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GeneArt™ *Synechococcus* Protein Expression Vector

For expression of recombinant proteins in *Synechococcus elongatus*

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Revision history

Revision	Date	Description
B.0	26 April 2017	Updated kit contents, reorganized protocols, and rebranded.
A.0	20 November 2013	Basis for the current revision.

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Kit contents

The GeneArt™ *Synechococcus* Protein Expression Vector contains the components listed below.

Component	Concentration	Amount
pSyn_6 vector	20 µL of vector at 0.5 µg/µL in TE buffer, pH 8.0*	10 reactions

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

Shipping and storage

The GeneArt™ *Synechococcus* Protein Expression Vector is shipped on dry ice. Upon receipt, store the vector at –20°C. When stored as directed, the vector is guaranteed for six months.

Description of the system

Introduction

The GeneArt™ *Synechococcus* Protein Expression System is a prokaryotic photosynthetic model system based on the unicellular cyanobacterium *Synechococcus elongatus* PCC 7942 (**http://cyanobacteria.web.pasteur.fr**), 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.

S. elongatus has a rod-shaped appearance and is oligotrophic, having the ability to survive in freshwater environments with low nutrients. *S. elongatus* genome consists of one chromosome (chromosome 1) and possibly up to two plasmids that have been sequence verified (Holtman et al. 2005, Chen et al. 2008, and Van der Plas et al. 1992). The circular chromosome of *S. elongatus* is ~2.7 Mb in length (fully sequenced) with 55.5% GC content (**https://www.ncbi.nlm.nih.gov/genome/430**). 718 of the organism's 2,723 genes have been identified as essential for survival under laboratory conditions (Rubin et al., 2017).

The cyanobacterium *S. 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 *S. 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 *S. 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 *S. elongatus* PCC 7942.

pSyn_6 vector

The pSyn_6 vector (4461 bp) is designed to facilitate rapid cloning of your gene of interest (GOI) 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 17.

- NS1 (neutral site 1) homologous recombination sites for the integration of the vector into the *Synechococcus elongatus* genome.
- The strong constitutive promoter of psbA gene (encoding photosystem II protein D1) from *Synechococcus elongatus* PCC 7942 driving the high level expression of your gene of interest.
- An N-terminal polyhistidine tag followed by a TEV recognition site for TEV protease-dependent cleavage of the N-terminal tag from your recombinant protein.
- A C-terminal V5 epitope followed by a polyhistidine tag for detection and purification of your recombinant protein.
- Two multiple cloning sites (MCS) that provide the flexibility to include either or both or none of the N-terminal and C-terminal tags using seamless, Type IIs, or restriction enzyme digestion-based cloning methods.

  Note: When cloning, make sure that your gene of interest is in frame with the tags you wish to include. For
- Spectinomycin resistance gene for selection in *E. coli* and *Synechococcus elongatus* PCC 7942.
- *rrnB* sequences for strong transcription termination.
- pUC origin for maintenance in *E. coli.*
- RP4/RK2 *bom* site (basis of mobility or *ori*T) for conjugation.

Experiment Outline

Workflow

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

Step	Action	Page
1	Clone your gene of interest (GOI) directly into pSyn_6 vector	5
2	Transform One Shot™ TOP10 *E. coli* with the pSyn_6 construct containing your GOI and select the transformants on LB plates containing spectinomycin	8
3	Analyze transformants by restriction digestion or PCR	9
4	Thaw and resuscitate *Synechococcus elongatus* PCC 7942 cells	12
5	Transform *Synechococcus elongatus* PCC 7942 cells and select transformants	14
7	Perform colony PCR to screen the transformed *Synechococcus elongatus* PCC 7942 colonies for full integration of the GOI	16

Methods

Clone into pSyn_6

General molecular biology techniques

For help with PCR amplification, DNA ligations, *E. coli* transformations, restriction enzyme analysis, DNA sequencing, and DNA biochemistry, refer to *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989) or *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

For help with seamless cloning, see **www.thermofisher.com/geneart** and our free, web-based GeneArt™ Primer and Construct Design Tool, which is available at **www.thermofisher.com/order/oligoDesigner**.

E. coli host

For cloning and transformation, use a recombination deficient (*rec*A) and endonuclease A-deficient (*end*A) strain such as TOP10. Note that other *rec*A, *end*A *E. coli* strains are also suitable.

Maintaining pSyn_6 vector

- To propagate and maintain the pSyn_6 vector, use 10 ng of the vector to transform a *rec*A, *end*A *E. coli* strain like TOP10, DH5α™, or equivalent.
- Select *E. coli* transformants on LB plates containing 100 µg/mL of spectinomycin.
- Prepare a glycerol stock of the plasmid for long-term storage (see page 9).

Cloning considerations

- Because the *Synechococcus elongatus* PCC 7942 genome has a high GC content (~55.5% GC; 1st letter GC ~64%, 2nd letter GC, ~44%, and 3rd letter GC ~60%), the expression levels of recombinant genes are significantly improved if the gene of interest is adapted to the preferred codon usage of highly expressed *Synechococcus elongatus* PCC 7942 genes. You can order synthesized codon-optimized genes from Invitrogen™ GeneArt™ Gene Synthesis services (**www.thermofisher.com/geneartgenesynthesis**).
- The pSyn6 vector contains two multiple cloning sites (MCS), which provide the option of adding N- and C-terminal polyhistidine tags to your recombinant fusion protein in any combination (either or both or none).
  - The N-terminal polyhistidine tag is followed by a TEV recognition site for TEV protease-dependent cleavage of the N-terminal tag from the recombinant protein after purification.
  - The C-terminal polyhistidine tag is flanked immediately upstream by the V5 epitope for the detection of the recombinant fusion protein with anti-V5 antibodies.

  When cloning, make sure that your gene of interest is in frame with the tags you wish to include.
- You can clone your gene of interest into the pSyn_6 vector using seamless, Type IIs, or restriction enzyme digestion-based cloning methods.
- pSyn_6 vector contains an ATG initiation codon only at the *Nde*I cloning site. You do not need include an initiation codon in your DNA insert if you:
  - use the *Nde*I site as your 5′ end cloning site

    or
  - add the N-terminal polyhistidine tag and the TEV recognition site to your insert by selecting the *Hin*dIII, *Bam*HI, *Eco*RI, or *Kpn*I site as your 5′ end cloning site.

- If you choose not to add the N-terminal tags to your recombinant protein and do not select the *Nde*I site as your 5′ end cloning site, you **must** design your insert to contain an ATG initiation sequence.

  You can use the native sequence containing the start codon or design your forward PCR primer so that your insert is amplified with the start codon in frame with your gene of interest.
- pSyn_6 vector contains a ribosome binding site (RBS), GAAGGAG, for efficient initiation of translation. For high level of protein expression, use the *Bgl*II or the *Nde*I site as the 5′ end cloning site.
- For best translation initiation, we recommend having a 7–9 nt spacer region between the RBS and the ATG initiation sequence (Markley et al., 2014).
- pSyn_6 vector contains a TAG stop codon 3′ of the C-terminal polyhistidine tag. Your insert does not need a stop codon if you wish to add the C-terminal tag to your recombinant fusion protein. However, you must design your gene of interest to be in frame with the C-terminal V5 epitope and polyhistidine tag.
- If you do not want to include the C-terminal tag in your recombinant protein, your insert must contain a stop codon for proper termination of your mRNA. Ensure that your insert is in-frame with the C-terminal tag.

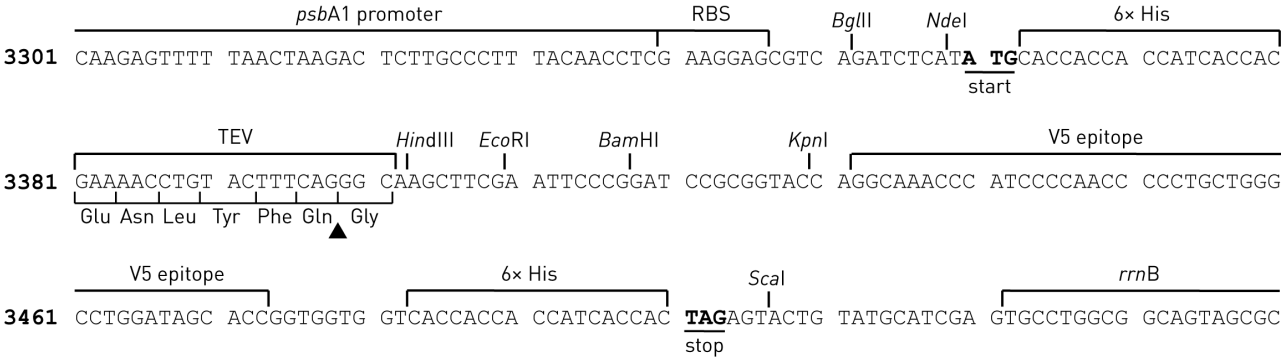
Multiple cloning sites of pSyn_6 vector

The two multiple cloning sites (MCS_1: *Bgl*II, *Nde*I; MCS_2: *Hin*dIII, *Bam*HI, *Eco*RI, *Kpn*I) of the pSyn_6 vector are shown below. Restriction sites are labeled to indicate the cleavage sites, and the start and stop codons are shown in bold and underlined. The cleavage site within the TEV recognition site is indicated by the black triangle. The multiple cloning sites have been confirmed by sequencing and functional testing.

Refer to Figure 1 to design suitable PCR primers to clone your PCR product into the pSyn_6 vector.

When cloning, make sure that your gene of interest is in frame with the tags you wish to include.

Figure 1. Multiple cloning site of the pSyn_6 vector.



Ligation

After you have determined a cloning strategy and PCR amplified your gene of interest, digest the pSyn_6 vector with the appropriate restriction enzymes and ligate your insert containing your gene of interest into pSyn_6 using standard molecular biology techniques.

Seamless cloning with GeneArt™ Seamless and Seamless PLUS Cloning and Assembly Kits

- The pSyn_6 vector is compatible with the GeneArt™ Seamless Cloning and Assembly technology, which allows the simultaneous and seamless assembly of up to 4 DNA inserts between 100 bp and 10 kb and a linearized *E. coli* vector, totaling up to 13 kb in length.
- After you have determined where to position your gene of interest in the pSyn_6 vector, use the diagram depicting the MCS of pSyn_6 vector (page 6) to design suitable PCR primers to clone and express your PCR product in the pSyn_6 vector. The primers should have at least 15-nt homology to the pSyn_6 vector at their 5′ end.
- After digesting the pSyn_6 vector with the appropriate restriction enzymes, PCR amplify your genes of interest, and use the GeneArt™ Seamless Cloning and Assembly Enzyme Mix (Cat. No. A14606) or the GeneArt™ Seamless PLUS Cloning and Assembly Kit (Cat. No. A14603) to seamlessly assemble into the digested pSyn_6 vector.
- For detailed instructions on how to use the GeneArt™ Seamless Cloning and Assembly Enzyme Mix or the GeneArt™ Seamless PLUS Cloning and Assembly Kit, see the relevant user guide at **www.thermofisher.com**.

  For help with primer design for seamless cloning, use our free, web-based GeneArt™ Primer and Construct Design Tool, available at **www.thermofisher.com/order/oligoDesigner**.

  For more information on seamless cloning and assembly technologies, see our website at **www.thermofisher.com/geneart**.

Seamless cloning with GeneArt™ Type IIs Assembly Kits

You can also use the GeneArt™ Type IIs Assembly Kit, *Aar* I (Cat. No. A15916) to seamlessly clone and assemble up to 8 DNA fragments and the pSyn_6 vector by simultaneous cleavage and ligation in a single reaction.

IMPORTANT! The GeneArt™ Type IIs Assembly Kit, *Bsa* I (Cat. No. A15917) and the GeneArt™ Type IIs Assembly Kit, *Bbs* I (Cat. No. A15918) **cannot** be used with the pSyn_6 vector, because the vector contains *Bsa* I and *Bbs* I restriction sites.

For more information, see the relevant user guide, which is available for download at **www.thermofisher.com**.

E. coli transformation method

- Chemical transformation is the most convenient for most researchers. You can use the chemical transformation method for seamless, Type IIs, and restriction enzyme digestion-based cloning. A protocol for the chemical transformation of One Shot™ TOP10 Chemically Competent *E. coli* cells is provided on page 8.
- You can also transform electrocompetent *E. coli* cells by electroporation, which is more efficient and the method of choice for large plasmids. You can use electroporation for Type IIs and restriction enzyme digestion-based cloning. Do **not** use electroporation for seamless cloning.

Transform One Shot™ TOP10 competent *E. coli* cells

Introduction

After you have performed the cloning reaction, transform your pSyn_6 construct into competent *E. coli*. The following protocol describes the transformation of OneShot™ TOP10 Chemically Competent *E. coli* cells, but you can also use other suitable cells.

If you are performing Type IIs or restriction enzyme digestion-based cloning, you can also transform electrocompetent cells using the protocol supplied with the electrocompetent cells.

Note: Do **not** use electroporation for seamless cloning.

Materials needed

- pSyn_6 construct containing your gene of interest
- One Shot™ TOP10 Chemically Competent *E. coli*
- S.O.C. Medium
- 42°C water bath
- LB plates containing 100 µg/mL spectinomycin (two for each transformation)
- 37°C shaking and non-shaking incubator

Prepare 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.
2. Warm the vial of S.O.C. medium to room temperature.
3. Warm LB plates containing 100 µg/mL of spectinomycin at 37°C for 30 minutes.
4. Thaw **on ice** 1 vial of One Shot™ TOP10 for each transformation.

One Shot™ chemical transformation protocol

1. Add 1–5 µL of the DNA (10 pg to 100 ng) 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 5–30 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 pre-warmed 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 **Analyze *E. coli* Transformants**, page 9.

Analyze *E. coli* transformants

Pick 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. K210001, see page 19).
3. Analyze the plasmids by restriction analysis or PCR to confirm the presence and correct orientation of the insert.

Analyze *E. coli* transformants by PCR

Use the protocol below (or any other suitable protocol) to analyze positive transformants using PCR. You will have to determine the primer sequences and amplification conditions based on your gene of interest. Design one of the primers to hybridize to the vector backbone flanking your insert and the other one to hybridize within your insert. If you are using this technique for the first time, we recommend that you perform restriction analysis in parallel. Artifacts may be obtained because of mispriming or contaminating template.

Materials needed

- PCR Super Mix High Fidelity (Cat. No. 10790020)
- Appropriate forward and reverse PCR primers (20 µM each)

Procedure

1. For each sample, prepare the following PCR mix in a 0.2-mL PCR tube:

Reagent	Amount
PCR SuperMix High Fidelity	48 µL
Forward primer (20 µM)	1 µL
Reverse primer (20 µM)	1 µL
Total volume:	**50 µL**

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 20–30 cycles.
5. For the final extension, incubate at 72°C for 10 minutes. Store at 2–8°C.
6. Visualize by agarose gel electrophoresis.

Analyze transformants by sequencing

After you have identified the correct clones, sequence your construct to confirm that your gene is cloned in the correct orientation and its sequence is in frame with the tags included. Design a primer that hybridizes to the vector backbone approximately 100 bp upstream of the cloning sites.

For the complete sequence of the pSyn_6 vector, see our website (**www.thermofisher.com**) or contact Technical support (see page 23).

Long-term storage

After you have identified the correct clone, make a glycerol stock of the clone for long-term storage at –80°C. We also recommend that you store a stock of plasmid DNA at –20°C.

Appendix A: Support protocols

Prepare 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 2–8°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 2–8°C, in the dark.

BG-11 agar plates

1. Add 15 g of agar to 200 mL of Gibco™ BG-11 medium in an autoclavable 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** over dry), invert them, and store at 2–8°C in the dark. Final agar concentration will be 1.5%.

Guidelines for *Synechococcus elongatus* PCC 7942 culture

General guidelines for *Synechococcus* culture

The following are culture guidelines for the cyanobacterium *Synechococcus elongates* PCC 7942 (**http://cyanobacteria.web.pasteur.fr**).

- *Synechococcus elongatus* PCC 7942 cells are not included in the GeneArt™ *Synechococcus* Protein Expression Vector Kit. They can be ordered from ATCC™ (ATCC No. 33912) (**http://www.atcc.org/products/all/33912.aspx**) or from the Pasteur Culture collection of Cyanobacteria (PCC No. 7942) (**http://cyanobacteria.web.pasteur.fr**).
- All solutions and equipment that may contact cells must be sterile. Always use proper aseptic technique and work in a laminar flow hood.
- Grow the cells using Gibco™ BG-11 medium (Cat. nos. A1379901, A1379902), which is specifically formulated for optimal growth and maintenance of *S. elongatus* cells.
- Grow *S. elongatus* liquid cultures 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: *S. elongatus* cultures can also be grown using light intensities of 50–400 µE m^{-2} s^{-1} with only atmospheric CO_2 (i.e., without additional CO_2).
- The presence of CO_2 is needed to obtain optimal growth of *S. 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, prepare the BG-11 medium with 50 mM $NaHCO_3$ and adjust the pH to 7.5. The presence of $NaHCO_3$ in the medium prevents it from becoming acidic.
- *S. 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 *S. 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 until the culture reaches OD_{750} of ≥1 before transformation.
- Take the OD measurements at 750 nm.
- *S. 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 is available for download from **www.cdc.gov**.

Thaw *Synechococcus elongatus* PCC 7942

Guidelines for thawing

- Do not thaw more than 3 vials at a time.
- Frozen *S. 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 34°C water bath. When the cells are completely thawed, immediately dilute them into Gibco™ BG-11 medium, pre-warmed to room temperature.
- You can count the *S. elongatus* cells using the Attune™ NxT Acoustic Focusing Cytometer (or equivalent) by detecting the endogenous orange-fluorescent phycoerythrin and red-fluorescent chlorophyll. For more information, contact Cell Analysis Technical Support Center (**www.thermofisher.com/support**).

Materials needed

- 34°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

Thaw 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 34°C water bath.
4. Quickly thaw the cells by placing the vial containing the cells in the 34°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 plates 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}.

   IMPORTANT! Do **not** incubate under light intensity of more than 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 14). If the culture has not yet reached OD_{750} = 1, return it to the algal growth chamber and continue the incubation.

Transform *Synechococcus elongatus* PCC 7942

Guidelines for transformation

- *Synechococcus elongatus* is naturally transformable with highest efficiencies of transformation when the culture is in the log phase of growth (OD_{750} of 1–2).
- Transform *S. 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 the plasmid DNA 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 19 for ordering information).
- Pre-warm the selective BG-11 + spectinomycin plates to room temperature for 1 hour before plating the transformants.

Materials needed

- pSyn_6 construct carrying your gene of interest (purified, supercoiled plasmid DNA from page 9)
- *Optional:* pSyn_6 positive control construct carrying the *S. elongatus* codon-optimized GUS gene

  Note: You can order synthesized codon-optimized genes from Invitrogen™ GeneArt™ Gene Synthesis services (**www.thermofisher.com/geneartgenesynthesis**).
- 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 10 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 1–2% CO_2, 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

Transformation procedure

1. Measure the optical density of the *Synechococcus elongatus* cultures (from step 10, page 13) 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_6 construct containing your gene of interest) into the resuspended cells.

   In a separate tube, add 100 ng of empty pSyn_6 vector as a negative control. Mix the DNA-cell suspension gently by flicking the tube.
8. Incubate the cell-DNA mixtures in the 34°C water bath with a dark lid for 4 hours. After the incubation is complete, remove the tubes 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 of 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. The results from the transformation with the pSyn_6 construct will depend on the nature of your gene of interest.

Screen 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 work.

Materials needed

- AccuPrime™ *Pfx* SuperMix (Cat. No. 12344040) or PCR SuperMix High Fidelity (Cat. No. 10790020)
- 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 colony

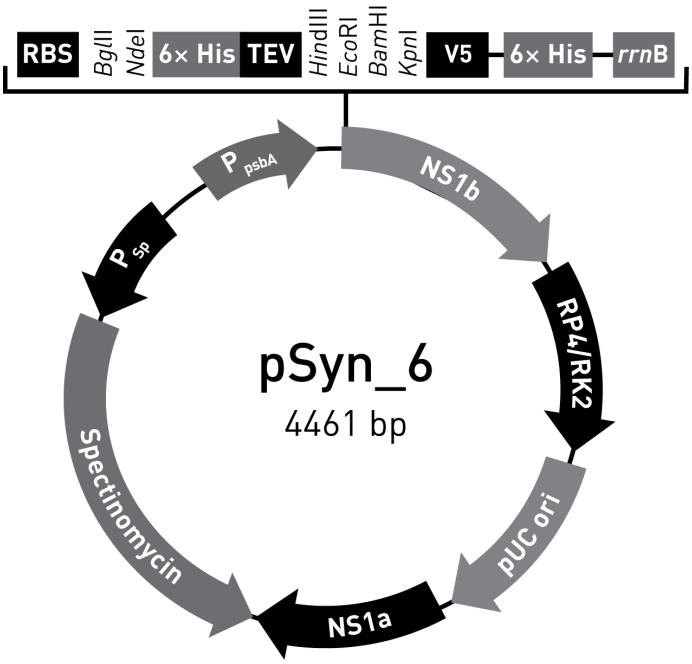
4. Mix the contents of the tubes and load into a thermal cycler.
5. Heat at 95°C for 5 minutes for the initial denaturation step before proceeding with the normal PCR cycling protocol.
6. Maintain reaction at 2–8°C after cycling. Samples can be stored at –20°C.
7. Analyze the results by agarose gel electrophoresis.

Appendix B: pSyn_6 Vector

Map and features of pSyn_6 vector

Map of pSyn_6 vector

The map below shows the features of the pSyn_6 vector. The complete sequence of the vector is available for download at **www.thermofisher.com** or by contacting Technical Support (page 23).



Features of pSyn_6 vector

4461 nucleotides

RP4/RK2 *bom* site:	1–304
pUC origin:	362–977
NS1a (neutral site 1a):	1049–1847
Spectinomycin resistance gene:	1970–2980 (c)
Spectinomycin promoter (P_{Sp}):	2981–3114 (c)
psbA promoter (P_{psbA}):	3117–3339
RBS (ribosome binding site):	3340–3346
6× His tag:	3363–3380
TEV recognition site:	3381–3401
V5 epitope:	3432–3473
6× His tag:	3483–3500
*rrn*B transcriptional termination region:	3522–3679
NS1b (neutral site 1b):	3680–4457

(c): complementary

Features of pSyn_6 vector

The pSyn_6 vector contains the following elements. All features have been functionally tested.

Feature	Benefit
PR4/RK2 site	*bom* site (basis of mobility or *oriT*) that facilitates bacterial conjugation (Burkhardt et al., 1979; Figurski and Helinski, 1979; Pansegrau et al., 1990; Thomas, 1981).
pUC origin	Allows high-copy replication and growth in *E. coli.*
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).
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).
psbA promoter (P_{psbA})	Strong constitutive promoter of psbA gene (encoding photosystem II protein D1) from *Synechococcus elongatus* driving the high level expression of your gene of interest.
2 multiple cloning sites with 6 unique restriction enzyme recognition sequences (*Bgl*II, *Nde*I, *Hin*dIII, *Eco*RI, *Bam*HI, *Kpn*I)	Allows insertion of your gene into pSyn_6 vector with the flexibility to include either or both or none of the N-terminal and C-terminal tags.
*rrn*B transcription termination region	Strong transcription termination region that protects the cloned gene from expression by vector-encoded promoters, thereby reducing possible toxicity (Brosius, 1984; Orosz *et al.*, 1991).
V5 epitope (Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr)	Allows detection of the fusion protein with the Anti-V5 Antibody or the Anti-V5-HRP Antibody (Southern *et al.*, 1991)
N-terminal and C-terminal polyhistidine (6× His) tags	Permits purification of your fusion protein on metal-chelating resins (i.e., ProBond™)
TEV recognition site	Allows TEV protease-dependent cleavage of the N-terminal 6× His tag from your recombinant protein upon purification

Appendix C: Ordering Information

Accessory Products

Proofreading DNA polymerases

We offer various proofreading, thermostable DNA polymerases for generating blunt-end PCR products. For more information, visit **www.thermofisher.com**.

Product	Quantity	Cat. No.
Platinum™ *Pfx* DNA Polymerase	100 units	11708013
AccuPrime™ *Pfx* DNA Polymerase	200 reactions	12344024
Pfx50™ DNA Polymerase	100 reactions	12355012
PCR SuperMix High Fidelity	100 reactions	10790020

Competent cells

Chemically competent and electrocompetent cells that can be used with the GeneArt™ *Synechococcus* Protein Expression Vector are also available separately. For more information, visit **www.thermofisher.com**.

Product	Quantity	Cat. No.
One Shot™ TOP10 Chemically Competent Cells	10 reactions 20 reactions	C404010 C404003
One Shot™ TOP10 Electrocomp™ *E. coli*	10 reactions 20 reactions	C404050 C404052
TOP10 Electrocomp™ Kits	20 reactions 40 reactions 120 reactions	C66455 C66411 C66424

Additional products

The following reagents are recommended for use with the GeneArt™ *Synechococcus* Protein Expression Vector. For more information, visit **www.thermofisher.com**.

Product	Quantity	Cat. No.
PureLink™ Growth Block	50 blocks	12256020
PureLink™ HQ Mini Plasmid Purification Kit	100 preps	K210001
PureLink™ HiPure Plasmid Miniprep Kit	25 preps 100 preps	K210002 K210003
LB Broth (1X), liquid	500 mL	10855021
V5 Epitope Tag Antibody	50 µL	R96025
AcTEV™ Protease	1000 units	12575015
GUSB Monoclonal Antibody	1 mL	MA1-35024
ImaGene Green™ C_{12}FDGlcU GUS Gene Expression Kit	1 kit	I2908
X-GlcU, CHA (5-Bromo-4-Chloro-3-Indolyl β-D-Glucuronide, Cyclohexylammonium Salt)	100 mg	B1691

Other GeneArt™ products for algae

The following GeneArt™ products for algal expression, culture, and maintenance are available from Thermo Fisher Scientific. For more information, visit **www.thermofisher.com**.

Product	Quantity	Cat. No.
Gibco™ BG-11 Media, optimized for cyanobacteria	1 L 6 L	A1379901 A1379902
GeneArt™ MAX Efficiency™ Transformation Reagent	250 mL	A24229
GeneArt™ Cryopreservation Kit for Algae	1 kit	A24228
GeneArt™ *Chlamydomonas* Protein Expression Vector	10 reactions	A24231
Gibco™ TAP Growth Media, optimized for *Chlamydomonas* culture	1 L 6 × 1 L	A1379801 A1379802

GeneArt™ Seamless Assembly products

The following GeneArt™ products can be used for seamless assembly of up to 10 DNA inserts and vector. For more information, visit **www.thermofisher.com** or contact Technical Support (see page 23).

Product	Amount	Cat. No.
GeneArt™ Type IIs Assembly Kit, *Aar*I	1 kit	A15916
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

Appendix D: Safety

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below, and consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological hazard safety

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

In the US:

- U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories found at:

  www.cdc.gov/biosafety

- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030), found at:

  www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html

- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.

- Additional information about biohazard guidelines is available at:

  www.cdc.gov

In the EU:

Check local guidelines and legislation on biohazard and biosafety precaution and refer to the best practices published in the World Health Organization (WHO) Laboratory Biosafety Manual, 3rd edition, found at:

www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/

Documentation and Support

Obtaining Support

Technical Support

For the latest services and support information for all locations, go to **www.thermofisher.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 (**thermofisher.com/support)**
- 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.thermofisher.com/sds**.

IMPORTANT! For the SDSs of chemicals not distributed by Thermo Fisher Scientific contact the chemical manufacturer.

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.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.

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