USER GUIDE

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GeneArt® *Synechococcus* TOPO® Engineering Kits

For directional TOPO® cloning and expression of recombinant proteins in *Synechococcus elongatus*

Catalog Numbers A14261, A14265

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Contents

Product Information

Contents and Storage

Types of kits

This manual is supplied with the products listed below. For a list of components supplied with each catalog number, see below.

Product	Catalog number
GeneArt® *Synechococcus* TOPO® Engineering Kit	A14261
GeneArt® *Synechococcus* TOPO® Engineering Kit with 6 L media	A14265

Kit components

Each GeneArt® *Synechococcus* TOPO® Engineering Kit contains the components listed below. See pages 3–4 for a detailed description of each of the components.

Box	Component	Catalog number A14261	Catalog number A14265
1	GeneArt® *Synechococcus elongatus* Cells	✓	✓
2	GeneArt® *Synechococcus* TOPO® Vector Set	✓	✓
3	One Shot® TOP10 Chemically Competent *E. coli*	✓	✓
4	Gibco® BG-11 Media		✓

Shipping/Storage

The GeneArt® *Synechococcus* TOPO® Engineering Kits are shipped in separate boxes as described below. Upon receipt, store each box as detailed below. All reagents are guaranteed for six months if stored properly.

Box	Component	Shipping	Storage
1	GeneArt® *Synechococcus elongatus* Cells	Dry ice	–80°C
2	GeneArt® *Synechococcus* TOPO® Vector Set	Dry ice	–20°C
3	One Shot® TOP10 Chemically Competent *E. coli*	Dry ice	–80°C
4	Gibco® BG-11 Media	Gel ice	4°C

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GeneArt® *Synechococcus elongatus* Cells

Each GeneArt® *Synechococcus* TOPO® Engineering Kit is supplied with 10 vials of GeneArt® *Synechococcus elongatus* PCC 7942 cells, with each vial containing 100 µL of frozen cells. Store the cells at –80°C upon receipt. Avoid repeated freeze/thaw cycles and temperature fluctuations.

GeneArt® *Synechococcus* TOPO® Vector Set

The table below lists the components of the GeneArt® *Synechococcus* TOPO® Vector Set (Box 2). Store the contents of Box 2 at –20°C.

Component	Concentration	Amount
pSyn_1/D-TOPO® Vector	10 µL at 20 ng/µL in: 50% glycerol 50 mM Tris-HCl, pH 7.4 (at 25°C) 1 mM EDTA 2 mM DTT 0.1% Triton X-100 100 µg/mL BSA 30 µM bromophenol blue	10 µL
pSyn_2/Control Vector	0.5 µg/µL in TE buffer, pH 8.0*	20 µL
Salt Solution	1.2 M NaCl 0.06 M $MgCl_2$	50 µL
Sterile Water	—	1 mL
Forward Sequencing Primer pSyn_1 vector	100 ng/ µL in TE buffer, pH 8.0	20 µL
Reverse Sequencing Primer pSyn_1 vector	100 ng/ µL in TE buffer, pH 8.0	20 µL
Control PCR primers, Directional (for TOPO® reaction insert control)	100 ng/ µL **each** in TE buffer, pH 8.0	10 µL
dNTP Mix	12.5 mM each dATP, dCTP, dGTP, and dTTP; neutralized at pH 8.0 in water	10 µL

***TE buffer, pH 8.0:** 10 mM Tris–HCl, 1 mM EDTA, pH 8.0

Continued on next page

Primer sequences

The "Forward Sequencing Primer pSyn_1 vector" and the "Reverse Sequencing Primer pSyn_1 vector" primers are used for verifying the sequence of your gene of interest after the *E. coli* transformation step (page 16). The sequences of these primers are provided below.

Forward Sequencing Primer pSyn_1 vector: 5′-AGT CGG CAA ATA ACC CTC GG-3′

Reverse Sequencing Primer pSyn_1 vector: 5′-CGT TTT ATT TGA TGC CTG GC-3′

One Shot® TOP10 Chemically Competent *E. coli*

The table below describes the items included in the One Shot® TOP10 Chemically Competent *E. coli* kit (Box 3). Store the contents of Box 3 at –80°C.

The transformation efficiency of One Shot® TOP10 Chemically Competent *E. coli* is 1×10^9 cfu/µg DNA.

Component	Concentration	Amount
TOP10 Cells	—	11 × 50 µL
S.O.C. Medium (may be stored at room temperature or 4°C)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	7 mL
pUC19 Transformation Control DNA	10 pg/µL in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8.0	20 µL

Genotype of TOP10

Use this strain to clone the PCR product into the pSyn_1/D-TOPO® vector.

Genotype: F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*Z M15 Δ*lac*74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

Gibco® BG-11 Media

Gibco® BG-11 Media, included in the GeneArt® *Synechococcus* TOPO® Engineering Kit with 6 L media (Cat. no. A14265), is supplied in 6 × 1 L bottles and is optimized for the growth and maintenance of GeneArt® *Synechococcus elongatus* PCC 7942 cells. Store the Gibco® BG-11 Medium at 4°C.

Note: Gibco® BG-11 Media (Cat. nos. A1379901, A1379902) are also available separately from Life Technologies. See page 27 for ordering information.

Description of the System

Introduction

The GeneArt® *Synechococcus* TOPO® Engineering Kit is a prokaryotic photosynthetic model system based on the unicellular cyanobacterium *Synechococcus elongatus* PCC 7942 (**http://genome.kazusa.or.jp/cyanobase/SYNPCC7942**) and the TOPO® cloning technology, which offers a simplified approach for cloning and expressing genes of interest for the study of circadian rhythms, nutrient regulation, environmental response, lipid metabolism, and protein expression.

Synechococcus elongatus PCC 7942

Synechococcus elongatus PCC 7942 is a freshwater unicellular cyanobacterium. Cyanobacteria, sometimes referred to as blue-green algae, are prokaryotes that are able to obtain their energy through photosynthesis. *Synechococcus elongatus* has a rod-shaped appearance and is oligotrophic, having the ability to survive in freshwater environments with low nutrients. This organism has a circular chromosome of ~2.7 Mb (fully sequenced) with a GC content of 55.5%, which contains the genes for 2,612 proteins and 53 RNAs (Vijayan *et al.*, 2011).

The cyanobacterium *Synechococcus elongatus* PCC 7942 is an excellent synthetic biology chassis and a model system for studying prokaryotic circadian rhythms, nutrient regulation, environmental responses, and lipid metabolism because of its small genome size and the ease with which it can be genetically manipulated by natural transformation or conjugation from *E. coli* (Atsumi *et al.*, 2009; Ducat *et al.*, 2011; Lan & Liao, 2011; Min & Golden, 2000; Simkovsky *et al.*, 2012; Taniguchi *et al.*, 2012).

Transformation of *Synechococcus elongatus* PCC 7942

The transformation of *Synechococcus elongatus* PCC 7942 relies on homologous recombination between the cell's chromosome and exogenous DNA that is not autonomously replicating and containing sequences homologous to the chromosome. The location of integration into the chromosome (neutral site, NS1) has been developed as a cloning locus (Clerico *et al.*, 2007) as it can be disrupted without any aberrant phenotype, thus allowing the homologous recombination of ectopic sequences. When transformed with vectors containing an antibiotic resistance cassette and neutral site sequences, a double homologous recombination event occurs between the neutral site vector and the *Synechococcus elongatus* chromosome. The selective marker (spectinomycin) and the gene of interest driven by a promoter are inserted into the neutral site and the vector backbone (pUC) is lost, allowing the expression of recombinant genes in *Synechococcus elongatus* PCC 7942.

Continued on next page

pSyn_1/D-TOPO® Vector

The pSyn_1/D-TOPO® Vector is designed to facilitate rapid, directional TOPO® cloning of blunt-end PCR products for expression in *Synechococcus elongatus* PCC 7942. Some of the features of the vector are listed below. For a map of the vector, see page 24.

- Directional TOPO® Cloning site for rapid and efficient directional cloning of a blunt-end PCR product
- NS1 (neutral site 1) homologous recombination sites for the integration of the vector into the *Synechococcus elongatus* genome
- The weak constitutive promoter of solanesyl diphosphate synthase gene from *Synechocystis* sp. strain PCC 6803 driving the basal expression of your gene of interest

  Note: Use of this weak constitutive promoter is a good fit for applications that are hindered by strong expression, such as pathway engineering or complementation of mutant genes normally expressed at low levels (Kaneko *et al.*, 1996; Simkovsky *et al.*, 2012).
- Spectinomycin resistance gene for selection in *E. coli* and *Synechococcus elongatus* PCC 7942
- pUC origin for maintenance in *E. coli*

How Topoisomerase I works

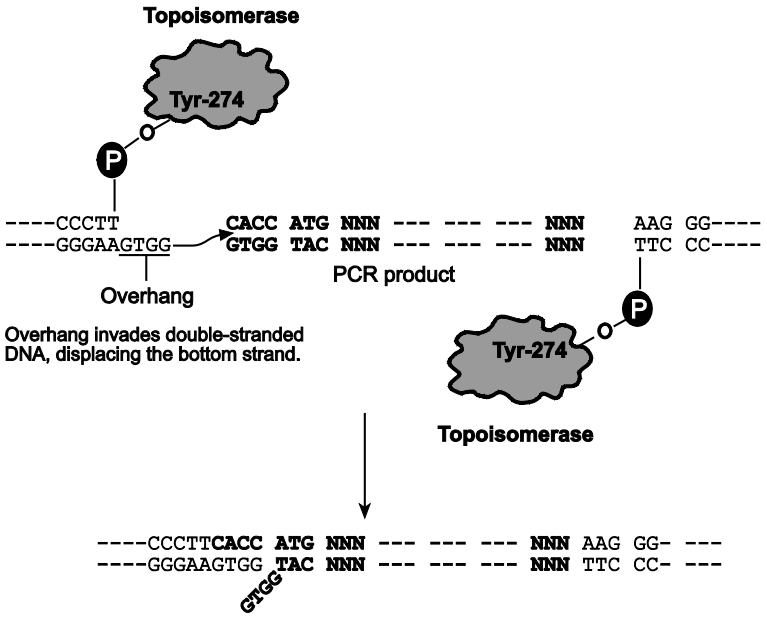
Topoisomerase I from *Vaccinia* virus binds to duplex DNA at specific sites and cleaves the phosphodiester backbone after 5′-CCCTT in one strand (Shuman, 1991). The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3′ phosphate of the cleaved strand 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). TOPO® Cloning exploits this reaction to efficiently clone PCR products.

Continued on next page

Directional TOPO® cloning

Directional joining of double-strand DNA using TOPO®-charged oligonucleotides occurs by adding a 3′ single-stranded end (overhang) to the incoming DNA (Cheng & Shuman, 2000). This single-stranded overhang is identical to the 5′ end of the TOPO®-charged DNA fragment. At Life Technologies, this idea has been modified by adding a 4 nucleotide overhang sequence to the TOPO®-charged DNA and adapting it to a 'whole vector' format.

In this system, PCR products are directionally cloned by adding four bases to the forward primer (CACC). The overhang in the cloning vector (GTGG) invades the 5′ end of the PCR product, anneals to the added bases, and stabilizes the PCR product in the correct orientation. Inserts can be cloned in the correct orientation with efficiencies equal to or greater than 90%.



Experiment Outline

Workflow

The table below describes the major steps needed to TOPO® clone and express your gene of interest in *Synechococcus elongatus* PCC 7942. For details, refer to the pages indicated.

Step	Action	Page
1	PCR amplify your gene of interest using the appropriate primers	9
2	TOPO® clone your gene of interest into pSyn_1/D-TOPO® Vector	12
3	Transform One Shot® TOP10 *E. coli* with pSyn_1/D-TOPO® vector containing your gene of interest and select the transformants on LB plates containing spectinomycin	14
4	Analyze *E. coli* transformants by restriction digestion or PCR	15
5	Thaw and resuscitate *Synechococcus elongatus* PCC 7942 cells	18
6	Transform *Synechococcus elongatus* PCC 7942 cells and select transformants	20
7	Screen *Synechococcus elongatus* PCC 7942 transformants by colony PCR for full integration of your gene of interest	22

PCR Amplifying the Gene of Interest

Design PCR primers

The pSyn_1/D-TOPO® Vector is a directional TOPO® cloning vector. Therefore, it is critical that the PCR primers to amplify your gene of interest contain the sequences required for directional cloning and expression. Consider the following when designing your PCR primers:

- To enable directional cloning, the forward PCR primer **must** contain the sequence CACC at the 5′ end of the primer (see **Example 1** below). The 4 nucleotides, CACC, base pair with the overhang sequence, GTGG, in the pSyn_1/D-TOPO® Vector.

  Note: The first three base pairs of the PCR product following the 5′ CACC overhang will constitute a functional codon.
- The ribosomal binding site GAAGGAG is optimally spaced for an ATG initiation codon, if placed immediately following CACC overhang.
- To ensure that your PCR product clones directionally with high efficiency, the reverse PCR primer **must not** be complementary to the overhang sequence GTGG at the 5′ end. A one base pair mismatch can reduce the directional cloning efficiency from 90% to 50%, increasing the likelihood of your ORF cloning in the opposite orientation (see **Example 2** on page 10). We have not observed evidence of PCR products cloning in the opposite orientation from a two base pair mismatch.
- Make sure to include the native sequence containing the stop codon in the reverse primer or ensure that the stop codon is upstream from the reverse PCR primer binding site.
- When synthesizing PCR primers, **do not** add 5′ phosphates to the primers, because 5′ phosphates prevent the synthesized PCR product from ligating into the pSyn_1/D-TOPO® Vector.
- We recommend that you gel-purify your oligonucleotides, especially if they are long (>30 nucleotides).

For a diagram of the TOPO® Cloning site of the pSyn_1/D-TOPO® Vector, refer to page 10. For more information on directional TOPO® cloning, refer to our website at **www.lifetechnologies.com**.

Example 1: forward primer design

Below is the DNA sequence of the N-terminus of a theoretical protein and the proposed sequence for your forward PCR primer:

DNA sequence: `5′-GTA GGA TCT GAT AAA`

Proposed Forward PCR primer: `5′-C ACC GTA GGA TCT GAT AAA`

Continued on next page

Example 2: reverse primer design

Below is the sequence of the C-terminus of a theoretical protein. You want to fuse the protein in frame with a C-terminal tag. The stop codon is underlined.

DNA sequence: **AAG TCG GAG CAC TCG ACG ACG GTG TAG-3′**

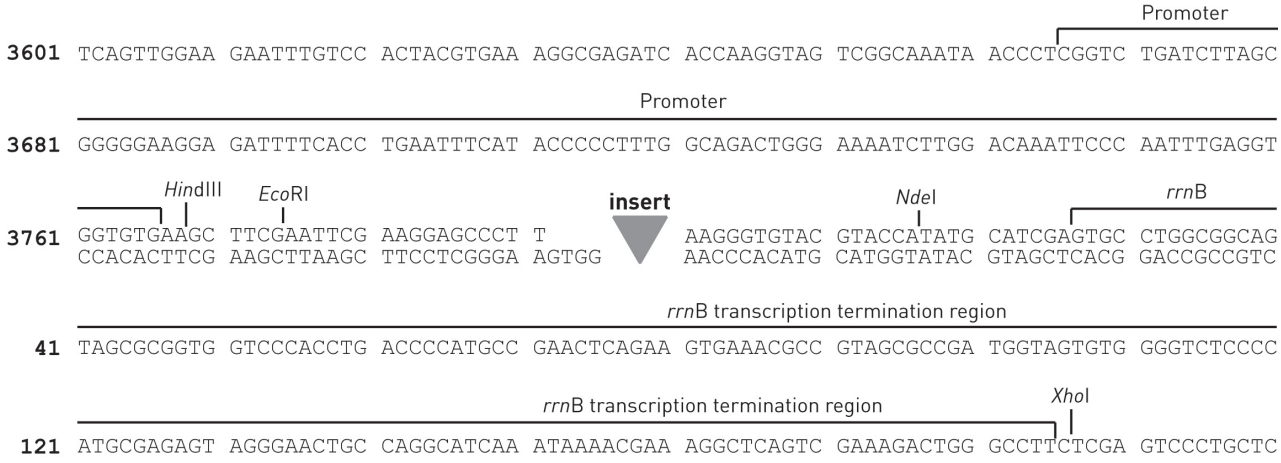
One solution is to design the reverse PCR primer to start with the codon just upstream of the stop codon, but the last two codons contain GTGG (underlined below), which is identical to the 4-bp overhang sequence. As a result, the reverse primer will be complementary to the 4-bp overhang sequence, increasing the probability that the PCR product will clone in the opposite orientation. You want to avoid this situation.

DNA sequence: **AAG TCG GAG CAC TCG ACG ACG GTG TAG-3′**
Proposed Reverse PCR primer sequence: **TG AGC TGC TGC CAC AAA-5′**

Another solution is to design the reverse primer so that it hybridizes just downstream of the stop codon, but still includes the C-terminus of the ORF. Note that you need to replace the stop codon with a codon for an innocuous amino acid such as glycine, alanine, or lysine.

TOPO® cloning site

Use the diagram below to design suitable PCR primers to clone and express your PCR product in the pSyn_1/D-TOPO® Vector. The sequence of pSyn_1/D-TOPO® Vector is available for downloading at **www.lifetechnologies.com** or by contacting Technical Support (page 29).



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Producing blunt-end PCR products

After you have decided on a PCR strategy and have synthesized the primers, produce your blunt-end PCR product using any thermostable, proofreading polymerase. We recommend Platinum® *Pfx* DNA Polymerase, AccuPrime™ *Pfx* DNA Polymerase, or *Pfx50*™ DNA Polymerase, available separately from Life Technologies (see page 27 for ordering information).

Follow the guidelines below to set up a 25 µL or 50 µL PCR for producing your blunt-end PCR product.

- Follow the instructions and recommendations provided by the manufacturer of your thermostable, proofreading polymerase to produce blunt-end PCR products.
- Use the cycling parameters suitable for your primers and template. Make sure to optimize PCR conditions to produce a single, discrete PCR product.
- Use a 7 to 30 minute final extension to ensure that all PCR products are completely extended.
- After cycling, place the tube on ice or store at –20ºC for up to 2 weeks. Proceed to **Checking the PCR product**, below.

Checking the PCR product

After you have produced your blunt-end PCR product, use agarose gel electrophoresis to verify the quality and quantity of your PCR product. Check for the following outcomes below.

- Be sure you have a single, discrete band of the correct size. If you do not have a single, discrete band, follow the manufacturer's recommendations to optimize your PCR with the polymerase of your choice. Alternatively, gel-purify the desired product.
- Estimate the concentration of your PCR product. Use this information when setting up your TOPO® cloning reaction (see **Amount of PCR product to use in the TOPO® cloning reaction**, page 12, for details).

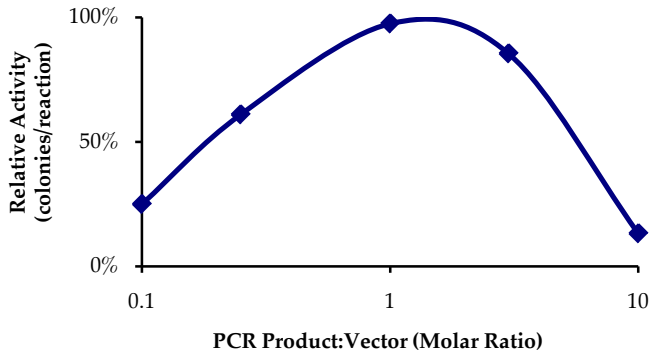
Performing the TOPO® Cloning Reaction

Introduction

Once you have produced the desired PCR product, you are ready to TOPO® clone it into the pSyn_1/D-TOPO® Vector and transform the recombinant vector into One Shot® TOP10 *E. coli*. It is important to have everything you need set up and ready to use to ensure best results. We suggest that you read this section and the section entitled **Transforming One Shot® TOP10 Competent *E. coli* Cells** (page 14) before beginning. If this is the first time you have TOPO® cloned, perform the control reactions in parallel with your samples.

Amount of PCR product to use in the TOPO® cloning reaction

When performing directional TOPO® cloning, we have found that the molar ratio of PCR product:TOPO® vector used in the reaction is critical to its success. **To obtain the highest TOPO® cloning efficiency, use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector** (see figure below). Note that the TOPO® cloning efficiency decreases significantly if the ratio of PCR product: TOPO® vector is <0.1:1 or >5:1. These results are generally obtained if too little PCR product is used (i.e., PCR product is too dilute) or if too much PCR product is used in the TOPO® cloning reaction. If you have quantitated the yield of your PCR product, you may need to adjust the concentration of your PCR product before proceeding to TOPO® cloning.



Using Salt Solution in the TOPO® cloning reaction

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 27 for ordering information).

- If you are transforming chemically competent *E. coli*, use the stock Salt Solution as supplied and set up the TOPO® cloning reaction as directed below.
- If you are transforming electrocompetent *E. coli*, **reduce** the amount of salt in the TOPO® cloning reaction 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 the page 13.

Continued on next page

TOPO® cloning reaction

Use the procedure below to perform the TOPO® cloning reaction. Set up the TOPO® cloning reaction depending on whether you plan to transform chemically competent *E. coli* or electrocompetent *E. coli.* **For optimal results, use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector in your TOPO® Cloning reaction.**

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

Reagents*	Chemically Competent *E. coli*	Electrocompetent *E. coli*
Fresh PCR product	0.5 to 4 µL	0.5 to 4 µL
Salt Solution	1 µL	—
Dilute Salt Solution (1:4)	—	1 µL
Sterile Water	add to a final volume of 5 µL	add to a final volume of 5 µL
TOPO® vector	1 µL	1 µL
Final volume	6 µL	6 µL

*Store all reagents at –20°C when finished. Store salt solutions and water at room temperature or 4°C.

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

   Note: For most applications, 5 minutes yields plenty of colonies for analysis. Depending on your needs, you can vary the length of the TOPO® cloning reaction from 30 seconds to 30 minutes. For routine subcloning of PCR products, 30 seconds may be sufficient. For large PCR products (> 1 kb) or if you are TOPO® cloning a pool of PCR products, increasing the reaction time may yield more colonies.

2. Place the reaction on ice and proceed to **Transforming One Shot® TOP10 Competent *E. coli* Cells**, page 14.

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

E. coli transformation method

You may use any method of your choice for transformation. Chemical transformation is the most convenient for most researchers. Electroporation is the most efficient and the method of choice for large plasmids. For your convenience, a protocol for chemical transformation using One Shot® TOP10 Chemically Competent *E. coli* is provided on page 14; however, you may also transform electrocompetent cells.

Transforming One Shot® TOP10 Competent *E. coli* Cells

Introduction

Once you have performed the TOPO® cloning reaction, you will transform your pSyn_1/D-TOPO® construct into competent *E. coli*. One Shot® TOP10 Chemically Competent *E. coli* are included with the kit to facilitate transformation; however, you may also transform electrocompetent cells. This section provides protocols to transform chemically competent *E. coli*.

Materials needed

- TOPO® Cloning reaction (from step 2, page 13)
- One Shot® TOP10 Chemically Competent *E. coli* (supplied with the kit, Box 3)
- S.O.C. Medium (supplied with the kit, Box 3)
- pSyn_2 positive control (to verify transformation efficiency, if desired; Box 2)
- 42°C water bath
- LB plates containing 100 µg/mL spectinomycin (two for each transformation)
- 37°C shaking and non-shaking incubator

Preparing for transformation

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

1. Equilibrate a water bath to 42°C (for chemical transformation) or set up your electroporator if you are using electrocompetent *E. coli*.
2. Warm the vial of S.O.C. medium from Box 3 to room temperature.
3. Warm LB plates containing 100 µg/mL spectinomycin at 37°C for 30 minutes.
4. Thaw **on ice** 1 vial of One Shot® TOP10 *E. coli* cells from Box 3 for each transformation.

One Shot® chemical transformation protocol

1. Add 2 µL of the TOPO® cloning reaction (from step 2, page 13) into a vial of One Shot® Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down**.
2. Incubate on ice for 5 to 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 and incubate on ice for 2 minutes.
5. Add 250 µL of room temperature S.O.C. Medium.
6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
7. Spread 50–200 µL from each transformation on a prewarmed selective plate and incubate overnight at 37°C. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
8. Proceed to **Analyzing *E. coli* Transformants**, page 15.

Analyzing *E. coli* Transformants

Picking positive clones

1. Pick 5–10 colonies and culture them overnight in LB medium containing 100 µg/mL spectinomycin.
2. Isolate plasmid DNA using your method of choice. If you need ultra-pure plasmid DNA for automated or manual sequencing, we recommend using the PureLink® HQ Mini Plasmid Purification Kit (Cat. no. K2100-01).
3. Analyze the plasmids by restriction analysis or PCR (see below) to confirm the presence and correct orientation of the insert.

Analyzing transformants by PCR

Use the protocol below (or any other suitable protocol) to analyze positive transformants using PCR. For PCR primers, use a combination of the the SEQF1 SYN1 (forward) or the SEQR1 SYN1 (reverse) primer (from Box 2) and a primer that hybridizes within your insert. You will have to determine the amplification conditions that best fits your GOI. If you are using this technique for the first time, we recommend performing restriction analysis in parallel. Artifacts may be obtained because of mispriming or contaminating template.

Materials Needed:

- PCR Super Mix High Fidelity (Cat. no. 10790-020)
- Appropriate forward and reverse PCR primers (20 µM each)

Procedure:

1. For each sample, aliquot 48 µL of PCR SuperMix High Fidelity into a 0.2 mL PCR tube. Add 1 µL each of the forward and reverse PCR primer.
2. Pick 5–10 colonies and resuspend them individually in 50 µL of the PCR SuperMix containing PCR primers (remember to make a patch plate to preserve the colonies for further analysis).
3. Incubate reaction for 10 minutes at 94°C to lyse the cells and inactivate the nucleases before proceeding to the normal PCR cycling protocol.
4. Amplify for 20 to 30 cycles.
5. For the final extension, incubate at 72°C for 10 minutes. Store at 4°C.
6. Visualize by agarose gel electrophoresis.

Continued on next page

Analyzing transformants by sequencing

Once you have identified the correct clone(s), you may sequence your construct to confirm that your gene is cloned in the correct orientation. Use the "Forward Sequencing Primer pSyn_1 vector" or the "Reverse Sequencing Primer pSyn_1 vector" (from Box 2) to help you sequence your insert. For the complete sequence of the pSyn_1/D-TOPO® Vector, refer to our website (**www.lifetechnologies.com**) or contact Technical Support (see page 29).

Long-term storage of *E. coli* clones

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

1. Streak the original colony out on LB plates containing 100 µg/mL spectinomycin.
2. Isolate a single colony, inoculate into 1–2 mL of LB containing 100 µg/mL spectinomycin, and grow until culture reaches stationary phase.
3. Mix 0.85 mL of culture with 0.15 mL of sterile glycerol and transfer to a cryovial, and store at –80°C.

Guidelines for Culturing *Synechococcus elongatus* PCC 7942

General guidelines for *Synechococcus* culture

- All solutions and equipment that may contact cells must be sterile. Always use proper aseptic technique and work in a laminar flow hood.
- *Synechococcus elongatus* liquid cultures should be grown at 34°C ± 1°C with CO_2 (1–2% in air) under continuous illumination using moderate light intensities of cool fluorescent white (50–100 µE m^{-2} s^{-1}) with agitation on a gyrotary shaker set to 100 rpm.

  Note: *Synechococcus elongatus* cultures can also be grown using light intensities of 50–400 µE m^{-2} s^{-1} using only atmospheric CO_2 (i.e., without the addition of CO_2).
- The presence of CO_2 is needed to obtain optimal growth of *Synechococcus elongatus* in liquid culture as it is a photosynthetic organism; however, additional CO_2 is not necessary during transformation where the cells are grown on agar support and are exposed to atmospheric CO_2.
- If you are bubbling the culture with CO_2 enriched air or CO_2 gas, you need to prepare the BG-11 with 50 mM $NaHCO_3$ and adjust the pH to 7.5. The presence of $NaHCO_3$ in the medium prevents the medium from becoming acidic.
- *Synechococcus elongatus* solid cultures on BG-11 agar plates can be grown at 34°C under continuous illumination using 100–200 µE m^{-2} s^{-1} of cool fluorescent white light.

  Note: You can also incubate the cultures at room temperature if sufficient light is provided; however, the growth will slower.
- The optimal equipment for culturing *Synechococcus elongatus* is an algal growth chamber (e.g., Percival Algal Chamber from Geneva Scientific) with regulatable light supply and a light meter to guide adjustments. If an algal growth chamber is not available, the cells can be grown in a standard cell culture incubator illuminated with cool fluorescent lights placed within 12 inches of the culture plates. Standard room lights and incubation at room temperature provide sub-optimal growth conditions.
- Do not stack the culture plates to allow continuous uniform illumination.
- Grow the cells using Gibco® BG-11 medium, which is specifically formulated for optimal growth and maintenance of *Synechococcus elongatus* cells.
- Grow the cells until the culture reaches OD_{750} of ≥1 before transformation.
- Take the OD measurements at 750 nm.
- *Synechococcus elongatus* is classified as a GRAS (generally regarded as safe) organism with no known viral or bacterial pathogens. However, we recommend following general safety guidelines under Biosafety Level 1 (BL-1) containment, similar to working with *E. coli* or yeast. For more information on BL-1 guidelines, refer to *Biosafety in Microbiological and Biomedical Laboratories*, 5th ed., published by the Centers for Disease Control, which isavailable for downloading at: **www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm**.

Thawing *Synechococcus elongatus* PCC 7942

Guidelines for thawing *Synechococcus elongatus*

- Do not thaw more than 3 vials at a time.
- Frozen *Synechococcus elongatus* cells are very sensitive to temperature fluctuations.
- Before the cells are thawed, the cells must be transferred from the –80°C freezer into a dry ice container as quickly as possible and the vials should be buried in dry ice.
- To maximize the recovery of the cells when thawing, warm the cells very quickly by placing the tubes directly from the dry ice container into a 35°C water bath. Once the cells are completely thawed, immediately dilute them into Gibco® BG-11 medium, pre-warmed to room temperature.

Materials needed

- 35°C water bath with a dark lid

  Note: If a dark lid is not available, cover the water bath with aluminum foil.
- Algal growth chamber (e.g., Percival Algal Chamber from Geneva Scientific) set to 34°C ± 1°C and 1% CO_2, optimal, under continuous illumination with 50 µE m^{-2} s^{-1}

  Note: If an algal growth chamber is not available, you can use a standard cell culture incubator under continuous illumination using moderate light intensities of cool fluorescent white (50 µE m^{-2} s^{-1}).
- Gyratory shaking platform set to 100 rpm
- 6-well clear-bottom culture plates
- Gibco® BG-11 medium (Cat. no. A1379901 or A1379902), pre-warmed to room temperature
- 70% ethanol
- Dry ice

Continued on next page

Thawing *Synechococcus elongatus* PCC 7942, continued

Thawing procedure

1. Remove the frozen cells from –80°C storage and immediately place them in a dry ice container. Bury the vial(s) containing the cells in dry ice to minimize temperature fluctuations before thawing.
2. Add 6 mL of Gibco® BG-11 medium, pre-warmed to room temperature, into each well of a 6-well plate.
3. Remove the cryovial containing the frozen cells from the dry ice storage and **immediately** place it into a 35°C water bath.
4. Quickly thaw the cells by placing the vial containing the cells in the 35°C water bath until the last ice crystal has melted (~2 minutes). Do not agitate the cells while thawing (i.e., do not swirl the vial).
5. After the cells have thawed, wipe the outside of the vial with 70% ethanol, and place the vial in a rack at room temperature. Proceed immediately to the next step
6. Transfer 100 µL of thawed cells from the vial into each well of the 6-well plate containing 6 mL of Gibco® BG-11 medium.
7. Place the 6-well plate(s) in the algal growth chamber set to set to 34°C ± 1°C with 1% CO_2 and illuminated with constant light of 50 µE m^{-2} s^{-1}. Do not incubate under light intensity of >50 µE m^{-2} s^{-1} because the cells are sensitive to light immediately after resuscitation. **Do not stack the plates.**
8. Incubate the cells with gentle agitation on a gyrotary shaker set to 100 rpm.
9. On Day 2, transfer 400 µL of the cell suspension and into a disposable plastic cuvette containing 400 µL of Gibco® BG-11 medium to measure the optical density.
10. Measure the cell density at 750 nm (OD_{750}). If the OD_{750} is greater than 1, proceed to the transformation step (page 20). If the culture has not yet reached OD_{750} = 1, return it to the algal growth chamber and continue the incubation.

Transforming *Synechococcus elongatus* PCC 7942

Guidelines for transforming *Synechococcus elongatus* PCC 7942

- *Synechococcus elongatus* PCC 7942 is naturally transformable with highest efficiencies of transformation when the culture is in the log phase of growth (OD_{750} of 1–2).
- Transform *Synechococcus elongatus* using circular, supercoiled DNA.
- Incubate the transformation reaction at 34°C, in the dark.
  Note: Darkness increases the transformation efficiency.
- The quality and the concentration of DNA used play a central role for the efficiency of transformation. Use a commercial kit such as the PureLink® HQ Mini Plasmid Purification or the PureLink® HiPure Plasmid Miniprep kits that deliver pure DNA (see page 27 for ordering information).
- Pre-warm the selective BG-11 + spectinomycin plates to room temperature for 1 hour before plating the transformants.

Materials needed

- pSyn_1/D-TOPO® construct carrying your gene of interest
- pSyn_2/Control Vector
- Gibco® BG-11 medium (Cat. no. A1379901 or A1379902), pre-warmed to room temperature
- BG-11 agar plates containing 10 µg/mL spectinomycin, pre-warmed to room temperature (see page 23 for recipe)
  Note: You will need 2 plates per transformation.
- 34°C water bath with a dark lid or covered with aluminum foil
- Algal Growth Chamber (e.g., Percival Algal Chamber from Geneva Scientific) set to 34°C ± 1°C and CO_2 (1–2% in air) under continuous illumination with 100 µE m^{-2} s^{-1}
  Note: If an Algal Chamber is not available, you can use a standard cell culture incubator under continuous illumination using moderate light intensities of cool fluorescent white (100 µE m^{-2} s^{-1}).
- 70% ethanol
- Sterile microcentrifuge tubes
- Disposable spreaders

Continued on next page

Transforming *Synechococcus elongatus* PCC 7942, continued

Transformation procedure

1. Measure the optical density of the *Synechococcus elongatus* cultures (from step 10, page 19) at 750 nm (i.e., OD_{750}).
   Note: For best performance, the OD_{750} of cultures should be greater than 1 and less than 2.
2. Harvest 1.5 mL of the cells (per transformation) by centrifugation at 14,000 rpm for 3 minutes at room temperature.
3. Remove the supernatant by pipetting.
4. Resuspend the cells in 1 mL of Gibco® BG-11 medium by gently pipetting up and down.
5. Centrifuge the cells at 14,000 rpm for 1 minute at room temperature, and remove the supernatant by pipetting.
6. Resuspend the cells in 100 µL of Gibco® BG-11 medium by gently pipetting up and down.
7. Add 100 ng of supercoiled plasmid DNA (i.e., pSyn_1/D-TOPO® construct containing your gene of interest) into the resuspended cells. Mix the DNA-cell suspension gently by flicking the tube. In a separate tube, prepare a control transformation with the pSyn_2/Control Vector.
8. Incubate the cell-DNA mixture(s) in the 34°C water bath with a dark lid for 4 hours. After the incubation is complete, remove the tube(s) from the water bath and wipe them with 70% ethanol.
9. Plate 80 µL and 5 µL of each transformation mixture on separate BG-11 agar plates containing 10 µg/mL spectinomycin and pre-warmed to room temperature.
10. Place the plates with agar side down on illuminated shelves at room temperature (25–30°C). Do not stack the plates to ensure continuous and even illumination.
11. Incubate the plates for 5–7 days or until the colonies are ready to pick. Control vector should produce a minimum of 100 transformants per transformation. The results from the transformation with the pSyn_1/D-TOPO® construct will depend on the nature of your gene of interest.

Screening for Integration by Colony PCR

Introduction

Use the protocols below to prepare cell lysates and perform colony PCR to screen the transformed *Synechococcus elongatus* colonies for full integration of the promoter and the gene of interest. You will have to design the forward and reverse PCR primers specific to your insert and determine the amplification conditions. We recommend using the AccuPrime™ *Pfx* Polymerase SuperMix or the PCR SuperMix High Fidelity for best results; however, other DNA polymerases may also be used.

Materials needed

- AccuPrime™ *Pfx* SuperMix (Cat. no. 12344-040) or PCR SuperMix High Fidelity (Cat. no. 10790-020)
- Appropriate forward and reverse primers (10 µM each)

Colony PCR procedure

1. Streak or patch colonies onto fresh BG-11 agar plates containing 10 µg/mL of spectinomycin and allow them to grow for 1–2 days or until you have sufficient growth before proceeding with the colony PCR protocol below.
2. Prepare the PCR reaction as shown in table below.
3. Pick up cells with a pipette tip from the re-streaked plates (lift enough material that is equivalent to about 1 small colony, too much material will inhibit the PCR reaction) and resuspend in the PCR reaction mix.

Reagent	Amount
AccuPrime™ *Pfx* SuperMix or PCR SuperMix High Fidelity	45 µL
Primer pre-mix (10 µM each of forward and reverse primers)	1 µL
Colony	1

4. Mix the contents of the tubes and load into a thermal cycler.
5. Heat at 95°C for 5 minutes for the initial denaturation step before proceeding with the normal PCR cycling protocol.
6. Maintain reaction at 4°C after cycling. Samples can be stored at –20°C.
7. Analyze the results by agarose gel electrophoresis.

Scale-up

We recommend initiating a seed culture first before scaling up your clones. To initiate a seed culture, scrape up as much of the streak or patch as possible (from step 1, **Colony PCR procedure**, above) and transfer it into each well of a 6-well plate containing 6 mL of Gibco® BG-11 medium with antibiotic, and grow them at 34°C ± 1°C with CO_2 (1–2% in air) and illuminated with constant light of 50 µE m^{-2} s^{-1}. Do not stack the plates.

Incubate the cells with gentle agitation on a gyrotary shaker set to 100 rpm until they reach the log phase of growth (OD_{750} of 1–2). Use these seed cultures to initiate larger cultures in shake flasks for further investigation by diluting them to 1/20 (v/v). We recommend the addition of antibiotic (10 µg/mL spectinomycin) to the culture, especially for genes that are toxic to the cells.

Appendix A: Support Protocols

Media and Plates

LB (Luria-Bertani) medium and plates

LB medium:

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 mL of deionized water.
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave the solution on liquid cycle for 20 minutes at 15 psi. Allow the solution to cool to 55°C and add the appropriate antibiotics, if needed.
   Note: Use spectinomycin at a final concentration of 100 µg/mL.
4. Store the medium at room temperature or at 4°C.

LB agar plates

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

Preparing BG-11 agar plates

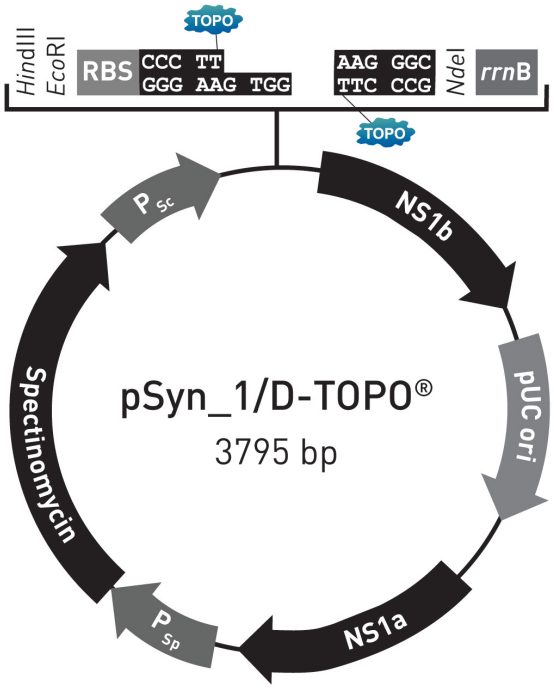
1. Add 15 g of agar to 200 mL of Gibco® BG-11 medium in an autoclaveable flask.
   Note: Use high quality agar, such as Calbiochem, Cat no.12177, or Sigma, Cat. no. A1296.
2. Autoclave on liquid cycle for 20 minutes.
3. Warm 800 mL of Gibco® BG-11 medium to 55–60°C in a water bath.
4. After autoclaving, cool the agar containing flask to ~55°C.
5. Combine the agar containing flask with 800 mL of Gibco® BG-11 medium.
6. Add spectinomycin to a final concentration of 10 µg/mL (if required), and pour into 10 cm plates.
7. Let the plates harden (do **not** overdry), invert them, and store at 4°C in the dark. Final agar concentration will be 1.5%.

Appendix B: Vectors

Map and Features of pSyn_1/D-TOPO® Vector

Map of pSyn_1/D-TOPO® Vector

The map below shows the features of pSyn_1/D-TOPO® vector. The complete sequence of the vector is available for downloading at **www.lifetechnologies.com** or from Technical Support (page 29).



Features of pSyn_1/D-TOPO® Vector

3795 nucleotides

TOPO® binding site 1:	1–5
*rrn*B transcriptional termination region:	28–185
NS1b (neutral site 1b):	186–967
pUC origin:	1025–1640
NS1a (neutral site 1a):	1712–2510
Spectinomycin promoter (P_{Sp}):	2517–2650
Spectinomycin resistance gene:	2651–3661
Promoter (P_{Sc}):	3666–3766
RBS (ribosome binding site):	3780–3786
TOPO® binding site 2:	3787–3791
GTGG overhang:	3792–3795

Continued on next page

Map and Features of pSyn_1/D-TOPO® Vector, continued

Features of pSyn_1/D-TOPO® Vector

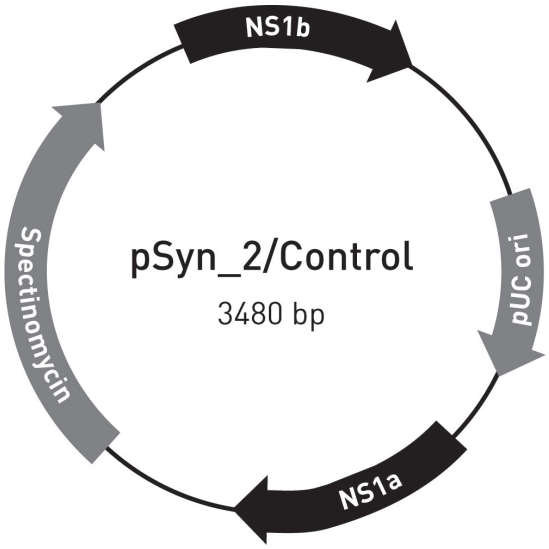
The pSyn_1/D-TOPO® vector contains the following elements. All features have been functionally tested.

Feature	Benefit
TOPO® binding sites (directional)	Allows rapid cloning of your PCR product for expression in *Synechococcus elongatus.*
*rrn*B transcription termination region	Strong transcription termination region.
NS1a and NS1b (neutral site 1)	Sites also present on *Synechococcus elongatus* genome to guide double homologous recombination of DNA contained between the neutral sites in the vector (Clerico *et al.,* 2007).
pUC origin	Allows high-copy replication and growth in *E. coli*.
Spectinomycin promoter (P_{Sp})	Allows expression of the spectinomycin resistance gene in *E. coli* and *Synechococcus elongatus.*
Spectinomycin resistance gene (*aad*A1)	Allows selection of the plasmid in *E. coli* and *Synechococcus elongatus* (Liebert *et al.,* 1999).
Promoter (P_{Sc})	Weak constitutive promoter from *Synechocystis* sp. strain PCC 6803 that allows a minimal level of expression of the GOI (Simkovsky *et al.,* 2012).
Ribosome binding site	Increases efficiency of recombinant protein expression.

Map of pSyn_2/Control Vector

pSyn_2/Control Vector

The map below shows the features of pSyn_2/Control Vector. The control vector is used as a positive control for *Synechococcus elongatus* PCC 7942 transformations and confers spectinomycin resistance to successfully transformed cells. When used as detailed on page 21, the control vector should produce a minimum of 100 transformants per transformation. The complete sequence of the vector is available for downloading at **www.lifetechnologies.com** or from Technical Support (page 29).



Features of pSyn_2/Control

3480 nucleotides

pUC origin:	58–673
NS1a (neutral site 1a):	745–1543
Spectinomycin resistance gene:	1550–2694
NS1b (neutral site 1b):	2699–3480

Appendix C: Ordering Information

Accessory Products

Proofreading DNA polymerases

Life Technologies offers a variety of proofreading, thermostable DNA polymerases for generating blunt-end PCR products. Ordering information is provided below. For details, visit **www.lifetechnologies.com**.

Product	Quantity	Cat. no.
Platinum® *Pfx* DNA Polymerase	100 units	11708-013
AccuPrime™ *Pfx* DNA Polymerase	200 reactions	12344-024
Pfx50™ DNA Polymerase	100 reactions	12355-012
PCR SuperMix High Fidelity	100 reactions	10790-020

Competent cells

Chemically competent and electrocompetent cells that can be used with GeneArt® *Synechococcus* TOPO® Engineering Kits are also available separately from Life Technologies. Ordering information is provided below. For details, visit **www.lifetechnologies.com**.

Product	Quantity	Cat. no.
One Shot® TOP10 Chemically Competent Cells	10 reactions 20 reactions	C4040-10 C4040-03
One Shot® TOP10 Electrocomp™ *E. coli*	10 reactions 20 reactions	C4040-50 C4040-52
TOP10 Electrocomp™ Kits	20 reactions 40 reactions 120 reactions	C664-55 C664-11 C664-24

Additional products

The following reagents are recommended for use with the GeneArt® *Synechococcus* TOPO® Engineering Kits. Ordering information for these reagents is provided below. For details, visit **www.lifetechnologies.com**.

Product	Quantity	Cat. no.
Gibco® BG-11 Media: Optimized for *Synechococcus*	1 L 6 × 1 L	A1379901 A1379902
PureLink® Growth Block	50 blocks	12256-020
PureLink® HQ Mini Plasmid Purification Kit	100 preps	K2100-01
PureLink® HiPure Plasmid Miniprep Kit	25 preps 100 preps	K2100-02 K2100-03
LB Broth (1X), liquid	500 mL	10855-021

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Other GeneArt® algal kits

In addition to the GeneArt® *Synechococcus* TOPO® Engineering Kits, Life Technologies offers the following products as model algal hosts. Ordering information is provided below. For details, visit **www.lifetechnologies.com**.

Product	Quantity	Cat. no.
GeneArt® *Synechococcus* Engineering Kit	1 kit	A14259
GeneArt® *Synechococcus* Engineering Kit with 6 L media	1 kit	A14263
GeneArt® *Chlamydomonas* Engineering Kit	1 kit	A14258
GeneArt® *Chlamydomonas* Engineering Kit with 6 L media	1 kit	A14262
GeneArt® *Chlamydomonas* TOPO® Engineering Kit	1 kit	A14260
GeneArt® *Chlamydomonas* TOPO® Engineering Kit with 6 L media	1 kit	A14264

Documentation and Support

Obtaining Support

Technical Support

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

At the website, you can:

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

Safety Data Sheets (SDS)

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

Limited Product Warranty

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

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

lifetechnologies.com
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