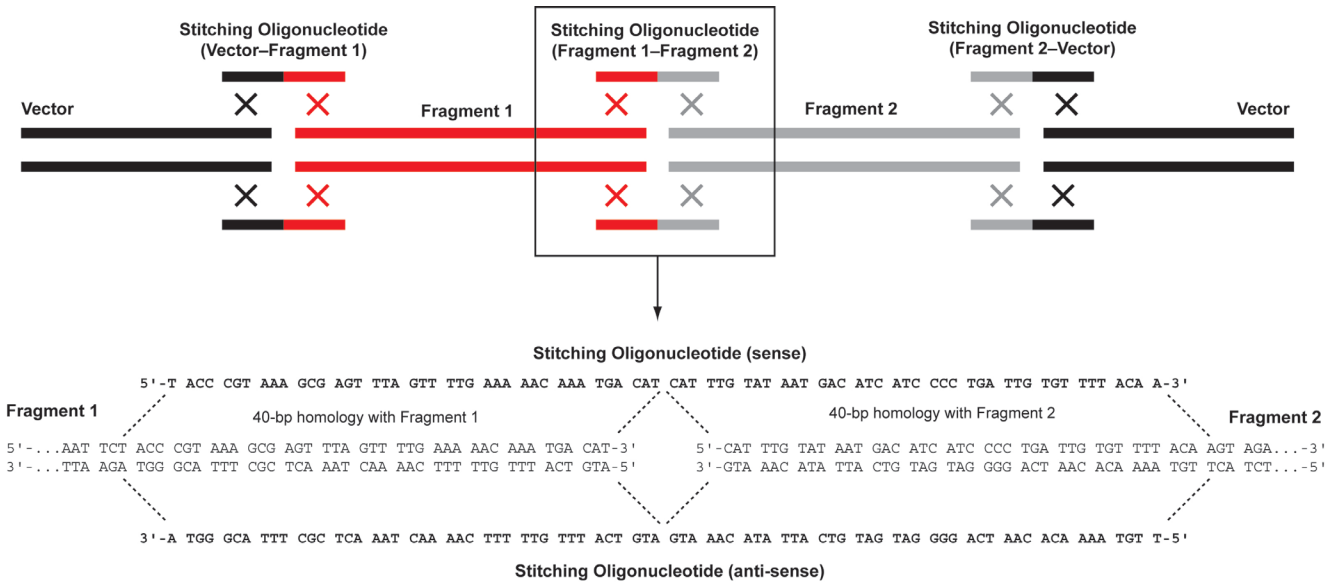
Using Stitching Oligonucleotides for Assembly, continued

Example for Stitching Oligonucleotide

The example below shows the assembly of two DNA fragments that do not share any homology into a vector using stitching oligonucleotides. The 80-bp double-stranded stitching oligonucleotide, shown in bold, shares 40-bp homology with each adjacent fragment (Fragments 1 and 2).



Using Stitching Oligonucleotides for Fragment Editing

Fragment Editing

You can use the oligonucleotide stitching procedure to perform fragment editing to introduce deletions, insertions, or other desired modifications to the final assembled recombinant DNA molecule. Fragment editing by means of oligonucleotide stitching is particularly useful when the removal of end sequences such as restriction sites or primer tails is required. Fragment editing also permits the opposite type of alteration, such as the insertion of restrictions sites, small tag coding regions, small desired watermarks, or modified nucleotides.

The GeneArt® Primer and Construct Design Tool does **not** support fragment editing. However, for deletion editing, you may enter the fragment sequence into the GeneArt® Primer and Construct Design Tool with the end deletions already applied. The tool then designs the correct oligonucleotides and provides the final assembled DNA sequence with the desired deletions.

For insertion editing, manually add the inserted nucleotides into the final oligonucleotides designed by the tool, then manually edit the final assembled DNA construct. For more information on GeneArt® Primer and Construct Design Tool, see pages 31–32.

Guidelines for Fragment Editing

- To perform fragment editing, follow the assembly procedure for non-homologous DNA fragments using modified stitching oligonucleotides.
- We recommend simultaneously assembling not more than 2 fragments using 3 stitching oligonucleotides.
- Stitching oligonucleotides used for **insertion editing** must have a 30-nucleotide overlap with each adjacent fragment in addition to the insertion bases (for a total length of up to 80-mer, including up to 20 insertion bases). See page 19 for an example of using stitching oligonucleotides for insertion editing.

  Note: This applies for a 2-fragment assembly and the insertion applies only to the internal junction. Use a 40-bp overlap (i.e., an 80-mer oligonucleotide) for the remaining seamless junctions.
- Stitching oligonucleotides used for **deletion editing** must have a 40-nucleotide overlap with each adjacent fragment, annealing up to 6 nucleotides from the junction into each fragment, thus leaving up to 6 bp at the end of each fragment to be deleted during transformation-associated recombination. See page 20 for an example of using stitching oligonucleotides for deletion editing.

  Note: This applies for a 2-fragment assembly and the deletion applies only to the internal junction. Use a 40-bp overlap (i.e., an 80-mer oligonucleotide) for the remaining seamless junctions.

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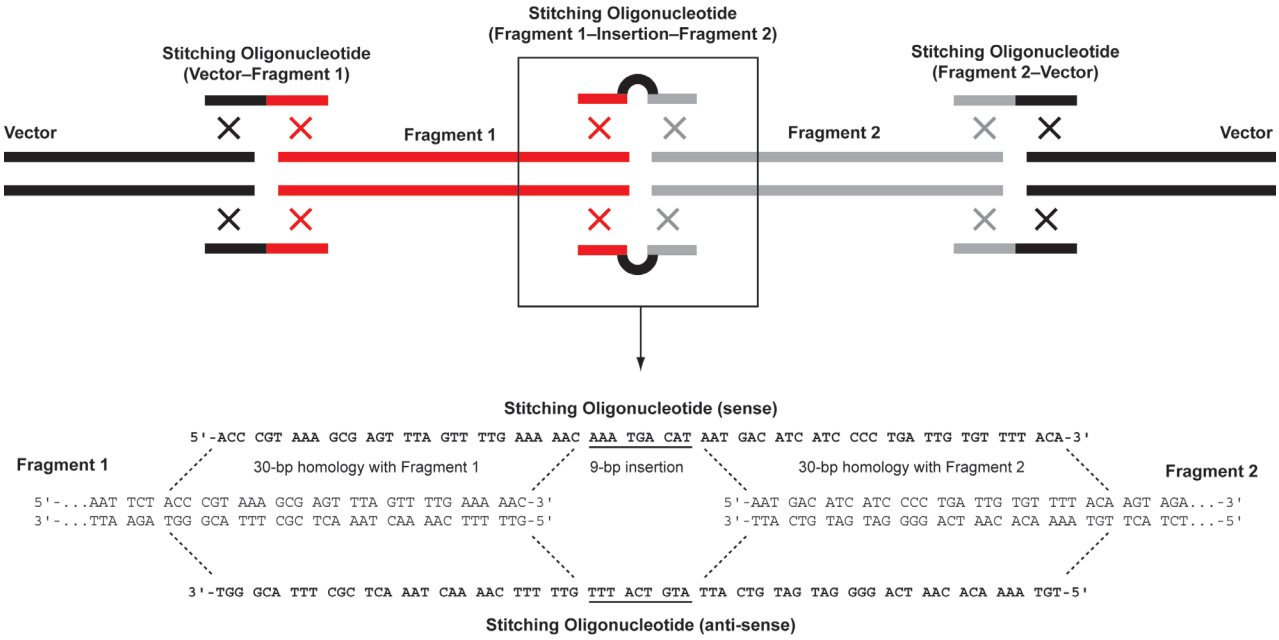
Using Stitching Oligonucleotides for Fragment Editing, continued

Example for Insertion Editing

The example below shows the assembly of two DNA fragments that do not share any homology into a vector using stitching oligonucleotides. The 69-bp double stranded stitching oligonucleotide, shown in bold, shares 30-bp homology with each adjacent fragment (Fragments 1 and 2); this oligonucleotide is used to insert 9 bp at the junction of the adjacent fragments. The insertion bases are shown underlined.

Note: You can insert up to 20 bp at the junction of the adjacent fragments using an insertion stitching oligonucleotides of up to 80-bp in length (30-bp overlap with each adjacent fragment plus up to 20 bp for insertion).



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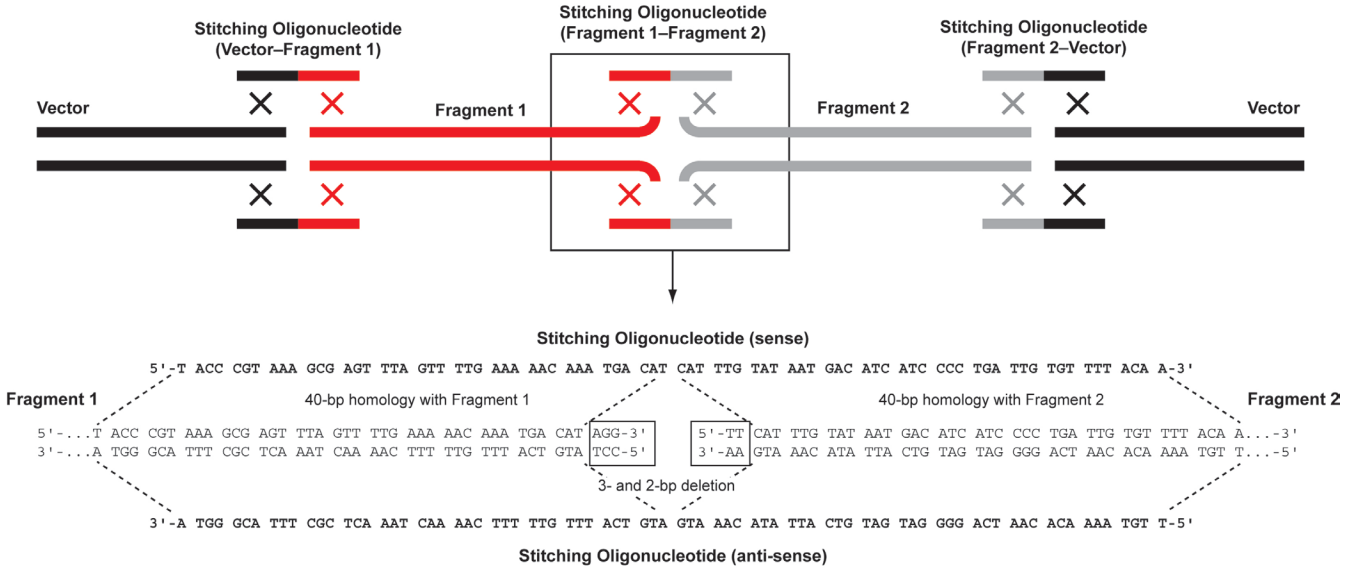
Using Stitching Oligonucleotides for Fragment Editing, continued

Example for Deletion Editing

The example below shows the assembly of two DNA fragments that do not share any homology into a vector using stitching oligonucleotides. The 80-bp double stranded stitching oligonucleotide, shown in bold, shares a 40-bp homology with each adjacent fragment (Fragments 1 and 2). However, this homology does not include the terminal 3 bp of Fragment 1 and the terminal 2 bp of Fragment 2 at the junction (shown in boxes), both of which will be deleted during transformation-associated recombination reaction.

Note: You can delete up to 6 bp from each adjacent fragment at their junction. The example below shows deletion of 3 bp from Fragment 1 and 2 bp from Fragment 2.



Performing High-Order DNA Assembly

Introduction

You can use the GeneArt® High-Order Genetic Assembly System to seamlessly assemble up to 10 DNA fragments (pre-existing or synthetic) and a vector, simultaneously. If your DNA fragments share the requisite end-terminal homology, simply co-transform them with your linearized, yeast-adapted cloning vector into MaV203 competent yeast cells. If the DNA fragments do not share any homology, include the appropriate stitching oligonucleotides in your transformation mix.

Note: *S. cerevisiae* has been shown to assemble more than 10 DNA fragments and molecules of up to 500 Kb (Gibson et al., 2008a; Gibson et al., 2008b; Gibson et al., 2010); however, the GeneArt® High-Order Genetic Assembly System has been optimized for best results in the lower range of the spectrum.

Before You Begin

Before you set up your high-order DNA 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 pages 31–32.).
- Prepared your DNA fragments and/or DNA oligonucleotide stocks according the guidelines on pages 13–15.
- Prepared CSM-Trp agar plates for plating transformed MaV203 yeast cells (see **Recipes**, page 36).
- *Optional*: Generated your yeast-adapted cloning vector (if you want to use your own vector; see **Generating Yeast-Adapted Cloning Vectors**, pages 33–35).

Materials Needed

- DNA fragments to assemble (up to 10 DNA fragments totaling up to 100 kb in length, not including the cloning vector)
- Stitching oligonucleotides (i.e., recombination linkers; if you are assembling DNA fragments that do not share end-terminal homology or if you want to carry out fragment editing)
- GeneArt® pYES1L Vector with Sapphire™ Technology or your own yeast-adapted cloning vector
- Control insert (for assessing the efficiency of the assembly reaction)
- MaV203 competent yeast cells
- pMAB37 DNA (control plasmid for assessing MaV203 transformation efficiency)
- PEG/LiAc solution
- DMSO
- 0.9% NaCl solution (sterile)
- DNase-, RNase-Free water
- CSM-Trp agar plates
- 30°C and 42°C water baths and a 30°C incubator
- Microcentrifuge
- SpeedVac (optional)

Continued on next page

Assembling Homologous DNA Fragments

Follow the procedure below to set up the high-order DNA assembly reaction when using DNA fragments that share end-terminal homology. If your fragments do **not** share end-terminal homology, see **Assembling Non-Homologous DNA Fragments by Oligonucleotide Stitching** on page 23.

1. Add the following components to a microcentrifuge tube and mix.

pYES1L Vector	100 ng
DNA fragments	100 ng each (if final construct is ≤ 25 kb) 200 ng each (if final construct is > 25 kb)

   If the total volume of the DNA mix is lower than 10 µL, proceed to Step 2.

   If the total volume of the DNA mix is higher than 10 µL, reduce total volume to 5–10 µL using a SpeedVac or a centrifugal filter device. Do **not** let the liquid dry completely.

   Note: Instead of pYES1L, you can also use your own yeast-adapted linear cloning vector.
2. Thaw the PEG/LiAc solution in a beaker containing room-temperature water. Mix the solution well before dispensing.
3. Thaw the competent MaV203 cells by placing them in a 30°C water bath for not longer than 90 seconds. Proceed immediately to Step 4. You can perform Steps 4, 5, and 6 at room temperature.

   Note: Do **not** freeze/thaw the MaV203 competent yeast cells. Competent yeast can only be thawed once without dramatic loss in competency.
4. Move the thawed cells to room temperature and invert the tube several times to mix the cells. **Do not vortex the cells.**
5. Add 100 µL of thawed MaV203 cells into the DNA mix (the volume of the DNA mix should be ≤ 10 µL). Mix well by tapping the tube.
6. Add 600 µL of the thawed PEG/LiAc solution to the DNA/competent cell mixture. Mix by inverting the tube 5–8 times until all of the components are homogeneous.
7. Incubate the mixture in the 30°C water bath for 30 minutes. Invert the tube occasionally (every 10 minutes) to resuspend the components.
8. Add 35.5 µL of DMSO to the tube. Mix by inverting the tube 5–8 times.

   Note: For best results, use fresh DMSO from an unopened bottle. DMSO stored at –20°C can also be used.
9. Heat-shock the cells by incubating the tube in the 42°C water bath for 20 minutes. Invert the tube occasionally to resuspend the components.
10. Centrifuge the tube at 1,800 rpm (200–400 × *g*) for 5 minutes.
11. Carefully discard the supernatant from the tube and resuspend the cell pellet in 1 mL of sterile 0.9% NaCl by gentle pipetting.
12. Plate 100 µL of the transformed cells onto CSM-Trp agar plates.

    Note: For final constructs of > 60 kb, we recommend that you centrifuge the remaining 900 µL of the transformation mixture, remove ~750 µL of the supernatant, resuspend the cell pellet in the remaining 100–150 µL of supernatant, and plate all cells onto another CSM-Trp agar plate to ensure that you have sufficient number of colonies to screen.
13. Incubate the cells at 30°C for 3 days and proceed to **Yeast Colony PCR**, page 26.

Continued on next page

Assembling Non-Homologous DNA Fragments by Oligonucleotide Stitching

Follow the procedure below to set up the high-order DNA assembly reaction when using DNA fragments that do **not** share end-terminal homology. You can also use this procedure to perform fragment editing with stitching oligonucleotides containing insertions, deletions, and other modifications. See pages 16–20 for DNA oligonucleotide requirements.

1. Add the following components to a microcentrifuge tube and mix.

pYES1L vector	100 ng
DNA fragments	100 ng each (if final construct is ≤ 25 kb) 200 ng each (if final construct is > 25 kb)
Stitching oligonucleotides	~500 ng each (20 pmol each)

   If the total volume of the DNA mix is lower than 10 µL, proceed to Step 2.

   If the total volume of the DNA mix is higher than 10 µL, reduce total volume to 5–10 µL using a SpeedVac or a centrifugal filter device. Do **not** let the liquid dry completely.

   Note: Instead of pYES1L, you can also use your own yeast-adapted, linearized vector.

2. Thaw the PEG/LiAc solution in a beaker containing room-temperature water. Mix the solution well before dispensing.
3. Thaw the competent MaV203 cells by placing them in a 30°C water bath for no more than 90 seconds. Proceed immediately to Step 4.

   Note: Do **not** freeze/thaw the MaV203 competent yeast cells. Competent yeast can only be thawed once without dramatic loss in competency.
4. Move the thawed cells to room temperature and invert the tube several times to mix the cells. **Do not vortex the cells.**
5. Add 100 µL of thawed MaV203 cells into the DNA mix (the volume of the DNA mix should be ≤ 10 µL). Mix well by tapping the tube.
6. Add 600 µL of the thawed PEG/LiAc solution to the DNA/competent cell mixture. Mix by inverting the tube 5–8 times until all of the components are homogeneous.
7. Incubate the mixture in the 30°C water bath for 30 minutes. Invert the tube occasionally (every 10 minutes) to resuspend the components.
8. Add 35.5 µL of DMSO to the tube. Mix by inverting the tube 5–8 times.

   Note: For best results, use fresh DMSO from an unopened bottle. DMSO stored at –20°C can also be used.
9. Heat shock the cells by incubating the tube in the 42°C water bath for 20 minutes. Invert the tube occasionally to resuspend the components.
10. Centrifuge the tube at 1,800 rpm (200–400 × *g*) for 5 minutes.
11. Carefully discard the supernatant from the tube and resuspend the cell pellet in 1 mL of sterile 0.9% NaCl by gentle pipetting.
12. Plate 100 µL of the transformed cells onto CSM-Trp agar plates.

    Note: We recommend that you also plate 100 µL of the transformed cells at a 1:10 dilution in 0.9% NaCl to ensure that at least one plate will have well-spaced colonies.
13. Incubate the cells at 30°C for 3 days and proceed to **Yeast Colony PCR**, page 26.

Control Experiments

IMPORTANT! The GeneArt® High-Order Genetic Assembly System contains 11 tubes of MaV203 Competent Yeast Cells. If you perform all the control experiments described below, you will only be able to perform 8 high-order assembly reactions.

Performing the High-Order Assembly Control Experiments

Follow the procedure below to set up the control experiments for the high-order DNA assembly reaction.

1. Prepare the following DNA mixtures for the control experiments in separate microcentrifuge tubes and mix.

   Positive control:

pYES1L vector	100 ng
Control Insert	100 ng

   Negative Control (*Optional*):

pYES1L vector only	100 ng

   Control for Transformation Efficiency:

pMAB37 control vector only	100 ng

2. Thaw the PEG/LiAc solution in a beaker containing room-temperature water. Mix the solution well before dispensing.
3. Thaw the competent MaV203 cells by placing them in a 30°C water bath for no more than 90 seconds. Proceed immediately to Step 4.

   Note: Do **not** freeze/thaw the MaV203 competent yeast cells. Competent yeast can only be thawed once without dramatic loss in competency.
4. Move the thawed cells to room temperature and invert the tube several times to mix the cells. **Do not vortex the cells.**
5. Add 100 µL of thawed MaV203 cells into the DNA mix (the volume of the DNA mix should be ≤ 10 µL). Mix well by tapping the tube.
6. Add 600 µL of the thawed PEG/LiAc solution to the DNA/competent cell mixture. Mix by inverting the tube 5–8 times until all of the components are homogeneous.
7. Incubate the mixture in the 30°C water bath for 30 minutes. Invert the tube occasionally (every 10 minutes) to resuspend the components.
8. Add 35.5 µL of DMSO to the tube. Mix by inverting the tube 5–8 times.

   Note: For best results, use fresh DMSO from an unopened bottle. DMSO stored at –20°C can also be used.
9. Heat shock the cells by incubating the tube in the 42°C water bath for 20 minutes. Invert the tube occasionally to resuspend the components.
10. Centrifuge the tube at 1,800 rpm (200–400 × *g*) for 5 minutes.

Continued on next page

Performing the High-Order Assembly Control Experiments, continued

11. Carefully discard the supernatant from the tube and resuspend the cell pellet in 1 mL of sterile 0.9% NaCl by gentle pipetting.
12. Plate the following volumes of transformed cells onto CSM-Trp plates and incubate the cells at 30°C for 3 days.

    Positive control: plate 100 µL of 1:10 dilution in 0.9% NaCl

    Negative control: plate 100 µL

    Control for Transformation Efficiency: plate 100 µL of 1:100 dilution in 0.9% NaCl
13. After 3 days growth at 30°C, screen the colonies by **Yeast Colony PCR** (pages 26–27).

Expected Results

You can expect the following results from your control experiments.

Control	Total Colony Output per Transformation (1mL)	% Cloning Efficiency*
Positive control	≥ 5,000	≥ 90%
Negative control	≤ 500	NA†
Control for transformation efficiency	≥ 10,000	NA†

***% cloning efficiency** = ("positive" colonies/total colonies) × 100, where "positive" colonies are those that are confirmed to contain the assembled control construct as validated by yeast colony PCR (see page 26) and sequencing.

†NA = not applicable

Yeast Colony PCR

Introduction

After you have performed the high-order DNA assembly reaction and obtained MaV203 colonies on your CSM-Trp agar plates, perform Yeast Colony PCR to screen for positive colonies containing your assembled recombinant DNA molecule.

Before You Begin

Design a pair of diagnostic primers (forward and reverse) at a distance of 200–250 bp from the ends of each DNA fragment (including the cloning vector) so that the colony PCR products are 400–500 bp in size and they span the junctions between the fragments.

Materials Needed

- MaV203 yeast cells transformed with your DNA mix
- Pair of diagnostic primers for each DNA junction including the cloning vector
- Lysis buffer
- Thermocycler and sterile PCR tubes or plates
- Platinum® PCR SuperMix High Fidelity (Cat. no. 12532-016)

Performing Yeast Colony PCR

Use the protocol below to analyze MaV203 transformants to screen for "positive" colonies containing your assembled recombinant DNA molecule.

Determine the amplification conditions appropriate for your diagnostic primers.

Do **not** use more than 0.5 µL of diluted yeast lysate in a 50 µL PCR volume.

1. Aliquot 15 µL of lysis buffer into PCR tubes or plates. Do **not** break the cells using the glass beads supplied with the kit.
2. Pick individual yeast colonies one at a time using a sterile 20 µL pipette tip. Leave the tip in the PCR tube or the well until all the colonies have been picked.
3. Resuspend the cells by pipetting up and down 3 times.
4. Transfer 5 µL of each cell suspension into fresh PCR tubes and store at 4°C until you have verified that the colony is positive (see below) and you are ready to transfer the assembled construct into *E. coli* (page 28).
5. Heat the remaining cells (10 µL) at 95°C for 5 minutes in a thermocycler and cool them down at 4°C or on ice. Briefly centrifuge the PCR tubes or plates to bring down all condensation.
6. Add 40 µL of nuclease-free water to into each lysate and pipet up and down 3–5 times to mix.

Continued on next page

Performing Yeast Colony PCR, continued

7. Set up a PCR master mix for each junction and aliquot 49.5 µL of it into fresh PCR tubes or plates.
8. Add 0.5 µL of each diluted yeast lysate (from step 4) into each PCR tube or well. Do **not** exceed 0.5 µL of lysed yeast cells for 50 µL of PCR volume.
9. **Vortex to mix the contents** and briefly centrifuge to bring down all liquid.
10. Perform PCR cycling in a thermocycler.
11. Load 10 µL onto an agarose gel to visualize the PCR products. If PCR products at expected sizes exist for all junctions, the colony is considered positive. You may also verify the sequence by using the PCR products as template for the sequencing reactions with one of the diagnostic primers.

Transferring the Assembled Construct from Yeast to *E. coli*

Introduction

After you have identified positive colonies containing your assembled construct, transfer the assembled construct into *E. coli* to prepare large scale plasmid preps for downstream applications.

Materials Needed

- CSM-Trp plates containing positive MaV203 colonies
- Lysis buffer
- Glass beads
- S.O.C. medium at room temperature
- One Shot® TOP10 Electrocomp™ *E. coli*
- pUC19 control DNA
- Electroporation cuvettes, chilled on ice
- Electroporator
- 15-mL snap cap tubes
- LB plates with the appropriate selection antibiotics, pre-warmed to 37°C

Transferring the Assembled Construct to *E. coli*

1. Aliquot 4–5 glass beads into a fresh PCR tube and add 10 µL of Lysis buffer.
2. Add 5 µL of the cell suspension that you have stored (from Step 4, page 26) into the Lysis Buffer/Glass beads mix. Pipet up and down 3–5 times to mix.
3. Vortex the cells at room temperature for 5 minutes. **Do not** heat the lysed cells.
4. For each transformation, thaw one vial of One Shot® TOP10 Electrocomp™ *E. coli* on ice.
5. Add 1 µL of the lysed cells (from Step 3, above) into a vial of One Shot® TOP10 Electrocomp™ *E. coli* (50 µL) and mix gently. Do **not** add more than 1 µL of the lysed cells to avoid arcing during electroporation.

   Note: For the pUC19 control, add 10 pg (1 µL) of DNA into a separate vial of One Shot® cells and mix gently.
6. Transfer the cells to the chilled electroporation cuvette on ice
7. Electroporate the cells following the manufacturer's recommended protocol.
8. Aseptically add 250 µL of pre-warmed S.O.C. Medium to each vial.
9. Transfer the solution to a 15 mL snap-cap tube and shake for at least 1 hour at 37°C to allow expression of the antibiotic resistance gene.
10. Spread 10 to 50 µL from each transformation on a pre-warmed LB plate supplemented with the appropriate selection antibiotic.

    Note: We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies. For the pUC19 control, dilute the transformation mix 1:50 into LB Medium (e.g. remove 20 µL of the transformation mix and add to 980 µL of LB Medium) and plate 20–100 µL.
11. Invert the selective plate(s) and incubate at 37°C overnight.

Troubleshooting

The table below lists some potential problems and solutions that help you troubleshoot your high-order DNA assembly experiments using the GeneArt® High-Order Genetic Assembly System.

Symptom	Cause	Solution
No yeast colonies after transformation and the control transformation did not work	Transformation unsuccessful	Perform the transformation procedure exactly as described on pages 22–23.
	MaV203 cells have been thawed and frozen multiple times	Do **not** freeze/thaw the MaV203 competent yeast cells. Competent yeast can only be thawed once without dramatic loss in competency.
	Incorrect yeast growth medium	Make sure that you grow transformed MaV203 cells on CSM-Trp agar plates prepared using the CSM Media for MaV203 Yeast Cells.
	DMSO was not fresh	For best results, use fresh DMSO from an unopened bottle. You may also use DMSO stored at –20°C.
	MaV203 cells were handled incorrectly	Competent yeast cells are very fragile. Handle the cells gently and resuspend them by pipetting up and down gently. **Do not vortex** the competent yeast cells.
No yeast colonies after transformation with DNA fragments, but the control transformation is successful	DNA fragments do not share the required end-terminal homology	Make sure that your DNA fragments and yeast-adapted cloning vector share the required end-terminal homology. Refer to pages 11–20 for the requirements on DNA oligonucleotides and PCR.
	Used single-stranded oligonucleotides for stitching	Make sure that you use two oligonucleotides, a sense and an anti-sense oligonucleotide (i.e., a double-stranded oligonucleotide) for each junction between adjacent fragments when performing oligonucleotide stitching.
	DNA fragments generated by PCR were impure	If you are using DNA fragments prepared by PCR, purify the DNA fragments through a PCR cleanup kit. We recommend that you use the PureLink® PCR Purification Kit, available separately from Life Technologies (Cat. no. K3100-01).
	Ends of the DNA fragments generated by PCR were damaged	Employ extra caution to minimize potential damage to the ends of your 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.

Continued on next page

Troubleshooting, continued

Symptom	Cause	Solution
No yeast colonies after transformation with DNA fragments, but the control transformation is successful	Incorrect amounts of DNA oligonucleotides, fragments, and/or vector were used	Make sure that you use the correct amounts of DNA oligonucleotides, fragments, and/or vector for high-order DNA assembly.
	Your cloning vector does not have the necessary genetic elements to propagate in yeast	• Use the the GeneArt® Vector Conversion Cassette with Sapphire™ Technology to adapt your vector to be a yeast-compatible cloning vector for use with the GeneArt® High-Order Genetic Assembly System (see pages 33–35). • Alternatively, use the GeneArt® pYES1L Vector with Sapphire™ Technology as a cloning vector to assemble your DNA fragments.
Small or no colonies after transformation, including the control transformation	Incorrect incubation temperature	• Incubate yeast transformations at 30°C for 3 days. • Incubate *E. coli* transformations at 37°C for 12–16 hours.
No "positive" colonies detected by Yeast Colony PCR	Cells lysed incorrectly	• Make sure to follow the lysing procedure on page 26 correctly. • Do not break the cells using the glass beads supplied with the kit.
	Too much lysate is used for PCR amplification	• Do **not** use more than 0.5 µL of diluted yeast lysate in a 50 µL PCR volume. • Re-streak the colony on a fresh plate while screening and repeat colony PCR.
Not all junctions are detected by Yeast Colony PCR	Diagnostic primers are not optimal	Change the location of the primers and repeat colony PCR.
	Too much lysate is used for PCR amplification	• Do **not** use more than 0.5 µL of diluted yeast lysate in a 50 µL PCR volume. • Re-streak the colony on a fresh plate while screening and repeat colony PCR.
Most or all of the clones are incorrect	Internal homology shared between the fragments, between fragment(s) and vector, or within the vector (not pYES1L)	Redesign your cloning construct and DNA fragments using the GeneArt® Primer and Construct Design Tool, which is available at **http://bioinfo.invitrogen.com/oligoDesigner**.

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® High-Order Genetic Assembly** as the product type under the **GeneArt® Seamless Cloning and Assembly** dropdown menu.
- 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.

  IMPORTANT! To perform the seamless cloning and assembly reaction, your empty cloning vector **must** be linear.

  Note: Your final assembled construct is displayed as a circular or linear molecule; it does not mean that you are starting with a circular or linear vector.
- 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.
- If you prefer using stitching oligonucleotides to assemble your DNA fragments without end-terminal homology, leave the PCR boxes unchecked.
- Click **Design My DNA Oligos**. 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 and/or recombination sites for your stitching oligonucleotides (see page 32 for an example). The tool also provides you with the sizes and sequences of PCR primers as well as their melting temperature (T_m) and GC content.

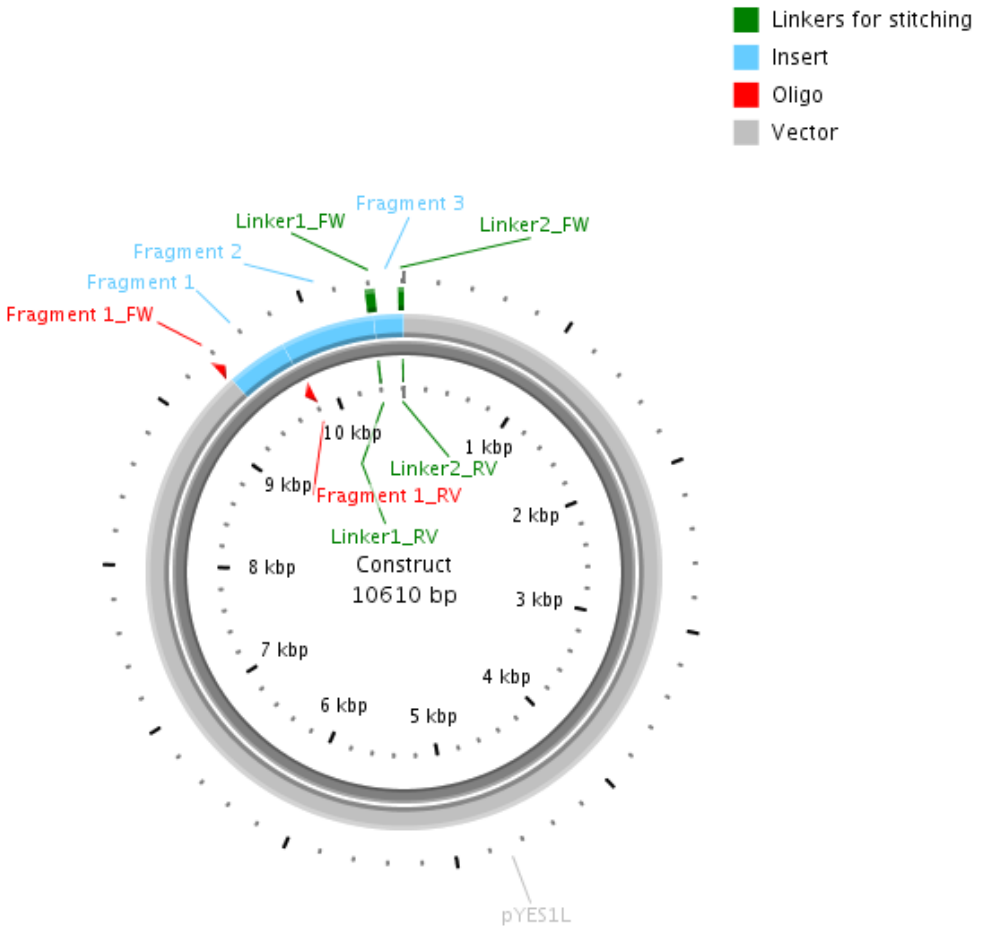
  Note: You can also assemble your final construct using any combination of PCR-amplified fragments, pre-existing DNA fragments, and stitching oligonucleotides.
- Click **Add to Cart** to order your PCR primers directly from Life Technologies.
- Click the **image icon** to save the output from the web tool as a GenBank file.

Continued on next page

GeneArt® Primer and Construct Design Tool, continued

Output example from the GeneArt® Primer and Construct Design Tool

The example below shows the output from the GeneArt® Primer and Construct Design Tool for a 3-fragment assembly into the GeneArt® pYES1L Vector with Sapphire™ Technology. In this example, Fragment 1 was PCR amplified to introduce the required end-terminal homology for recombination (i.e., PCR box checked when uploading its sequence), whereas Fragments 2 and 3 were assembled using stitching oligonucleotides (i.e., corresponding PCR boxes were unchecked).



IMPORTANT! The GeneArt® Primer and Construct Design Tool does **not** support fragment editing.

- For **deletion editing**, enter the fragment sequence into the GeneArt® Primer and Construct Design Tool with the end deletions already applied. This way, the tool will design the correct stitching oligonucleotides and provide the final assembled DNA sequence with the desired deletions.
- For **insertion editing**, manually add the inserted nucleotides into the final stitching oligonucleotide designed by the tool and then manually edit the final assembled DNA construct.

Appendix B: Additional Protocols

Generating Yeast-Adapted Cloning Vectors

Introduction

Using the GeneArt® Vector Conversion Cassette with Sapphire™ Technology (available separately from Life Technologies, Cat. no. A13291), you can adapt any *E. coli* vector to be a yeast-compatible cloning vector for use with the GeneArt® High-Order Genetic Assembly System. The GeneArt® Vector Conversion Cassette with Sapphire™ Technology is a **blunt-ended** DNA fragment of about 3.8 Kb. For a map and features of the conversion cassette, see page 38.

Materials Needed

- Single- or low-copy-number vector to be adapted to yeast
- GeneArt® Vector Conversion Cassette with Sapphire™ Technology (Cat. no. A13291)
- Appropriate restriction enzyme for linearizing the vector, preferably a blunt-end cutter
- T4 DNA Ligase (Cat. no. 15224-017)
- *Asc*I or *Asi*SI restriction enzymes
- PureLink® Quick Gel Extraction Kit (Cat. no. K2100-12) or equivalent
- PureLink® PCR Purification Kit (Cat. no. K3100-01) or equivalent
- PureLink® Quick Plasmid Miniprep Kit (Cat. no. K2100-10) or equivalent
- Competent *E. coli* cells
- LB agar plates supplemented with chloramphenicol and the appropriate selection antibiotic for your custom vector
- LB agar plates supplemented with spectinomycin
- 0.9% NaCl solution (sterile)
- DNase-, RNase-Free water
- CSM-Trp agar plates
- 1X TE buffer, pH 8.0
- 30°C and 42°C water baths
- 30°C incubator
- Thermocycler (PCR machine)
- Microcentrifuge
- SpeedVac (optional)

Continued on next page

Generating Yeast-Adapted Cloning Vectors, continued

Guidelines for Generating Yeast-Adapted Cloning Vectors

When generating a yeast-adapted cloning vector for use with the GeneArt® High-Order Genetic Assembly System, follow the guidelines below:

- Using the GeneArt® Primer and Construct Design Tool, verify that your vector and the GeneArt® Vector Conversion Cassette with Sapphire™ Technology do **not** share internal homology to prevent potential rearrangements when using your adapted vector with the GeneArt® High-Order Genetic Assembly System.
- Use vector with a single- or low-copy-number origin for a final construct of >15 kb, if the final plasmid construct will be transferred into *E. coli*. Usually, low-copy-number *E. coli* vectors have significantly higher capacity than high-copy-number vectors.
- Avoid chloramphenicol selection markers on the custom vector.
- If your custom vector contains the spectinomycin selection marker, screen more colonies during the adaptation step.

Generating the Yeast-Adapted Cloning Vector

Follow the procedure below to generate a yeast adapted-cloning vector using the GeneArt® Vector Conversion Cassette with Sapphire™ Technology.

1. Cut your vector with appropriate enzyme(s).
2. Clean up the restriction reaction with a PCR cleanup kit (e.g., PureLink® PCR Purification Kit) or by phenol/chloroform extraction and ethanol precipitation.
3. Ligate ~10 ng of your linearized vector backbone with the GeneArt® Vector Conversion Cassette with Sapphire™ Technology at a 1:10 (vector: insert) molar ratio at 14°C overnight using T4 ligase.
4. Transform competent *E. coli* cells with the ligation mixture and plate on double-selection LB plates (chloramphenicol plus the antibiotic marker on your custom vector backbone). Incubate the plates at 37°C overnight.
5. Re-streak 6–12 colonies onto the double-selection LB plate and an LB plate supplemented with spectinomycin (assuming that the custom vector is not spectinomycin resistant).
6. Pick the resultant colonies (i.e., transformants) that grow only on the double-selection plate (but not on spectinomycin selection) and grow them overnight at 37°C in LB medium supplemented with chloramphenicol and the appropriate selection antibiotic for your custom vector.
7. The next day, harvest the cells and isolate the plasmid DNA using PureLink® Quick Plasmid Miniprep Kit or equivalent.
8. Analyze the plasmid DNA by restriction enzyme digestion and/or sequencing for verification. This is your **adapted vector**.

Continued on next page

Generating the Yeast-Adapted Cloning Vector, continued

9. Linearize the adapted vector using two different restriction enzymes in the multiple cloning region.

   Note: Double enzyme digestion is required to avoid background caused by residual palindromic end sequences resulting from a single enzyme digestion. We recommend using the following combination of enzymes: *Not*I/*Pac*I, *Asc*I/*Pac*I, *Asc*I/*Not*I, *Asi*SI/*Pac*I, *Asi*SI/*Not*I, or other combination of restriction enzymes that you prefer (refer to the map of your adapted vector).

   IMPORTANT! Make sure that the restriction enzymes do **not** cut anywhere else in the adapted vector.

10. Clean up the digestion reaction with a PCR cleanup kit (e.g., PureLink® PCR Purification Kit) or by phenol/chloroform extraction and ethanol precipitation. Resuspend the linearized converted vector in 10 mM Tris, pH 8.0.

11. Proceed to fragment assembly (pages 21–23).

    Note: Use 50–100 ng of linear vector if your custom vector has a high-copy-number origin for *E. coli*. Use 100 ng of linear vector if your custom vector has a low- or single-copy-number origin for *E. coli*.

Appendix C: Recipes

Media and Solutions

CSM Media for MaV203 Yeast Cells

For your convenience, the CSM Media for MaV203 Yeast Cells is included prepackaged in the GeneArt® High-Order Genetic Assembly System (*with Yeast Growth Media*), and it is also available separately from Life Technologies (Cat. no. A13292, see page 39 for ordering information).

CSM Media for MaV203 Yeast Cells is used for preparing CSM-Trp agar plates. Because MaV203 yeast cells are auxotrophic for tryptophan (*trp1-901*), only cells that have been successfully transformed with GeneArt® pYES1L Vector with Sapphire™ Technology (or your own yeast-compatible cloning vector prepared using the GeneArt® Vector Conversion Cassette with Sapphire™ Technology) will grow on CSM-Trp agar plates.

To prepare 1 liter of CSM-Trp agar plates, use only 1 pouch of CSM-Trp Agar Yeast Media minus Glucose and 1 bottle of 20% Glucose.

1. Dissolve the contents of the CSM-Trp Agar Yeast Media minus Glucose pouch from the CSM Media for MaV203 Yeast Cells in 900 mL of distilled water.
2. Autoclave the solution for 20 minutes on liquid cycle.
3. Allow the solution to cool to 50°C, then add 100 mL of sterile 20% Glucose (i.e., the entire bottle), pre-warmed to 50°C.
4. Swirl the autoclave bottle to mix and pour into 10-cm plates.
5. Let the plates harden, then invert and store them at 4°C, in the dark. The shelf life of CSM-Trp agar plates is several months.

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 the medium into 10 cm plates.
4. Let the agar harden, then invert the plates and store them at 4°C, in the dark.

Appendix D: Vectors

GeneArt® pYES1L Vector with Sapphire™ Technology

Map of GeneArt® pYES1L Vector with Sapphire™ Technology

The figure below summarizes the features of the linear GeneArt® pYES1L Vector with Sapphire™ Technology (9,380 bp). **The complete sequence for the vector is available for downloading at www.lifetechnologies.com or by contacting Technical Support (see page 41).**

Comments for GeneArt® pYES1L Vector with Sapphire Technology™:

9,380 nucleotides

TRP1 gene: bases 172–846 (c)
ARS4/CEN5: bases 1,491–1,759
Spectinomycin promoter (P_{Spn}): bases 2,161–2,294
Spectinomycin resistance gene: bases 2,295–3,305
COS ORF: bases 3,347–3,508
repE gene: bases 4,677–5,432
sopA gene: bases 6,011–7,186
sopB gene: bases 7,186–8,157
ori T 1: bases 8,995–9,284

(c) = complementary strand

Features of the Vector

The linear GeneArt® pYES1L Vector with Sapphire™ Technology (9,380 bp) contains the following elements. All features have been functionally tested.

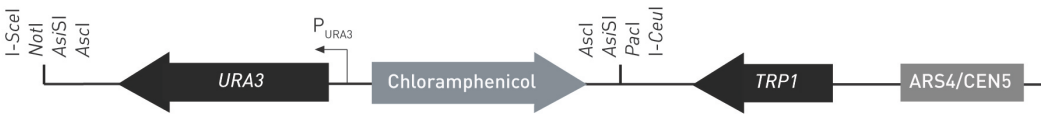
Feature	Benefit
ARS4/CEN5 origin	Allows the stable maintenance of the plasmid in yeast
TRP1 gene	Permits the selection of yeast transformants in tryptophan-free medium
COS site	Allows packaging in phage λ
Spectinomycin resistance gene	Allows selection in *E. coli*
resE, *sopA*, and *sopB*	Function as accessory genes to F′ ori
*Not*I, *Pac*I sites	Rare restriction enzyme recognition sites
I-*Ceu*I, I-*Sce*I	Homing endonucleases sites for restriction mapping of the final assembled construct

GeneArt® Vector Conversion Cassette with Sapphire™ Technology

Map of GeneArt® Vector Conversion Cassette with Sapphire™ Technology

The figure below summarizes the features of the GeneArt® Vector Conversion Cassette with Sapphire™ Technology (3,834 bp), available separately from Life Technologies (Cat. no. A13291). The vector conversion cassette is a blunt-ended, linearized DNA that can be used to adapt any *E. coli* vector to be yeast compatible (see **Generating Yeast-Adapted Cloning Vectors**, page 33).

The complete sequence of the vector conversion cassette is available for downloading at www.lifetechnologies.com or by contacting Technical Support (see page 41).



Comments for GeneArt® Vector Conversion Cassette with Sapphire™ Technology:

3,834 nucleotides

URA3 gene: bases 148–1,029 (c)
URA3 promoter (P_{URA3}): bases 1,030–1,251 (c)
Chloramphenicol resistance gene: bases 1,318–1,998
TRP1 gene: bases 3,347–3,508 (c)
ARS4/CEN5: bases 3,457–3,725

(c) = complementary strand

Features of the Vector Conversion Cassette

The linear GeneArt® Vector Conversion Cassette with Sapphire™ Technology (3,834 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
ARS4/CEN5 origin	Allows the stable maintenance of the plasmid in yeast
TRP1 gene	Permits the selection of yeast transformants in tryptophan-free medium
URA3 gene	Optional counter-selectable marker
Chloramphenicol resistance gene (Cm^R)	Allows selection in *E. coli*
*Asc*I, *Asi*SI sites	Rare restriction enzyme recognition sites to linearize the final construct for high-order genetic assembly
*Not*I, *Pac*I sites	Alternative rare restriction enzyme recognition sites
I-*Ceu*I, I-*Sce*I	Homing endonuclease sites for mapping the final assembled construct

Appendix E: Ordering Information

GeneArt® Products

GeneArt® High-Order Genetic Assembly System Products

Some of the components of the GeneArt® High-Order Genetic Assembly System 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® pYES1L Vector with Sapphire™ Technology	10 reactions	A13287
MaV203 Competent Yeast Cells, Subcloning Scale	4 × 0.1 mL	11445-012
CSM Media for MaV203 Yeast Cells	1 kit	A13292
One Shot® TOP10 Electrocomp™ *E. coli*	10 reactions	C4040-50
GeneArt® Vector Conversion Cassette with Sapphire™ Technology	10 reactions	A13291

Other GeneArt® Products

Life Technologies also offers other GeneArt® products that can be used for seamless *in vitro* or *in vivo* assembly of DNA fragments 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® 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
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® High-Order Genetic Assembly System. For more information, refer **www.lifetechnologies.com** or contact Technical Support (see page 41).

Product	Amount	Cat. no.
Platinum® *Taq* DNA Polymerase	100 reactions 500 reactions	10966-018 10966-034
Platinum® PCR SuperMix High Fidelity		12532-016
PureLink® PCR Purification Kit	50 preps	K3100-01
PureLink® Quick Gel Extraction Kit	1 kit	K2100-12
PureLink® Quick Plasmid Miniprep Kit	50 preps 250 preps	K2100-10 K2100-11
T4 DNA Ligase (5 U/µL)	250 units	15224-017
DNA Polymerase I (Klenow fragment)	100 units	18012-021
LB Broth	500 mL	10855-021
LB Agar	500 g	22700-025

Custom DNA Oligos

To order custom primers, visit **www.lifetechnologies.com/oligos** or contact Technical Support (see page 41). You can also purchase synthesized DNA fragments at **www.lifetechnologies.com/geneart** or using our GeneArt® Primer and Construct Design Tool, which is available at **http://bioinfo.invitrogen.com/oligoDesigner**.

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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