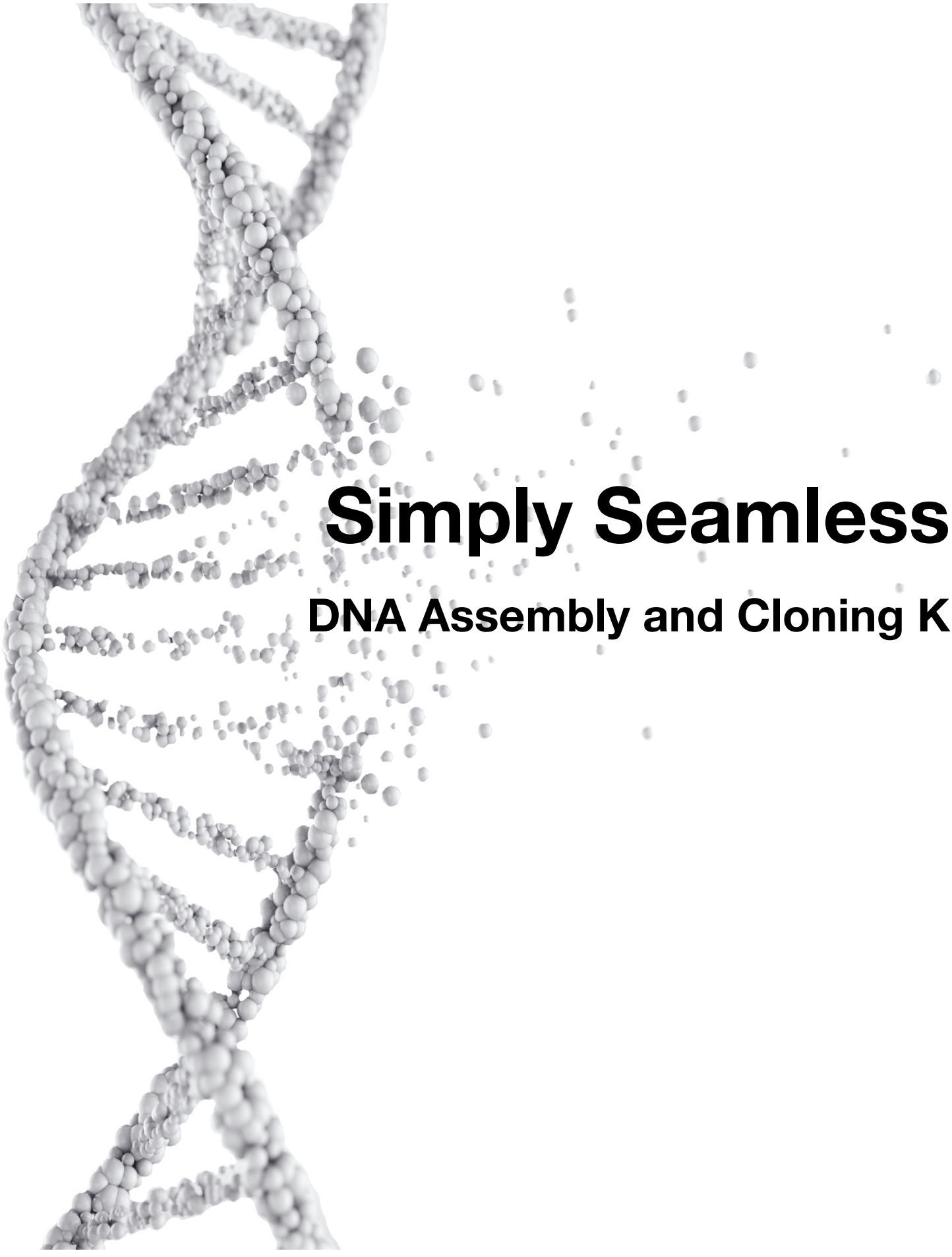

Simply Seamless

DNA Assembly and Cloning Kit

Technical Services: For technical assistance, please contact technical services at info@synovance.com.

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

www.synovance.com

Catalog #

Component	S93-01	S93-02	Storage Temp
Simply Seamless DNA Assembly Enzyme Mix (10X) part # S92	10 reactions	40 reactions	-80°C
10X Reaction Buffer part # S94	13 µl	55 µl	-20°C
Simply Seamless Positive Control: part # S95	3 reactions 24 µl	12 reactions 96 µl	-20°C
Purchased separately			
Chemically Competent DH5α cells: part # S96	10 reactions	40 reactions	-80°C

The kit is shipped on dry ice (-80°C). Components in the kit are stable for 1 year after production date if properly stored.

- Required Materials Not Included:

DNA Polymerase: For generating PCR Products, we recommend using a High-Fidelity DNA Polymerase to reduce the chance of incorporating errors within DNA fragments.

Thermocycler

DNA fragments (PCR fragments or synthetic DNA): For the Simply Seamless cloning reaction.

LB (Luria-Bertani) media and plates: For outgrowth and selection of transformed competent cells, we recommend using LB plates supplemented with appropriate antibiotic.

- Additional Optional Materials:

Electroporator, Electroporation Competent cells and electroporation cuvettes: If electroporation is used to introduce the assembled reaction into cell.

Introduction:

The Simply Seamless technology was developed to improve the speed, efficiency and accuracy of DNA assembly and cloning. This method allows for seamless assembly of multiple DNA fragments, regardless of fragment length or end compatibility. This method has been used to assemble either single-stranded oligonucleotides or different sizes of DNA fragments with 25 bp overlaps. Ideal for the Synthetic Biology community, Simply Seamless makes one-step cloning of multiple fragments easy, fast, and reliable. The reaction includes a proprietary mix of enzymes that work together in the same buffer (see Figure 1):

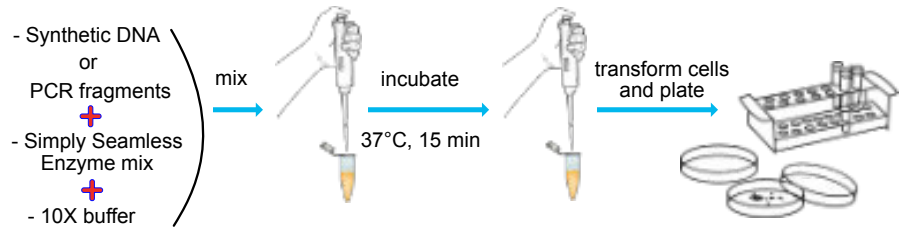

Figure 1) Overview of the DNA Assembly reaction. (for detailed protocol, see page 6)

- Fragments to be assembled can be synthetic DNA, PCR fragments or a combination of these.
- Mix DNA with the buffer and enzyme mix.
- Incubate from 15-30 minutes at 37°C.
- Reaction is complete, ready to transform.

The end result is a double-stranded fully sealed DNA molecule that can serve as template for PCR, RCA or a variety of other molecular biology applications, including direct transformation of E coli.

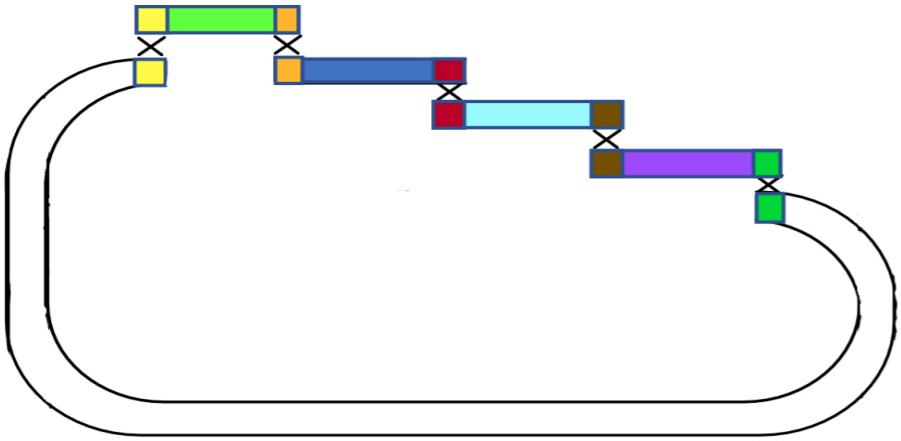

Figure 2) Cartoon diagram illustrating the homology regions and assembly of 4 DNA fragments into a vector. The 25 bp homologous ends are depicted by colored squares.

Overview of Simply Seamless DNA Assembly

DNA Assembly and Cloning Workflow:

- Design primers to amplify fragments (and/or vector) with appropriate overlaps. Alternatively, DNA can be purchased from a commercial provider of synthetic DNA.
- Amplify fragments using a high-fidelity DNA polymerase.
- Prepare linearized vector by Inverse PCR using a high-fidelity DNA polymerase or by restriction enzyme digestion.
- Determine concentration of fragments and linearized vector using agarose gel electrophoresis, a NanoDrop® instrument or other method.
- Add insert fragments, linearized vector, reaction buffer, enzyme mix and incubate at 37°C for 15 to 30 minutes (60 minutes can be used for multiple inserts).
- Transform into Competent DH5-alpha *E. coli* (provided with cloning kit or purchased separately from Simply Seamless) or use directly in other applications. Alternatively, electroporation can be used to transform cells.

Design and PCR of Fragments for DNA Assembly:

Structure of the Overlapping Primers

PCR primers must have two sequence components:
- an overlap homology sequence, required for the assembly of adjacent fragments;
- a gene-specific sequence, required for template priming during PCR.

The 25 bp non-priming overlap sequence is added at the 5´ end of the primer. This sequence is homologous to the 3´-terminal sequence of the adjacent fragment to be assembled.

The gene-specific priming sequence added to the 3´ end of the primer should meet the criteria required for efficient template annealing during PCR.

The Tm for the 3´ gene-specific sequence can be calculated using one of several Tm calculators that are found online or by using a commercial DNA analysis software.

The secondary structure of the 25 bp homologous overlap region is critical to the success of the assembly reaction. If there is a strong secondary structure (stem-loop) in this 25 bp region it will reduce the homology and inhibit the assembly reaction. It is strongly suggested to confirm that there is no secondary structure within the 25 bp homologous sequences using an online tool similar to the one similar to the one found at. http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi

General Recommendations for Design of Overlapping Primers

To prevent errors in primer design it is recommended to first construct an *in silico* map of the region to be assembled and create a final sequence file displaying both DNA strands.

This virtual sequence may then be used as a template to design overlapping primers.
- First, mark the junctions between the adjacent fragments.
- Next, adjacent to each junction, choose the 25 nucleotides from this sequence region that will serve as the overlap homology between the two adjacent fragments. This overlap region can belong to only one fragment or
  it can be splitbetween the two adjacent fragments.
- Define the priming region for the 3' end of the oligonucleotide. It is recommended that the Tm = 60°-65°C.

Primer Design for PCR-Generated Vector

An inverse PCR strategy produces a linear vector fragment. Here the primers are designed to amplify the vector only. If the vector is quite large, it is possible to amplify the vector in 2 – 4 fragments that would be re-assembled using the Simply Seamless reaction. Generally, 0.1 – 1.0 ng of a vector is recommended as a template in the PCR reaction.

Assembly of Restriction Enzyme Digested Vector

Restriction enzyme-treated DNA fragments can have 5´ overhangs, 3´ overhangs or blunt ends. Care must be taken to consider the overhang sequences when calculating the 25 bp homology sequences required for assembly. In general, the cloning vector can be linearized by any restriction endonuclease or pair of different restriction endonucleases. By using a combination of two unique cut sites the background can be reduced significantly.

Non-homologous overlap sequences at this restriction site will be removed, resulting in a seamless assembly product. **Simply Seamless enzyme mix has the ability to remove both 3´ and 5´ end flap sequences upon fragment assembly.**

NOTE :

• Some restriction endonucleases cannot efficiently digest supercoiled DNA. This uncut vector DNA leads to high levels of background transformants. In this case, the digested vector should be gel purified before use in the assembly reaction.

• Some restriction endonucleases might have a reduced activity on plasmid DNA purified using various plasmid purification kits. This may be the result of contaminants remaining in the DNA prep. It is strongly advised to use DNA that has an OD 260/280 (>1.8) and 260/230 (>2.0) ratios > 1.8.

• When generating the linearized vector by restriction digest, we recommend that you digest the vector with two restriction enzymes to reduce the amount of background.

• Simply Seamless reactions are co mpatible with restriction enzymes that leave 3' protruding, 5' protruding, or blunt ends. It is very important to have a complete digest (very low background of uncut vector).

• High levels of salts (particularly sodium chloride) in the Simply Seamless reaction can inhibit the assembly and reduce efficiency.

• The presence of high concentrations of NaCl in the Assembly reaction will significantly reduce efficiency of the reaction.

Useful Recommendations for PCR

• The use of a high-fidelity DNA polymerase reduces error rates and the incorporation of mutations.

• Use a minimal amount of circular plasmid DNA as a template in the PCR reaction (e.g., 0.1–0.5 ng of plasmid template per 50 µl PCR reaction) to reduce the background on transformation plates.

• If using a PCR generated vector, it is recommended to digest the PCR product with DpnI restriction endonuclease in order to destroy the plasmid template before setting up the Simply Seamless Assembly reaction (reduces circular template carryover). DpnI cleaves only *E. coli* Dam methylase-methylated plasmid DNA, but does not cleave the PCR product, since it is not methylated.

• Verify PCR product purity and yield by gel electrophoresis. Non-specific DNA fragments will reduce the efficiency of the assembly reaction. If multiple bands are observed, you will need to purify the target fragment from the agarose gel to ensure the correct product assembly is produced during the Simply Seamless assembly reaction.

• PCR product purification is not necessary as long as the product is > 90% pure. You can add unpurified PCR product directly from the PCR reaction into the assembly reaction, for up to 20% of the total reaction volume. Larger volumes of unpurified PCR products could significantly inhibit both the assembly and the transformation.

• If the concentration of the PCR product is low, it is recommended to column purify PCR products and, if necessary, to concentrate DNA by ethanol precipitation or by use of a speed vac.

Simply Seamless DNA Assembly Reaction Protocol:

Suggested DNA quantities to use in a simple single-fragment cloning reaction:

	User Reaction	Positive Control
Linear Vector DNA	50–100 ng	-
Insert DNA	a 1:1 to 10:1 molar ratio of insert to vector	-
10X reaction buffer	1 µl	1 µl
Simply Seamless Enzyme Mix	1 µl	1 µl
H2O	Add to a total volume of 10 ul	-
Positive control DNA	-	8 µl

NOTES: 1) For multi-fragment cloning it is recommended to use equimolar ratio for vector and all inserts (1:1)
2)Total volume of unpurified PCR fragments in the assembly reaction should not exceed 20%

pmols = (weight in ng) x 1,000 / (base pairs x 650 daltons)

for example : 50 ng of 5000 bp dsDNA is about 0.015 pmols
50 ng of 500 bp dsDNA is about 0.15 pmols

The mass of each fragment can be measured using the NanoDrop instrument, absorbance at 260 nm or estimated from agarose gel electrophoresis followed by ethidium bromide staining.

Simply Seamless DNA Assembly Protocol

1. Set up the assembly reaction in a microtube on ice: (keep all components on ice and transfer stocks back to freezers promptly after use)

2. Incubate the samples in a thermocycler, water bath or a hot block at 37°C for 15-30 minutes. (incubation time may be extended to 60 minutes when more than 4 fragments are being assembled)

3. Transfer the completed reaction to ice and use immediately or store at -20°C for subsequent transformation.

3. Transform Competent *E. coli* cells DH5α (purchased separately) with 3 µl of the assembled product, following the transformation protocol.

25 ng of (1160 bp) 25 ng of (749 bp) The ends of the two fragments have 25 bp homologies to facilitate assembly. These two fragments are pre-mixed at the correct ratio so that 8 ul of the (+) control mixture is used. Transformation of 3 ul of the positive control reaction and plating 200 ul from the transformation recovery mixture onto LB plates containing 100 µg/ml ampicillin should yield more than 150 colonies.

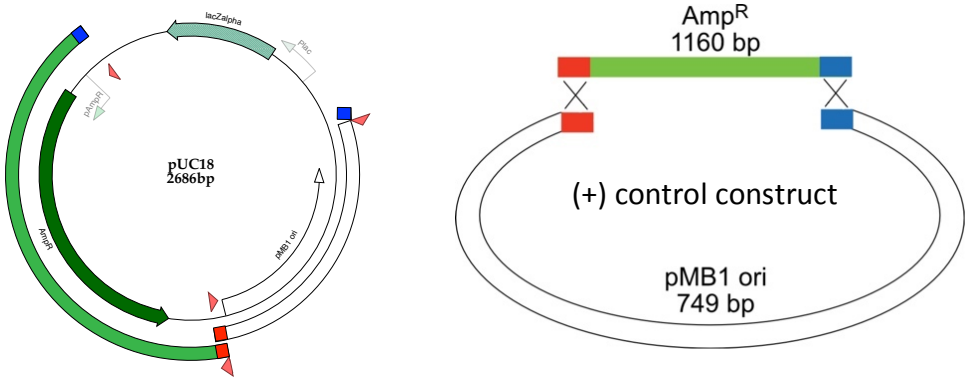

Figure 3) Diagram of the positive control DNA assembly. The two DNA regions were PCR amplified from pUC18 and combined in an equimolar ratio. There are 25 bp of homology on the ends of the ampicillin gene. Drawing not to scale.

Transformation Protocol:

DH5α chemical competent cells (Genotype: F- Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 phoA supE44 thi-1 gyrA96 relA1 λ-) can be purchased separately. The transformation efficiency of these cells is ≈ 10^6 / ug DNA.

1. Thaw chemically-competent cells on ice.
2. Add 3 µl of the chilled assembled product to 100 µl competent cells. Mix gently by pipetting up and down or by flicking the tube 4–5 times. Do not vortex.
3. Place the mixture on ice for 30 minutes. Do not mix.
4. Heat shock in a thermocycler, water bath or a hot block at 42°C for 60 seconds.
5. Transfer tubes to ice for 5 minutes.
6. Transfer cells to a 13ml tube and add 950 µl of room-temperature LB media.
7. Incubate shaking vigorously, 37°C for 1 hour.
8. Plate cells onto LB media containing the appropriate antibiotic. **Use LB with Amp plates for the positive control sample.**

If transforming by electroporation we recommend to dilute the reaction mixture 1:5 in nuclease-free water and that 1 µl of this diluted sample be used for electroporation.

Expected Results:

Following the protocols defined in this manual, hundreds of transformants should be expected for the assembly of 1-3 inserts. In our hands, assembly has worked for up to seven fragments with a reduced transformation efficiency.

Positive clones can be identified by either restriction digestion of isolated plasmids or by colony PCR. Typically, only 4-10 colonies need to be screened.

Troubleshooting:

Observation	Possible cause	Recommended action
Several transformants contain wrong insert.	Impure insert DNA used.	Check inserts on a gel. A single band should be seen. If multiple bands are present, gel purify the correct insert.
Several transformants contain no insert.	Cloning vector was not completely linearized.	It is essential to completely linearize the vector and remove uncut plasmid. Clean your digested vector with a column and repeat the restriction digest.
	If vector was a PCR product then either too much plasmid template was used in the PCR reaction or the DpnI digest was incomplete.	Clean the vector PCR that has been digested with DpnI with a column and repeat the DpnI restriction digest.
Positive control did not work.	Competent cells were handled incorrectly.	Do not freeze/thaw competent cells. Cells can only be thawed once.
		Store competent cells at -80°C.
	Not enough cells were plated	Increase quantity of cells plated from the recovery mix.
	Transformants were plated on plates that contained the wrong antibiotic	Use the appropriate antibiotic for selection
Positive control works but the assembly reaction has no inserts	DNA fragments do not share end-terminal homology	Re-check your DNA fragments to confirm that there are 25 bp of homologous sequence at the ends to be assembled.
	Insert PCR products were not pure.	Clean your PCR reaction using a column.
	DNA was damaged.	Minimize exposure to UV light when gel purifying DNA fragments.
	Incorrect ratio of insert to vector was used.	For best efficiency, use a 1:1 molar ratio
	Simply Seamless Enzyme Mix was handled incorrectly.	Do not leave the enzyme mix at room temperature. Return the mix to freezer promptly after use.