USER GUIDE

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GeneArt® Gene Synthesis Kit

A complete workflow solution for do-it-yourself gene synthesis using the CorrectASE™ Error Correction Technology

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Product Information

Kit Contents and Storage

Shipping/Storage

The GeneArt® Gene Synthesis Kit is shipped in separate boxes as described below, and contains sufficient reagents to perform 10 gene synthesis reactions. Upon receipt, store each box as detailed below. All reagents are guaranteed for six months, if stored properly.

Box	Component	Storage
1	GeneArt® Gene Synthesis Kit	–20°C
2	DNA Quantitation Module	4°C
3	One Shot® TOP10 Chemically Competent *E. coli*	–80°C

GeneArt® Gene Synthesis Kit

The table below lists the components of the GeneArt® Gene Synthesis Kit (Box 1). Store the individual components of Box 1 as described below. For convenience, you may also store the entire Box 1 at –20°C.

Item	Amount	Storage
Platinum® *Pfx* DNA Polymerase, 2.5 U/µL	12 µL	–20°C
10 mM dNTP Mix	50 µL	–20°C
5X Platinum® *Pfx* PCR Buffer	400 µL	–20°C
50 mM Magnesium Sulfate	1 mL	–20°C
CorrectASE™ Reagent	10 µL	–20°C
10X CorrectASE™ Reaction Buffer	500 µL	–20°C
5 mM EDTA	1.3 mL	4°C or RT*
pCR™-Blunt II-TOPO®	20 µL	–20°C
TOPO® Salt Solution	50 µL	4°C or RT
Sterile Water	1 mL	4°C or RT
Control Template – T800 (0.1 µg/µL)	10 µL	–20°C
CAT Oligo Set	50 µL	–20°C
CAT PCR Primers (10 mM each)	10 µL	–20°C
M13 forward sequencing primer (10 mM)	20 µL	–20°C
M13 reverse sequencing primer (10 mM)	20 µL	–20°C

* RT: Room temperature

Continued on next page

Kit Contents and Storage, continued

Primers

The GeneArt® Gene Synthesis Kit contains the following primers to sequence your synthetic gene or DNA fragment after it has been cloned into the pCR™-Blunt II-TOPO® vector.

Primer	Sequence
M13 forward	5′– CCC AGT CAC GAC GTT GTA AAA CG –3′
M13 reverse	5′–AGC GGA TAA CAA TTT CAC ACA GG–3′

DNA Quantitation Module

The table below lists the components of the DNA Quantitation Module (Box 2). Store the individual components of Box 2 as described below. For convenience, you may also store the entire Box 2 at 4°C, or at –20°C for long term storage.

Item	Amount	Storage
Quant-iT™ PicoGreen® dsDNA Reagent	100 µL	–20°C or 4°C, **in the dark**
20X TE	2 × 1 mL	4°C or RT*
Lambda DNA Standard (100 ng/µL)	100 µL	4°C

One Shot® TOP10 Reagents

The following reagents are included in the One Shot® TOP10 Chemically Competent *E. coli* kit (Box 3). Transformation efficiency is ≥1 × 10^9 cfu/µg plasmid DNA. Store the individual components of Box 3 as described below. For convenience, you may also store the entire Box 3 at –80°C.

Item	Amount	Storage
One Shot® TOP10 Chemically Competent *E. coli*	21 × 50 µL	–80°C
S.O.C. Medium	6 mL	4°C or RT
pUC19 Control DNA	50 µL	–20°C

Genotype of TOP10 Strain

F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*χ74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

Product Use

For Research Use Only. Not for use in diagnostic procedures.

Description of the System

GeneArt® Gene Synthesis Kit

The GeneArt® Gene Synthesis Kit is a complete do-it-yourself gene synthesis kit that includes all the reagents necessary to perform 10 gene synthesis reactions from oligonucleotide assembly to gene cloning. The kit relies on polymerase cycling assembly of synthetic oligonucleotides into a full-length gene or a DNA fragment (see below). The mismatch and frameshift mutations introduced into the assembled gene or DNA fragment as a result of the errors present in the starting synthetic oligonucleotides are removed in the CorrectASE™ error correction step before the final amplification and cloning (see **GeneArt® Gene Synthesis Workflow**, page 7).

The GeneArt® Gene Synthesis Kit provides the following advantages:

- Increases the success rate of do-it-yourself gene synthesis by providing standardized reagents and protocols for the complete gene synthesis workflow.
- Increases the probability of isolating a synthetic gene with the correct sequence 3–10 fold by including an error correction step in the workflow.
- Enables gene synthesis in 3 days, from oligonucleotide assembly to the isolation of the sequence verified clone.
- Reduces the labor time and sequencing costs by allowing the isolation of the clone with the correct sequence by screening of only 2–4 instead of 10–16 clones.
- Allows the custom addition of promoters, RBS, fusion or expression tags, and terminators.
- Enables the designing of genes that are codon optimized for your host and without the unwanted sequences such as duplicate restriction sites, unstable repeats, internal start sites, etc. for optimal expression.
- Allows the introduction of multiple desired point mutations and truncations in one step.

Components of the GeneArt® Gene Synthesis Kit

The GeneArt® Gene Synthesis Kit includes the following major components:

- Platinum® *Pfx* DNA Polymerase: a high-fidelity DNA polymerase with automatic hot-start for oligonucleotide assembly and amplification
- DNA Quantitation Module: an ultra sensitive fluorescent nucleic acid stain for measuring the concentration of double-stranded DNA (dsDNA)
- CorrectASE™ Enzyme: an error correction enzyme for preventing the unwanted mutations introduced during gene synthesis
- pCR™-Blunt II-TOPO® Vector: a TOPO®-adapted cloning vector for cloning the blunt-end synthetic genes generated by the Platinum® *Pfx* DNA Polymerase
- One Shot® TOP10 Chemically Competent *E. coli*: chemically competent *E. coli* cells that are ideally suited for transformation with the pCR™-Blunt II-TOPO® construct containing your synthetic gene

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Platinum® *Pfx* DNA Polymerase

Platinum® *Pfx* DNA Polymerase is a proprietary enzyme preparation containing recombinant DNA polymerase from *Thermococcus* species strain KOD (Nishioka *et al.*, 2001; Takagi *et al.*, 1997). Platinum® *Pfx* DNA Polymerase possesses proofreading 3′ to 5′ exonuclease activity and is a highly processive enzyme with fast chain extension capability (Cline *et al.*, 1996). Platinum® *Pfx* DNA Polymerase is provided in inactive form, due to specific binding of the Platinum® antibody. Polymerase activity is restored after a PCR denaturation step at 94°C, providing an automatic "hot start" for increased specificity, sensitivity, and yield (Sharkey *et al.*, 1994).

DNA Quantitation Module

The DNA Quantitation Module contains the Quant-iT™ PicoGreen® dsDNA reagent, which is an ultra sensitive fluorescent nucleic acid stain for quantitating double-stranded DNA (dsDNA) in solution. The reagent enables the quantitation of as little as 25 pg/mL of dsDNA (50 pg dsDNA in a 2 mL assay volume) with a standard spectrofluorometer and fluorescein excitation and emission wavelengths. In the GeneArt® Gene Synthesis workflow, the Quant-iT™ PicoGreen® dsDNA reagent is used for measuring the concentration of the DNA fragments assembled from oligonucleotides prior to the CorrectASE™ error correction reaction.

Note: Quant-iT™ PicoGreen® dsDNA reagents and kits are also available separately from Life Technologies. For ordering information, see page 36.

CorrectASE™ Enzyme

CorrectASE™ is an error correction enzyme that reduces the mutations caused by oligonucleotide errors during gene synthesis by 3–10-fold and substantially decreases the number of colonies that have to be screened to isolate the construct with the correct sequence.

GeneArt® Gene Synthesis (or any gene synthesis by a PCR-based method) starts with synthetic oligonucleotides that have an error rate of 1/300 to 1/1000 bp, depending on the source. During the initial PCR assembly, these errors are introduced into the assembled gene or DNA fragment. The subsequent denaturation and reannealing of the assembled DNA strands generate mismatches and/or frameshift mutations (insertions and deletions), which are then be removed by the 3′ to 5′ exonuclease activity of CorrectASE™ enzyme.

A final PCR with a proofreading polymerase then assembles the corrected fragments, thus increasing the likelihood of isolating clones with correct sequences. Depending on the incoming oligonucleotide quality, only 2–4 clones have to be screened compared to 10–16 clones in a workflow that does not include the CorrectASE™ correction step.

Note: CorrectASE™ enzyme is also available separately from Life Technologies. For ordering information, see page 36.

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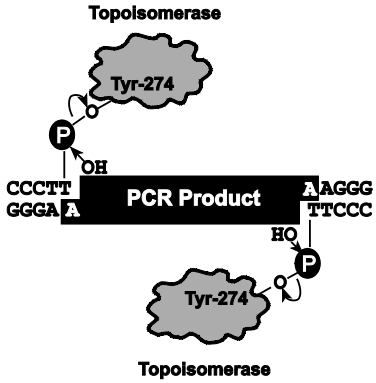
pCR™-Blunt II-TOPO® Vector

pCR™-Blunt II-TOPO® vector is designed to facilitate rapid TOPO® cloning of blunt-end PCR products (e.g., your synthetic gene or DNA fragments). In addition, pCR™-Blunt II-TOPO® vector allows direct selection of recombinants via disruption of the lethal *E. coli* gene, *ccd*B (Bernard *et al.*, 1994), fused to the C-terminus of the LacZα fragment. Ligation of the PCR product disrupts the expression of the *lac*Zα-*ccd*B gene fusion, permitting growth of only positive recombinants upon transformation. Cells that contain non-recombinant vector are killed upon plating. Therefore, blue/white screening is not required. For a map and additional features of the vector, see page 30.

- *ccdB* gene for positive selection
- *Eco*R I sites flanking the TOPO® cloning site for easy excision of your synthetic genes or DNA fragments
- Kanamycin and Zeocin™ resistance genes for your choice of selection in *E. coli*
- M13 forward and reverse primer sites for sequencing or PCR screening

How Blunt-End TOPO® cloning Works

Topoisomerase I from *Vaccinia* virus binds to duplex DNA at specific sites and cleaves the phosphodiester backbone after 5′-CCCTT in one strand (Shuman, 1991). The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3′ phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5′ hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase (Shuman, 1994).



The plasmid vector (pCR™-Blunt II-TOPO® vector) is supplied linearized with *Vaccinia* virus DNA topoisomerase I covalently bound to the 3´ end of each DNA strand (referred to as "TOPO®-activated" vector). The TOPO® cloning Reaction can be transformed into chemically competent cells (included in the kit) or electroporated directly into electrocompetent cells (available separately). Once the PCR product is cloned into the pCR™-Blunt II-TOPO® vector and the transformants are analyzed for correct orientation and reading frame, the expression plasmid may be transfected into your cell line of choice.

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Other Cloning Options

While the GeneArt® Gene Synthesis Kit contains the pCR™-Blunt II-TOPO® vector as a general cloning vector for your convenience, the system is independent of any specific cloning technology. When you design your gene, just add the necessary cloning sequences (homology, *att* sites for Gateway® cloning, or restriction enzyme recognition sites) to the 5′ and 3′ ends of the sequence before submitting for oligonucleotide design.

- GeneArt® Seamless Cloning and Assembly – Add 20 bases of homology between the vector and the ends of your designed sequence. GeneArt® Seamless Cloning and Assembly Kits (Cat. nos. A13288, A14603) also allow for the assembly of multiple fragments into larger genes or expression cassettes.
- Gateway® Cloning – Add the *att*B and *att*P sites to the ends of your designed sequence.
- Restriction Enzyme Cloning – Add the desired unique restriction enzyme recognition sites to your designed sequence. Make sure the sites are not currently in the sequence and are compatible with the reaction buffers.

After assembling your synthetic gene of interest, you may clone it into any plasmid vector, including Gateway® vectors, provided that the gene and the vector contain the appropriate sequence elements necessary for cloning. If you wish to express your synthetic gene in a particular expression system, ensure that your gene or the cloning vector contains the genetic elements required for expression specific to the host system.

Suggested Size for Synthetic Genes

Polymerase cycling assembly technology of the GeneArt® Gene Synthesis Kit is best suited for assembling synthetic oligonucleotides into a full-length gene of <1 kb in length. Although genes up to 1.2 kb can be assembled using this system, the efficiency of the assembly will be lower.

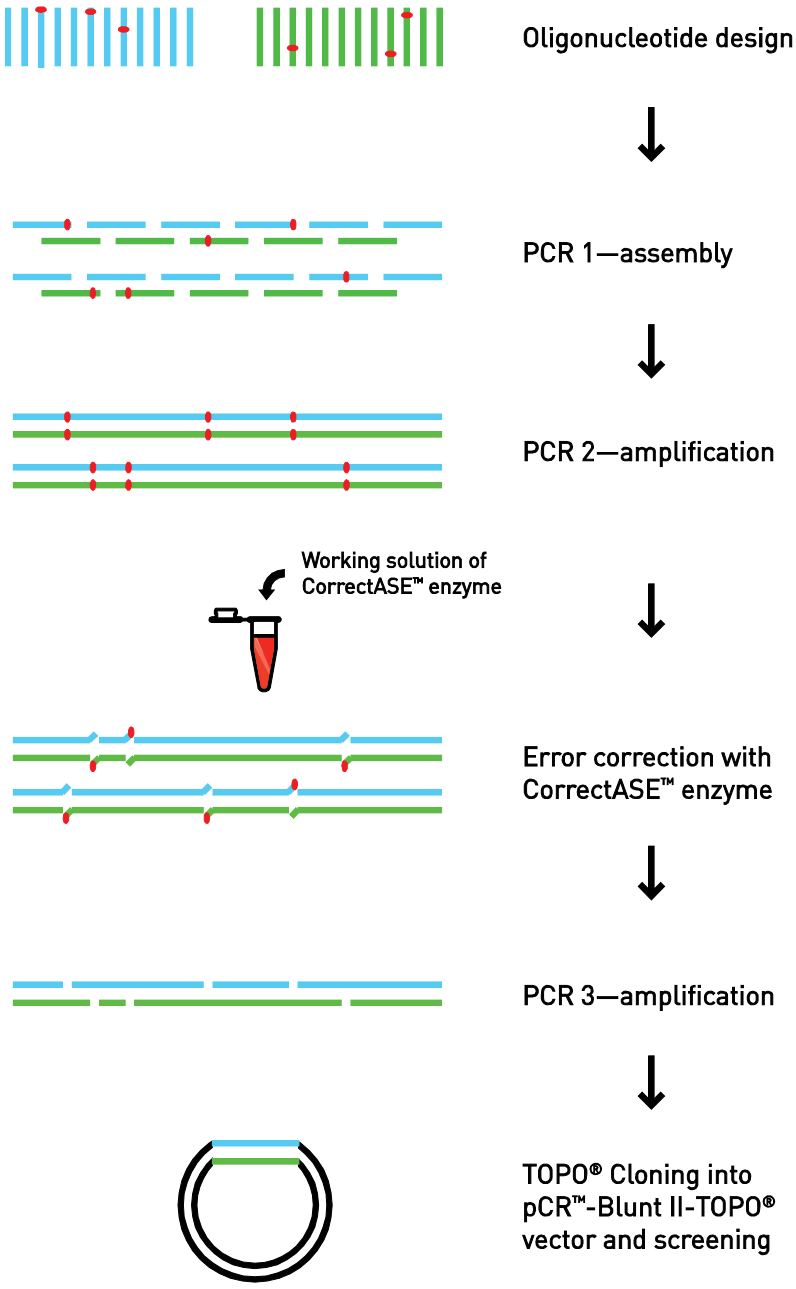
If you wish to generate a gene that is >1.2 kb in length, we recommend synthesizing your gene in several smaller fragments that are <1 kb each, and then assembling these fragments into a full-length gene using the GeneArt® Seamless Cloning and Assembly Kit (Cat. no. A13288) or the GeneArt® Seamless PLUS Cloning and Assembly Kit (Cat. no. A14603), which are available separately from Life Technologies (see page 36 for ordering information).

GeneArt® Gene Synthesis Workflow

Workflow

The figure below summarizes the comprehensive workflow for the GeneArt® Gene Synthesis Kit.

Note: The red dots denote errors in the DNA sequence.



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GeneArt® Gene Synthesis Workflow, continued

Experimental Outline

The table below describes the major steps required to assemble your synthetic gene using the GeneArt® Gene Synthesis Kit. Refer to the specified pages for details to perform each step.

Step	Action	Page
1	Develop your DNA assembly strategy and design your oligonucleotides	10
2	Perform the primary PCR assembly and amplification (i.e., PCR 1 and PCR 2)	13
3	Measure the concentration of your initial assembled synthetic gene or DNA fragment	15
4	Perform CorrectASE™ error correction reaction	17
5	Perform the final PCR amplification of your synthetic gene or DNA fragment (i.e., PCR 3)	18
6	*Optional*: Purify your synthetic gene or DNA fragment	19
7	TOPO® clone your synthetic gene or DNA fragment into pCR™-Blunt II-TOPO® vector	21
8	Transform into One Shot® TOP10 Chemically Competent *E. coli*	22
9	Pick 2–4 transformants and screen for the correct clone by sequencing	24

Methods

Designing Oligonucleotides

Introduction

GeneArt® Gene Synthesis relies on polymerase cycling assembly of synthetic oligonucleotides into a full length gene or a DNA fragment. The first step in the workflow is the design of the oligonucleotides that will be assembled into the synthetic gene or the DNA fragment. This section provides guidelines for the design and assembly strategy of the oligonucleotides.

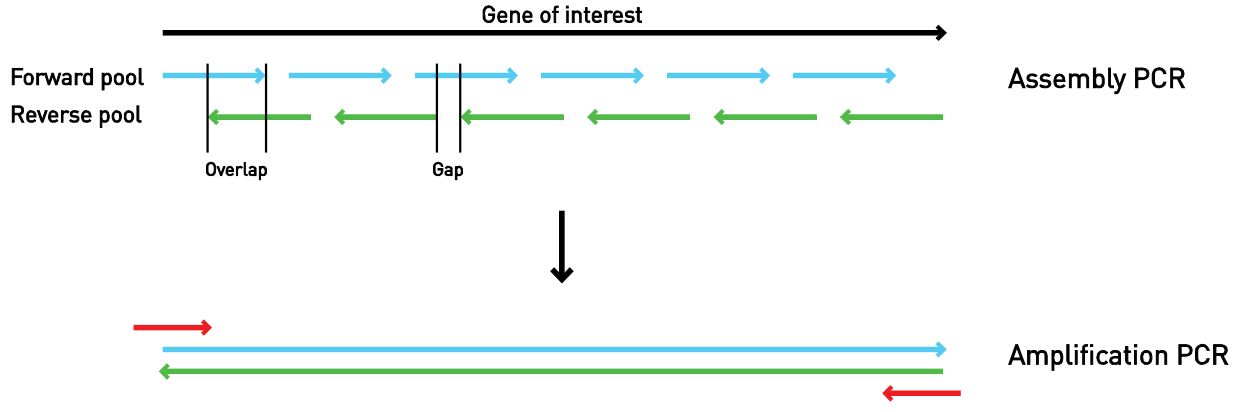
Overview of Polymerase Cycling Assembly

Synthetic genes can be generated from the polymerase cycling assembly of a pool of overlapping oligonucleotides, followed by PCR amplification of the full length gene using unique forward and reverse primers.

During "Assembly PCR", synthetic oligonucleotides from the forward pool are annealed to their complimentary oligonucleotides in the reverse pool and amplified to generate the full length gene or DNA fragment. During Assembly PCR, each oligonucleotide in the forward or reverse pool acts as a PCR primer for the corresponding oligonucleotide in the complementary pool.

Assembly PCR is followed with "Amplification PCR", during which the full length gene of DNA fragment is amplified using unique forward and reverse primers.

Note: The Assembly and Amplification PCR steps may be combined into a single Assembly/Amplification PCR step using the appropriate conditions.



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Designing Oligonucleotides, continued

Points to Consider When Designing Oligonucleotides

- Generally, oligonucleotides should be designed to be 35–60 bases long and share at least 18 bases of overlap with the corresponding complementary oligonucleotides (see image on page 10).
- Gaps are not required (see image on page 10), but they can reduce the cost of oligonucleotides if you are using longer oligonucleotides for assembly. For example, a 60-base oligonucleotide could have 20 bases of overlap, 20 bases of gap, and 20 bases of overlap.
- Codon optimization is important for increasing the expression levels of your synthetic gene. This is especially true for trans-genes, which may contain codons uncommon in your host organism.
- Ensure that the oligonucleotides used in the assembly PCR have similar annealing temperatures.
- When designing oligonucleotides, you must consider the potential for mispriming and the effect of repeat regions. Note that oligonucleotides that have a high GC content are more difficult to assemble.
- With longer genes, it becomes increasingly difficult to monitor all the variables for oligonucleotide design. To minimize the time required for designing your oligonucleotides and to identify potential pitfalls linked to your specific sequences, we recommend using design software such as the DNAWorks web-tool (see below) or equivalent.

DNAWorks Oligonucleotide Design Tool

The Helix Systems' DNAWorks web-tool, available free-of-charge by NIH at **http://helixweb.nih.gov/dnaworks**, automates the design of oligonucleotides for gene synthesis by PCR-based methods. The program requires simple input information, i.e., nucleotide sequence of the desired gene or the amino acid sequence of the target protein, and outputs a set of oligonucleotide sequences composing the gene of interest that have been optimized to match the codon bias of the chosen host for expression and highly homogeneous melting temperatures of all overlapping oligonucleotide sections (Hoover & Lubkowski, 2002).

For more information and guidelines on using the DNAWorks web-tool, see page 31.

Continued on next page

Purity and Concentration of Oligonucleotide Stocks

- For general gene synthesis applications, you can use standard purity oligonucleotides (i.e., desalted or cartridge-purified).
- Prepare oligonucleotide stocks, including the unique PCR primers for amplifying the assembled gene, at a final concentration of 100 µM in 1X TE buffer, pH 8. Store oligonucleotide stocks at –20°C.
- Before use, prepare a 0.15 µM oligonucleotide pool for gene assembly by combining and diluting the forward and reverse oligonucleotide stocks (but not the PCR primers) in DNase- and RNase-free water. These pools can be stored at –20°C. Generally, 5 µL of each 10 µM primer added together, brought to a total volume of 330 µL in TE buffer, and then vortexed to mix will result in a 0.15 µM pool.
- Dilute the forward and reverse PCR primers used for amplifying the assembled gene or DNA fragment to 10 µM in DNase- and RNase-free water.
- For custom DNA oligonucleotide synthesis options available from Life Technologies, visit **www.lifetechnologies.com/oligos** or contact Technical Support (see page 38).

Primary PCR Assembly and Amplification

Introduction

During the primary PCR assembly and amplification step, oligonucleotides in the pools are annealed to their complements with which they share an overlap, and act both as a primer and a template in the PCR to generate the full length gene.

Note: The protocols below provide instructions to perform the gene assembly and amplification steps separately in two PCRs. You may also adjust the reaction parameters to perform both steps in a single PCR, if desired.

Materials Needed

- Platinum® *Pfx* DNA Polymerase (2.5 U/µL)
- 5X Platinum® *Pfx* PCR Buffer
- 50 mM Magnesium Sulfate ($MgSO_4$)
- 10 mM dNTP Mix
- Forward and reverse oligonucleotides for gene assembly; combined and diluted to 0.15 µM (i.e., oligonucleotide pool, see page 12)
- Forward and reverse primers at 10 µM (for PCR amplifying the assembled gene)
- Sterile water
- Thermocycler

PCR 1 – Assembly

1. Set up the following assembly reaction in a 50 µL volume.

Oligonucleotide pool (0.15 µM)	10 µL
5X Platinum® *Pfx* PCR Buffer	10 µL
50 mM $MgSO_4$	1 µL
10 mM dNTPs	1.5 µL
Sterile Wate	to final volume of 49.6 µL
Platinum® *Pfx* DNA Polymerase (2.5 U/µL)	0.4 µL

2. Perform assembly using the cycling parameters below.

Temperature	Time	Cycles
94°C	30 seconds	1X
94°C	15 seconds (ramp rate 40%)*	15X
55°C	30 seconds	
68°C	60 seconds**	
68°C	2 minutes	1X
4°C	hold†	1X

* The 40% ramp rate between 94°C and 55°C can improve assembly but is not required.

** For genes >1 kb, increase the 68°C cycle to 90 seconds.

† Remove the product when ready to proceed to next step.

3. *Optional checkpoint*: At this point you can confirm the assembly by running 5 µL of the PCR product against a size marker on a 1% agarose gel. There should be an upward smear visible from where the unextended primers run up to the target size (see page 26).
4. Proceed to **PCR 2 – Amplification**, page 14.

Continued on next page

Primary PCR Assembly and Amplification, continued

PCR 2 – Amplification

1. Using the product of the assembly PCR (page 13), set up the following assembly reaction in a 50 µL volume.

Product from PCR 1 – Assembly (page 13)	5 µL
5X Platinum® *Pfx* PCR Buffer	10 µL
50 mM $MgSO_4$	1 µL
10 mM dNTPs	1.5 µL
Forward primer (10 µM)	1.25 µL
Reverse primer (10 µM)	1.25 µL
Sterile Water	to final volume of 49.6 µL
Platinum® *Pfx* DNA Polymerase (2.5 U/µL)	0.4 µL

2. Perform assembly using the cycling parameters below.

Temperature	Time	Cycles
94°C	30 seconds	1X
94°C	15 seconds (ramp rate 40%)*	30X
60°C	30 seconds	
72°C	60 seconds	
72°C	5 minutes	1X
4°C	hold**	1X

* The 40% ramp rate between 94°C and 60°C can improve assembly but is not required.

** Remove the product when ready to proceed to next step.

3. *Optional checkpoint:* At this point you can confirm the assembly by running 5 µL of the PCR product against a size marker on a 1% agarose gel. There should be a clear band at the correct size (see page 26).

4. Measure the concentration of the PCR product (i.e., the "initial assembly") using the DNA Quantitation Module (page 15) before proceeding to CorrectASE™ error correction.

Quantitating the Initial DNA Assembly

Introduction

The DNA Quantitation Module contains the ultra sensitive fluorescent nucleic acid stain Quant-iT™ PicoGreen® dsDNA reagent. Using this reagent to accurately measure the concentration of the initial assembly ensures that the amount of CorrectASE™ enzyme is optimized for the error correction reaction. Keep the Quant-iT™ PicoGreen® dsDNA reagent in the dark.

Materials Needed

- Product from the primary PCR assembly and amplification step ("initial assembly", page 14)
- Quant-iT™ PicoGreen® dsDNA reagent
- 20X TE
- Lambda DNA Standard
- Sterile, distilled, DNase-free water

Prepare Assay Buffer and Reagent

1. Prepare a 1X TE working solution by diluting the concentrated 20X TE buffer (included in the kit) 20-fold with sterile, distilled, DNase-free water.
   Note: Because the Quant-iT™ PicoGreen® dye is an extremely sensitive detection reagent for dsDNA, it is imperative that the TE solution used be free of contaminating nucleic acids.
2. On the day of the experiment, prepare an aqueous working solution of the Quant-iT™ PicoGreen® reagent by making a 200-fold dilution of the concentrated DMSO solution in 1X TE. Use the diluted reagent within a few hours of its preparation.
   Note: We recommend preparing this solution in a plastic container rather than glass, as the reagent may adsorb to glass surfaces. Protect the working solution from light by covering it with foil or placing it in the dark.

Prepare DNA Standard Curve

1. Prepare a 2 µg/mL working dsDNA solution by diluting the Lambda DNA Standard (included in the kit) 50-fold in 1X TE.
2. Create a four-point standard curve from 1 ng/mL to 100 ng/mL by diluting the 2 µg/mL working dsDNA solution into disposable cuvettes (or plastic test tubes for transfer to quartz cuvettes) as shown in the table (page 16). Mix well and incubate for 2–5 minutes at room temperature, protected from light.
3. After incubation, measure the sample fluorescence using standard fluorescein wavelengths (excitation ~485 nm, emission ~525 nm).
   Note: To ensure that the sample readings remain in the detection range of the fluorometer, the instrument's gain should be set so that the sample containing the highest DNA concentration yields fluorescence intensity near the fluorometer's maximum. To minimize photobleaching effects, keep the time for fluorescence measurement constant for all samples.
4. Subtract the fluorescence value of the reagent blank from that of each of the samples. Use corrected data to generate a standard curve of fluorescence versus DNA concentration

Continued on next page

Volume of 1X TE	Volume of 2 µg/mL DNA stock	Volume of diluted Quant-iT™ PicoGreen® reagent	Final DNA concentration in the assay
900 µL	100 µL	1000 µL	100 ng/mL
990 µL	10 µL	1000 µL	10 ng/mL
999 µL	1 µL	1000 µL	1 ng/mL
1000 µL	0 µL	1000 µL	blank

Quantitate the Sample

1. Dilute 5 µL of the product from the primary PCR assembly and amplification step ("initial assembly", page 14) in 995 µL of 1X TE in disposable cuvettes or test tubes.
2. Add 1.0 mL of the aqueous working solution of the Quant-iT™ PicoGreen® reagent to each sample. Incubate for 2–5 minutes at room temperature, protected from light.
3. After incubation, measure the sample fluorescence using standard fluorescein wavelengths (excitation ~485 nm, emission ~525 nm).

   Note: To minimize photobleaching effects, keep the time for fluorescence measurement constant for all samples.
4. Subtract the fluorescence value of the reagent blank from that of the sample and determine the DNA concentration of the sample from the standard curve generated.

We recommend using the Qubit® Fluorometer to measure the DNA concentration in your initial assembly reaction. The Qubit® Fluorometer provides a user-friendly, benchtop design for simple, fast, and highly accurate quantitation of DNA, in less than 5 seconds per sample (with sample incubation times of 2 minutes for DNA). For more information on the Qubit® Fluorometer, refer to our website at **www.lifetechnologies.com/qubit**.

CorrectASE™ Error Correction

Introduction

CorrectASE™ enzyme is used to remove the mismatches in the initial assembly, which mostly originate from synthesis errors present in the oligonucleotides that compose it. Ensure that the concentration of the initial assembly is accurately quantified (page 15) before proceeding with the CorrectASE™ error correction.

Materials Needed

- Initial DNA assembly, quantitated using the DNA Quantitation Module
- CorrectASE™ enzyme
- 10X CorrectASE™ Reaction Buffer
- Sterile, distilled, DNase-free water
- 5 mM EDTA
- Thermocycler

CorrectASE™ Error Correction Reaction

1. Prepare a 1X CorrectASE™ Reaction Buffer by diluting the concentrated 10X buffer 10-fold with sterile, distilled, DNase-free water.
2. In a PCR tube, dilute the initial DNA assembly to 20–25 ng/µL in 1X CorrectASE™ Reaction Buffer in a final volume of 50 µL (i.e., mix the appropriate amount of DNA with 5 µL of 10X CorrectASE™ Reaction Buffer and bring the volume up to 50 µL with deionized water).
3. Denature and re-anneal the diluted DNA to create mismatches using the following conditions:

Temperature	Time
98°C	2 minutes
4°C	5 minutes
37°C	5 minutes
4°C	hold

4. Place the re-annealed DNA dilution **on ice** and transfer 10 µL to a separate PCR tube.
5. To 10 µL of the re-annealed DNA dilution, add 1 µL of CorrectASE™ enzyme and mix thoroughly by pipetting up and down 10 times.
6. Incubate the reaction mix at 25°C for 1 hour. Do **not** overincubate.
7. After the incubation, immediately place the reaction mix **on ice** and add 1 µL of 5 mM EDTA to slow the CorrectASE™ reaction.
8. Proceed **immediately** to the final PCR amplification step (page 18).

IMPORTANT! It is crucial that the final PCR amplification step is commenced immediately after the CorrectASE™ error correction reaction. To minimize the time between the error correction reaction and the final PCR amplification, set up the components of the PCR (minus the primers and the error-corrected DNA) during error correction. The CorrectASE™ enzyme is not completely stopped until the denaturation step in the final PCR.

Final PCR Amplification

Introduction

After the errors in the initial assembly are removed by the 3′ to 5′ exonuclease activity of CorrectASE™ enzyme, the corrected fragments are assembled and amplified in a final PCR. It is crucial that you start this final PCR amplification immediately after error correction, because the CorrectASE™ enzyme is not completely stopped until the denaturation step in the final PCR.

Materials Needed

- Error-corrected DNA assembly (from Step 6, page 17)
- Platinum® *Pfx* DNA Polymerase (2.5 U/µL)
- 5X Platinum® *Pfx* PCR Buffer
- 50 mM Magnesium Sulfate ($MgSO_4$)
- 10 mM dNTP Mix
- Forward and reverse primers at 10 µM
- Sterile water
- Thermocycler

PCR 3 – Assembly

1. Set up the following assembly reaction in a 50 µL volume.

Error-corrected DNA	2 µL
5X Platinum® *Pfx* PCR Buffer	10 µL
50 mM $MgSO_4$	1 µL
10 mM dNTPs	1.5 µL
Forward primer (10 µM)	1.25 µL
Reverse primer (10 µM)	1.25 µL
Sterile Water	to final volume of 49.6 µL
Platinum® *Pfx* DNA Polymerase (2.5 U/µL)	0.4 µL

2. Perform assembly using the cycling parameters below.

Temperature	Time	Cycles
98°C	30 seconds	1X
98°C	10 seconds (ramp rate 40%)	25X
55°C	30 seconds	
72°C	60 seconds	
72°C	5 minutes	1X
4°C	hold*	1X

* Remove the product when ready to proceed to next step.

3. *Optional checkpoint:* At this point you can confirm the presence of the assembled gene by running 2 µL of the PCR product on a 1% agarose gel (page 26). There should be a clear band at the correct size. If there is a significant amount of primer-dimers or truncated PCR products, you can purify the PCR product (see page 19) before proceeding to the cloning step.
4. Proceed to TOPO® cloning reaction (page 20).

Optional: Purifying the Assembled Gene

Purifying the Assembled Gene

- TOPO® cloning into the pCR™-Blunt II-TOPO® vector can work with any double- stranded blunt DNA, but smaller products will be preferentially cloned over large products. Normally, the assembly and amplification PCR steps result in a single band of the correct, full-length product. In these cases, purification is not necessary, and you can proceed directly to TOPO® cloning.
- If you observe significant amounts of primer-dimers as indicated by the presence of a smear around the 100-bp size marker, we recommend purifying the PCR product using the PureLink® PCR Micro Kit (see page 37 for ordering information). The PureLink® PCR Micro Kit is designed for rapid and efficient purification of DNA from PCR products ranging in size from 125 bp–12.3 kb. This kit allows you to isolate and purify high concentrations of DNA from PCR products with low elution volumes (10 µL). Using the PureLink® PCR Micro Kit, >90% of dsDNA/primer dimmers less than 50 bp, as well as dNTPs, enzymes, and salts are removed from your PCR products in approximately 6 minutes.
- If you observe a significant amount of truncated products as indicated by the presence of a smear reaching up the expected size for the final assembled gene, we recommend gel purifying the full-length PCR product using the PureLink® Quick Gel Extraction Kit (see page 37 for ordering information).
- For an example of a diagnostic gel showing the products from various stages of the assembly workflow, see page 26.

TOPO® Cloning Reaction

Introduction

Once you have produced the desired PCR product, you are ready to TOPO® clone it into the pCR™-Blunt II-TOPO® vector and use this plasmid for transformation of competent *E. coli*. It is important to have everything you need to set up the reaction so that you can obtain the best results.

We have found that including salt (200 mM NaCl, 10 mM $MgCl_2$) in the TOPO® cloning reaction increases the number of transformants 2- to 3-fold. In addition, incubating the reaction mixture for greater than 5 minutes in the presence of salt can also increase the number of transformants. This is in contrast to experiments **without salt** where the number of transformants decreases as the incubation time increases beyond 5 minutes.

Including salt in the TOPO® cloning reaction allows for longer incubation times because it prevents topoisomerase I from rebinding and potentially nicking the DNA after ligating the PCR product and dissociating from the DNA. The result is more intact molecules leading to higher transformation efficiencies.

Using Salt in the TOPO® Cloning Reaction

You will perform TOPO® cloning in a reaction buffer containing salt (i.e., using the stock salt solution provided in the kit). Note that the amount of salt added to the TOPO® cloning reaction varies depending on whether you plan to transform chemically competent cells (provided) or electrocompetent cells (see page 37 for ordering information).

- If you are transforming chemically competent *E. coli* (included with the kit), use the stock Salt Solution as supplied, and set up the TOPO® cloning reaction as directed on the next page.
- If you are transforming electrocompetent *E. coli* (available separately from Life Technologies; see page 37), the amount of salt in the TOPO® cloning reaction must be reduced to 50 mM NaCl, 2.5 mM $MgCl_2$ to prevent arcing during electroporation. Dilute the stock Salt Solution 4-fold with water to prepare a 300 mM NaCl, 15 mM $MgCl_2$ Dilute Salt Solution. Use the Dilute Salt Solution to set up the TOPO® cloning reaction as directed on page 21.

Continued on next page

TOPO® Cloning Reaction, continued

Materials Needed

- Your PCR product (i.e., assembled gene)
- pCR™-Blunt II-TOPO® vector
- Salt Solution or Dilute Salt Solution (see previous page)
- Sterile water

Performing the TOPO® Cloning Reaction

The table below describes how to set up your TOPO® cloning reaction (6 µL) to use for transformation of either chemically competent or electrocompetent *E. coli*.

Note: The red color of the TOPO® vector solution is normal and is used to visualize the solution.

Reagent	Chemically Competent *E. coli*	Electrocompetent *E. coli*
PCR Product	0.5–4 µL	0.5–4 µL
Salt Solution	1 µL	—
Dilute Salt Solution	—	1 µL
Sterile Water	Add to total volume of 5 µL	Add to total volume of 5 µL
TOPO® Vector	1 µL	1 µL
Final Volume	**6 µL**	**6 µL**

*Store all reagents at –20°C when finished. Salt solution and water can be stored at room temperature or 4°C.

1. Mix reaction gently and incubate for 5 minutes at room temperature (22°C–23°C).

   Note: For most applications, 5 minutes will yield a sufficient number of colonies for analysis. The length of the TOPO® cloning reaction can be varied from 30 seconds to 30 minutes. For routine subcloning of PCR products, 30 seconds may be sufficient. For larger PCR products (>1 kb), increasing the reaction time may yield more colonies.

2. Place the reaction on ice and proceed to Transforming One Shot® Competent *E. coli*, next page.

   Note: You may store the TOPO® cloning reaction overnight at –20°C.

Transforming One Shot® Competent *E. coli*

Introduction

After you have performed the TOPO® cloning reaction, you are ready to use your construct gene to transform competent *E. coli*. One Shot® TOP10 Chemically Competent *E. coli* are included with the kit (Box 3) to facilitate transformation. You may also transform One Shot® Electrocompetent *E. coli* cells, if desired (see page 37 for ordering information).

Materials Needed

In addition to general microbiological supplies (i.e., plates, spreaders), you will need the following:

- TOPO® cloning reaction (from Step 2, previous page)
- One Shot® TOP10 *E. coli*, either chemically competent (supplied with the kit, Box 3)
- S.O.C. Medium (supplied with the kit, Box 3)
- pUC19 positive control (supplied with the kit, Box 3)
- 42°C water bath
- LB plates containing 50 µg/mL kanamycin or Low Salt LB plates containing 25 µg/mL Zeocin™ selective antibiotic (use two plates per transformation; see page 35 for recipes)
- 37°C shaking and non-shaking incubators

Preparing for Transformation

For each transformation, you will need one vial of One Shot® competent cells and two selective LB plates.

- Equilibrate a water bath to 42°C if using chemically competent *E. coli*
- Warm the vial of S.O.C. Medium to room temperature
- Warm selective LB plates at 37°C for 30 minutes
- Thaw one vial of One Shot® cells **on ice** for each transformation

Continued on next page

One Shot® Chemical Transformation Protocol

Use the following protocol to transform One Shot® TOP10 chemically competent *E. coli*.

1. Add 2 µL of the TOPO® cloning reaction into a vial of One Shot® Chemically Competent *E. coli* with a sterile pipette tip and mix gently. Do not mix by pipetting up and down.

   Note: If you are using the pUC19 control plasmid for transformation, use 1 µL (10 pg).
2. Incubate cells/plasmid mix on ice for 5–30 minutes.

   Note: Longer incubations on ice seem to have a minimal effect on transformation efficiency.
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice.
5. Add 250 µL of room temperature S.O.C. Medium.
6. Cap the tube tightly and shake the tube horizontally at 200 rpm in a 37°C shaking incubator for 1 hour.
7. Spread 10–50 µL from each transformation on a pre-warmed selective LB plate. To ensure even spreading of small volumes, you may add 20 µL of S.O.C. Medium to the transformation mixture. We recommend that you plate two different volumes to ensure that at least one plate contains well-spaced colonies. Incubate plates overnight at 37°C.

Analyzing Positive Clones

Introduction

After transformation of your pCR™-Blunt II-TOPO® construct containing your synthetic gene into *E. coli*, select and analyze 2–4 colonies by sequencing using the specific primers included in the kit to determine the orientation of the insert. For genes <600 bp, 2 colonies should be sufficient; for genes >600 bp, we recommend picking 4 colonies.

Rapid Screening by Colony PCR

For rapid screening of clones, you can use the M13 primers to amplify the gene directly from a colony. Set up 2–4 PCR mixes and a corresponding number of tubes with just LB medium containing the appropriate selective antibiotic.

Materials Needed

- Platinum® Blue PCR SuperMix (see page 37 for ordering information)
- M13 forward and reverse PCR primers (10 µM each), included in the kit
- LB medium containing 50 µg/mL Kanamycin or 25 µg/mL Zeocin™ selective antibiotic

Procedure

1. For each sample, aliquot 48 µL PCR SuperMix into a 0.5 mL microcentrifuge tube. Add 1 µL each of the forward and reverse PCR primer.
2. With a pipette tip, pick a colony and dip into the PCR mix 3 times, followed by dipping the tip into the corresponding media tube. Repeat for at least 3 additional colonies (for a total of 4 colonies or more).

   Note: Be sure to save the original colony by patching to a fresh plate, if needed.
4. Perform assembly using the cycling parameters below.

Temperature	Time	Cycles
94°C	5 minutes	1X
94°C	15 seconds	35X
55°C	30 seconds	
72°C	60 seconds/kb	
72°C	5 minutes	1X
4°C	hold*	1X

   * Remove the product when ready to proceed to next step.
3. Visualize the results by running 5 µL of the product against a size standard on an agarose gel to confirm the presence of the correct sized clone. Correct sized clones can be spin column purified and submitted for sequencing with the M13 forward and reverse primers.
4. Incubate the inoculated cultures corresponding to the clones being sequenced overnight at 37°C in LB medium containing 50 µg/mL Kanamycin or 25 µg/mL Zeocin™ selective antibiotic so that the clone can be isolated the next day using a miniprep kit (e.g., PureLink® Quick Plasmid Miniprep Kit). After sequence analysis, you only need to isolate the correct clone for downstream applications.

Continued on next page

Analyzing Positive Clones, continued

Analyzing Positive Clones by Restriction Digestion

1. Culture 2–4 colonies overnight in LB medium containing 50 µg/mL Kanamycin or 25 µg/mL Zeocin™ selective antibiotic. Be sure to save the original colony by patching to a fresh plate, if needed.
2. Isolate the plasmid DNA containing your synthetic gene using PureLink® Quick Plasmid Miniprep Kit, following the instructions provided in the kit (see page 37 for ordering information). Other kits for plasmid DNA purification are also suitable for use.
3. Analyze the plasmids by restriction analysis to confirm the presence and correct orientation of the insert (i.e., synthetic gene). Use a restriction enzyme or a combination of enzymes that cut once in the vector and once in the insert.

Sequencing

Sequence your construct to confirm that your synthetic gene is assembled correctly and cloned in the correct orientation. The M13 Forward (–20) and M13 Reverse primers are included to help you sequence your insert. Refer to the map on page 30 for the sequence surrounding the TOPO Cloning® site. For the full sequence of pCR™-Blunt II-TOPO® vector, refer to **www.lifetechnologies.com** or contact Technical Support (page 38).

Long-Term Storage

Once you have identified the correct clone, be sure to purify the colony and make a glycerol stock for long-term storage. We also recommend that you store a stock of plasmid DNA at –20°C.

1. Streak the original colony out on LB plates containing 50 µg/mL Kanamycin or 25 µg/mL Zeocin™ selective antibiotic.
2. Isolate a single colony and inoculate into 1–2 mL of LB containing 50 µg/mL Kanamycin or 25 µg/mL Zeocin™ selective antibiotic.
3. Grow with shaking to log phase (OD_{600} = ~0.5).
4. Mix 0.85 mL of culture with 0.15 mL of sterile glycerol and transfer to a cryovial.
5. Store at –80°C.

Checkpoints

Introduction

The design of the oligonucleotides and proper pooling are key factors for a successful assembly. If one of the oligonucleotides is omitted from the assembly reaction or it is at very low concentration compared to the rest, the assembly will not work. There are several check points in the protocol where you can run the reaction on an agarose gel to see the progress of your assembly workflow. Not all of these need to be performed; usually, running a gel after the second and third PCRs is sufficient.

Checkpoints

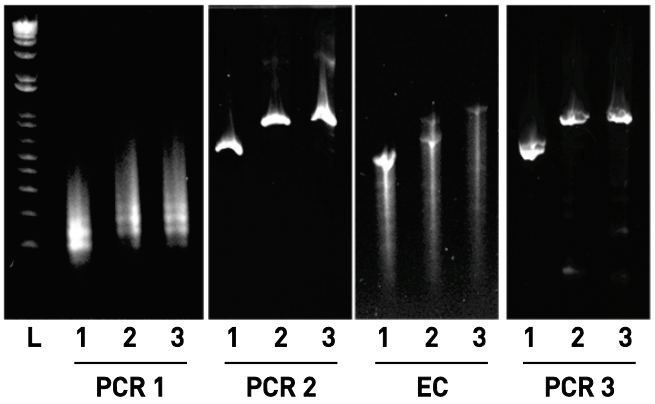
1. Run gel after first PCR; should see rising smear of DNA up to the size of the gene
2. Run gel after second PCR (recommended); should see specific full-length product
3. Measure the concentration of DNA after the second PCR (required); should get 10–200 ng/µL of dsDNA (usually ~50–100 ng/µL)
4. Run gel after error correction; should see a smear coming down from full-length product, where the band corresponding to the full-length product has disappeared or is very faint
5. Run gel after third PCR (recommended); should see same full length product as the second PCR

Example of a Diagnostic Agarose Gel

The images below show examples of the products from three separate assembly reactions obtained at various stages of the GeneArt® Gene Synthesis workflow.

When 10 µL of the product from the first PCR (i.e., assembly PCR) is run against a 1-kb ladder on a 1% agarose gel, a smear spanning from ~100 bp to ~700 bp is observed. When 2 µL of the product from the second PCR (i.e., amplification PCR) is run on the gel, clean full-length bands are observed running at the expected positions. 5 µL of CorrectASE™ reaction (EC) show degraded smears compared to the results from the second PCR. Finally, 2 µL of the product from the third PCR (i.e., final amplification PCR) show the clean full-length PCR product again.

L = 1kb ladder, **1** = 630-bp gene 1, **2** = 913-bp gene 2, **3** = 1020-bp gene 3.



Troubleshooting

Introduction

The table below lists some potential problems solutions that may help you troubleshoot your assembly, cloning, and the expression of your gene of interest.

Problem	Possible Cause	Solution
No PCR product after second PCR	PCR set up incorrectly	Ensure that the PCR mix contains $MgSO_4$, dNTPs, and the polymerase.
	Incorrect primers	Verify that correct primers are used.
	Primers have a low T_m	Lower the annealing temperature of PCR to match primer T_m.
	Oligonucleotide pool not complete	• Run 5 µL of the product from the first PCR; should see a smear. • If no smear is observed, then the oligonucleotides have not been designed correctly or not all of the oligonucleotides are present in the mix.
	Poor oligo design	• If GC content of the oligonucleotide is >70% or < 30%, or there are large areas of direct repeats, proper assembly may not occur. • If possible, change the GC content by modifying the wobble position. • To avoid repeats, fragment the sequence into smaller parts.
PCR product is of the wrong size or truncated products are observed	Poor oligo design	Recheck the oligonucleotides to see if they contain long repeats or have high GC content.
	Mispriming	Raise the annealing temperature or lower the amount of primer pool from 10 µL to 3 µL in first PCR.
No PCR product after third PCR	Failed second PCR	Confirm that second PCR gave correct product.
	Not enough DNA used	• Recheck DNA concentration of the second PCR and ensure that you are using 200 ng. • Repeat the error correction from the remaining denatured DNA tube, if necessary

Continued on next page

Problem	Possible Cause	Solution
No product after third PCR	Overdigestion with CorrectASE™ enzyme	• Make sure that the reaction was performed for no longer than 60 minutes. If the reaction mix is left at room temperature for longer than 60 minutes or left on ice for a long period before starting third PCR, the CorrectASE™ enzyme will continue to degrade template. • Run 5 µL of remaining error correction assay on a gel. The DNA should be degraded compared to second PCR, with a smear going from the full-length to ~200bp. • If no smear is observed, then the CorrectASE™ enzyme has lost its activity or it was omitted from the reaction. • If the smear is all below 200 bp marker, then too much CorrectASE™ enzyme was added to the reaction and the incubation was performed for too long.
No detectable error correction	Underdigestion with the CorrectASE™ enzyme	• If the reaction was incubated for significantly less than 60 minutes, then the reaction may not be complete. • Repeat the error correction from the remaining denatured DNA tube, if necessary.
	Too much DNA used	• Recheck the DNA concentration of second PCR to ensure that you are using 200 ng of the product. • Repeat the error correction from the remaining denatured DNA tube, if necessary.
	Enzyme not stored properly	CorrectASE™ enzyme should be kept on ice when in use and stored at –20°C. If left out, the enzyme will lose its activity.

Continued on next page

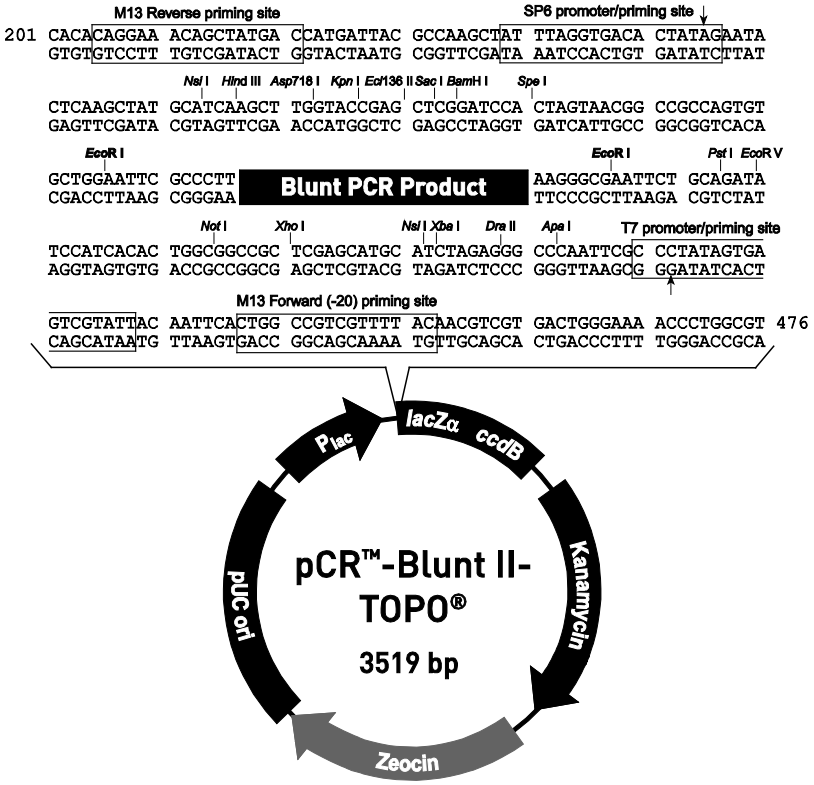
Problem	Possible Cause	Solution
Few or no colonies obtained from sample reaction, but transformation control yielded colonies	Incomplete extension during PCR	Include a final extension step of 7–30 minutes during PCR. Longer PCR products will need a longer extension time.
	Excess or dilute PCR product used in the TOPO® Cloning reaction	Reduce or concentrate the amount of PCR product.
	PCR primers contain 5′ phosphates	Do not add 5′ phosphates to your PCR primers.
	Large PCR product	• Increase the amount of PCR product used in the TOPO® cloning reaction. • Increase the incubation time of TOPO® cloning reaction from 5 minutes to 30 minutes. • Gel-purify the PCR product to remove primer-dimers or other artifacts.
	PCR reaction contains artifacts (i.e., not a single band on an agarose gel)	• Optimize your PCR conditions. • Gel-purify your PCR product.
Large number of incorrect inserts cloned	PCR cloning artifacts	• Gel-purify your PCR product to remove primer-dimers and other artifacts. • Optimize your PCR conditions.
Few or no colonies obtained from sample reaction and the transformation control gave no colonies	One Shot® competent *E. coli* stored incorrectly	• Store One Shot® competent *E. coli* at –80°C. • If you are using another *E. coli* strain, follow the manufacturer's instructions.
	Insufficient amount of *E. coli* plated	Increase the amount of *E. coli* plated.
	Transformants plated on selective plates with the wrong antibiotic	Use LB + ampicillin plates for selection.

Appendix A: Vectors

pCR™-Blunt II-TOPO® Vector

Map and Features of pCR™-Blunt II-TOPO® Vector

The map below shows the features of pCR™-Blunt II-TOPO® vector and the sequence surrounding the TOPO® cloning site. Restriction sites are labeled to indicate the actual cleavage site. The arrows indicate the start of transcription for the T7 and SP6 polymerases. The complete sequence of pCR™-Blunt II-TOPO® vector is available for downloading from **www.lifetechnologies.com** or by contacting Technical Support (page 38).



Features of the pCR™-Blunt II-TOPO®
3519 nucleotides

lac promoter/operator region:	95–216
M13 reverse priming site:	205–221
LacZα ORF:	217–576
SP6 promoter priming site:	239–256
Multiple cloning site:	269–399
TOPO® cloning site:	336-337
T7 promoter priming site:	406–425
M13 (–20) forward priming site:	433–448
Fusion joint:	577–585
ccdB lethal gene:	586–888
kan promoter:	1099–1236
Kanamycin resistance ORF:	1237–2031
Zeocin resistance ORF:	2238–2612
pUC origin:	2724–3397

Appendix B: Tools for Oligonucleotide Design

DNAWorks

Introduction

The Helix Systems' DNAWorks is a free, web-based design tool that automates the design of oligonucleotides for gene synthesis by PCR-based methods. After the appropriate information regarding the gene of interest is input (e.g., nucleotide sequence of the desired gene or the amino acid sequence of the target protein), the program outputs a set of codon-optimized oligonucleotide sequences with highly homogeneous melting temperatures that compose the gene of interest (Hoover & Lubkowski, 2002). The design tool is available free-of-charge by NIH at **http://helixweb.nih.gov/dnaworks**.

Using the DNAWorks Oligonucleotide Design Tool

1. Click **http://helixweb.nih.gov/dnaworks** to access the DNAWorks oligonucleotide design tool at Helix Systems on NIH servers.
2. Enter the following field on the user interface:
   - **Job name**: Enter a name for your design job. Acceptable characters are A–Z, a–z, 0–9, and _ (i.e., underscore).
   - **e-mail address** (*optional*): The design tool provides the results online results, but they will be deleted after two days. Enter your e-mail address if you want the results sent to you.
   - **Mutant run**: Check **Mutant Run** box if you want the parameters from the original logfile to override those that are output by the program.
   - **Codon Frequency Table**: Choose the organism for which you want your oligonucleotide sequences to be codon-optimized (i.e., match the codon bias of the chosen host for expression). Alternatively, you can manually enter or upload the desired codon frequency table using the GCG Codon Frequency format.
   - **Parameters**: Enter the desired parameters for your oligonucleotides, such as the oligonucleotide length, annealing temperature, etc. The default parameters provided by the program are acceptable, but recommend the oligonucleotides to be up to 60 nucleotides long.
   - **Restriction Site Screen** (*optional*): Enter the restriction sites that you want to be excluded from the protein coding region of the synthetic gene.
   - **Custom Site Screen** (*optional*): Input your own site to be excluded from the protein coding region(s) of the synthetic gene.
   - **Sequence(s)**: Select **protein** if you want your oligonucleotide sequences to be optimized for the host organism's codon bias and unwanted restriction enzyme sites to be removed from the synthetic gene.

     Select **nucleotide** if you have a defined sequence and you do not want any sequence optimization.
3. Upload the desired sequence of the synthetic gene or enter it manually. We recommend a sequence length up to 1600 bp, preferably no larger than 1200 bp. You may split longer sequences into smaller fragments, if necessary.

Continued on next page

DNAWorks, continued

Output from the DNAWorks Oligonucleotide Design Tool

- Once submitted, the DNAWorks server processes the sequence using the parameters you have entered and returns the results in a logfile. For standard sequences, it usually takes 1–10 minutes to get the results.
- The logfile contains all the necessary information regarding your oligonucleotides and assembly strategy, including the starting sequence, codon optimization strategy, oligonucleotide overlap design, any potential misprimes or repeats.
- The Final Summary provides a score for the designed oligonucleotides. The lower the score, the better; a score of 0 is best. Generally, a score below 50 is sufficient for most gene synthesis applications, but some attempts at optimization are recommended. Changing the oligonucleotide length and rerunning the program can help lower the score.
- Occasionally a sequence will result in a high score due to high or low GC content or long repeat regions. These issues can be addressed by fragmenting the sequence into smaller parts. For difficult sequences, we recommend that you consider the GeneArt® Custom Synthesis Service for generating your synthetic gene-of-interest.
- The logfile also provides a list of primers that are required for your gene synthesis reaction. These can be ordered through Life Technologies. For custom DNA oligonucleotide synthesis options available from Life Technologies, visit **www.lifetechnologies.com/oligos** or contact Technical Support (see page 38).
- Depending on your cloning strategy regarding the synthetic the gene, you may want to design specific forward and reverse oligonucleotides to include unique restriction sites or homology for seamless cloning.

Appendix C: Support Protocols

GeneArt® Gene Synthesis Controls

Introduction

We recommend performing the GeneArt® gene synthesis control reactions using the CAT Oligo Set and CAT PCR Primers (included in the kit) to help you evaluate your gene assembly and CorrectASE™ error correction results. This control is especially useful for confirming that a failed assembly reaction is caused by poor oligonucleotide design, because this set of control oligonucleotides and PCR primers have been verified to correctly generate the 810-bp chloramphenicol acetyltransferase (CAT) gene.

Performing this set of controls involves assembling and amplifying the CAT gene with and without CorrectASE™ error correction. The assembly reaction is then evaluated by confirming the presence of the full-length CAT gene by gel electrophoresis or sequencing, and the efficiency of error correction is ascertained by comparing the error rates of the genes synthesized with and without CorrectASE™ error correction.

GeneArt® Gene Synthesis Control Reactions

1. Perform the primary PCR assembly and amplification steps as detailed on pages 13 and 14, but substituting CAT Oligo Set and CAT PCR Primers for the oligonucleotide pool and forward and reverse amplification primers, respectively.
2. Confirm proper assembly and amplification after the second PCR by running 5 µL of the product against a 1-kb marker on a 1% agarose gel. The CAT control should give a single band product at 810 bp.
3. Measure the concentration of the PCR product (i.e., the "initial assembly") using the DNA Quantitation Module as detailed on page 15.
4. Prepare a 1X CorrectASE™ Reaction Buffer by diluting the concentrated 10X buffer 10-fold with sterile, distilled, DNase-free water.
5. In a PCR tube, dilute the initial DNA assembly to 20–25 ng/µL in 1X CorrectASE™ Reaction Buffer in a final volume of 50 µL (i.e., mix the appropriate amount of DNA with 5 µL of 10X CorrectASE™ Reaction Buffer and bring the volume up to 50 µL with deionized water).
6. Denature and re-anneal the diluted DNA to create mismatches using the same denaturing and re-annealing conditions as on page 17.
7. Place the re-annealed DNA dilution **on ice** and transfer to two separate PCR tubes (labeled "+" and "–") 10 µL of the re-annealed DNA dilution each.
8. To the sample labeled "+", add 1 µL of CorrectASE™ enzyme. To the sample labeled "–", add 1 µL of sterile, deionized water. Incubate both samples at 25°C for 1 hour. Do **not** overincubate.
9. After the incubation, immediately place the samples **on ice** and add to each tube 1 µL of 50 mM EDTA.
10. Proceed **immediately** to the final PCR amplification as detailed on page 18, but using the CAT PCR Primers.

Continued on next page

GeneArt® Gene Synthesis Control Reactions, continued

11. Purify the PCR products using the PureLink® PCR Micro Kit, and then TOPO® clone the assembled control genes into the pCR™-Blunt II-TOPO® vector as detailed on page 21.
12. Place the TOPO® cloning reactions on ice and use 2 µL of each TOPO® cloning reaction mix to transform two separate vials of One Shot® competent cells using the procedure on page 22.
13. Spread 10–50 µL from each transformation mix onto two LB plates containing 50 µg/mL kanamycin. Be sure to plate 2 different volumes to ensure that at least 1 plate has well-spaced colonies. For plating small volumes, add 20 µL of S.O.C. medium to allow even spreading.
14. Incubate the plates overnight at 37°C.
15. For a quick screening of errors correction, randomly pick 10–20 error-corrected colonies and an equal number of colonies without error correction from the LB + kanamycin plates, and restreak them on separate LB + chloramphenicol plates. Incubate the plates overnight at 37°C.
16. Compare the number of streaks that have grown on chloramphenicol with or without CorrectASE™ treatment to evaluate the efficiency of error correction.
17. Alternatively, the CAT gene can be isolated from colonies with or without CorrectASE™ error correction and their sequences compared to the correct sequence.

What You Should See

pCR™-Blunt II-TOPO® constructs with a functional CAT gene in the correct orientation will have a higher percentage of colonies growing on plates with chloramphenicol (50% without error correction vs. 90% with error correction).

Sequencing

Sequence your construct to confirm that your synthetic gene is assembled correctly and cloned in the correct orientation. The M13 Forward (–20) and M13 Reverse primers are included to help you sequence your insert. Refer to the map on page 30 for the sequence surrounding the TOPO Cloning® site. For the full sequence of pCR™-Blunt II-TOPO®, refer to **www.lifetechnologies.com** or contact Technical Support (page 38). The sequence of the CAT gene is given below.

Sequence of the CAT gene (810-bp)

```
GGGCGTATTTTTTGAGTTATCGAGATTTTCAGGAGCTAAGGAAGCTAAAATGGAAAAAAAG
ATCACCGGCTACACGACCGTTGACATCTCTCAGTGGCATCGTAAAGAGCACTTTGAAGCGT
TCCAGTCCGTTGCGCAGTGCACCTACAACCAAACTGTTCAGCTCGACATCACCGCATTCCT
CAAGACTGTCAAGAAAAACAAGCACAAATTCTACCCGGCCTTCATCCACATCCTGGCGCGT
CTCATGAACGCCCATCCGGAATTTCGTATGGCGATGAAAGACGGCGAACTGGTTATCTGGG
ACTCTGTTCACCCGTGCTACACCGTTTTTCACGAACAGACCGAAACCTTCTCTAGCCTGTG
GAGCGAATACCACGACGACTTCCGTCAGTTCCTGCACATCTACTCTCAGGACGTTGCGTGT
TACGGTGAGAATCTCGCGTATTTCCCAAAAGGCTTCATCGAAAATATGTTCTTCGTATCTG
CGAACCCTTGGGTATCCTTCACTAGCTTCGACCTGAATGTAGCGAACATGGACAACTTTTT
CGCACCGGTCTTCACCATGGGTAAATACTACACCCAGGGTGACAAAGTTCTGATGCCGCTG
GCGATCCAGGTTCACCACGCGGTTTGCGATGGTTTCCACGTTGGTCGTATGCTGAATGAAC
TGCAACAGTATTGCGACGAGTGGCAGGGTGGTGCGTAGTAAGAATTCTTGG
```

Appendix D: Recipes

Media and Solutions

LB (Luria-Bertani) Medium and Plates

Do not use Luria-Bertani Medium with Zeocin™ selective antibiotic. See **Low Salt LB medium** below or use **imMedia™** (see page 37 for ordering information).

Composition:

1.0% Tryptone

0.5% Yeast Extract

1.0% NaCl

pH 7.0

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 mL deionized water.
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave on liquid cycle for 20 minutes at 15 psi. Allow the solution to cool to 55°C and add antibiotic (50 µg/mL kanamycin or 30 µg/mL chloramphenicol), if needed.
4. Store 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 on liquid cycle for 20 minutes at 15 psi.
3. After autoclaving, cool to ~55°C, add antibiotic (50 µg/mL kanamycin or 30 µg/mL chloramphenicol), and pour into 10-cm plates.
4. Let the plates harden, then invert and store at 4°C in the dark.

Low Salt LB Medium

Reduce the salt in LB medium if you are using Zeocin™ selective antibiotic for selection.

Composition:

1.0% Tryptone
0.5% Yeast Extract
0.5% NaCl
pH 7.5

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and **5 g NaCl** in 950 mL deionized water. For plates, be sure to add 15 g/L agar.
2. Adjust the pH of the solution to 7.5 with NaOH and bring the volume up to 1 liter.
3. Autoclave on liquid cycle for 20 minutes at 15 psi. Allow solution to cool to 55°C and add Zeocin™ selective antibiotic to a final concentration of 25 µg/mL.
4. Store at room temperature or at 4°C.

Appendix E: Ordering Information

GeneArt® Products

GeneArt® Gene Synthesis Kit Products

Some of the components of the GeneArt® Gene Synthesis 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 38).

Product	Amount	Cat. no.
CorrectASE™ Reagent	50 reactions	A14972
	200 reactions	A14973
Quant-iT™ PicoGreen® dsDNA Assay Kit	1 kit	P7589
Platinum® *Pfx* DNA Polymerase	100 reactions	11708-013
	250 reactions	11708-021
	500 reactions	11708-039
One Shot® TOP10 Chemically Competent *E. coli*	10 reactions	C4040-10
	20 reactions	C4040-03

Other GeneArt® Products

Life Technologies also offers other GeneArt® products for gene synthesis and site directed mutagenesis. For more information, refer to **www.lifetechnologies.com** or contact Technical Support (see page 38).

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
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® High-Order Vector Conversion Cassette	10 reactions	A13291
CSM Media for MaV203 Yeast Cells	1 kit	A13292
GeneArt® Site-Directed Mutagenesis System	1 kit	A13282
GeneArt® Site-Directed Mutagenesis PLUS Kit	1 kit	A14551

Additional Products

Accessory Products

The following reagents are suitable for use with the GeneArt® Gene Synthesis Kit and are available separately from Life Technologies. Ordering information is provided below. For more information, refer to **www.lifetechnologies.com** or contact Technical Support (see page 38).

Item	Amount	Cat. no.
Platinum® *Taq* DNA Polymerase	100 reactions 250 reactions 500 reactions	10966-018 10966-026 10966-034
Platinum® *Taq* DNA Polymerase High Fidelity	100 units	11304-011
Platinum® Blue PCR SuperMix	100 reactions	12580-015
One Shot® TOP10 Chemically Competent *E. coli*	10 reactions 20 reactions	C4040-10 C4040-03
One Shot® TOP10 Electrocompetent *E. coli*	10 reactions 20 reactions	C4040-50 C4040-52
Zero Blunt® TOPO® PCR Cloning Kit with One Shot® TOP10 Chemically Competent *E. coli*	20 reactions 40 reactions	K2800-20 K2800-40
LB Media	500 mL	10855-021
Kanamycin	5 g 25 g	11815-024 11815-032
Kanamycin, liquid (10 mg/mL)	100 mL	15160-054
Zeocin™ Selection Reagent	8 × 1.25 mL 50 mL	R250-01 R250-05
Geneticin® Selective Antibiotic, liquid	20 mL	10131-035
S.O.C. Medium	10 × 10 mL	15544-034
imMedia™ Zeo Liquid	20 each	Q62020
PureLink® PCR Micro Kit	10 preps 50 preps 250 preps	K310010 K310050 K310250
PureLink® Quick Gel Extraction Kit	50 preps 250 preps	K2100-12 K2100-25
PureLink® Quick Plasmid Miniprep Kit	50 preps 250 preps	K2100-10 K2100-11

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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