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Video Article

Genetic Modification of Cyanobacteria by Conjugation Using the CyanoGate Modular Cloning Toolkit

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Abstract

Cyanobacteria are a diverse group of prokaryotic photosynthetic organisms that can be genetically modified for the renewable production of useful industrial commodities. Recent advances in synthetic biology have led to development of several cloning toolkits such as CyanoGate, a standardized modular cloning system for building plasmid vectors for subsequent transformation or conjugal transfer into cyanobacteria. Here we outline a detailed method for assembling a self-replicating vector (e.g., carrying a fluorescent marker expression cassette) and conjugal transfer of the vector into the cyanobacterial strains *Synechocystis* sp. PCC 6803 or *Synechococcus elongatus* UTEX 2973. In addition, we outline how to characterize the performance of a genetic part (e.g., a promoter) using a plate reader or flow cytometry.

Video Link

The video component of this article can be found at https://www.jove.com/video/60451/

Introduction

Cyanobacteria are autotrophic bacteria that can be used for the biosynthesis of a wide variety of natural and heterologous high value metabolic products[1,2,3,4,5,6]. Several hurdles still need to be overcome to expand their commercial viability, most notably, the relatively poor yields compared to heterotrophic bio-platforms (e.g., *Escherichia coli* and yeast)[7]. The recent expansion of available genetic engineering tools and uptake of the synthetic biology paradigm in cyanobacterial research is helping to overcome such challenges and further develop cyanobacteria as efficient biofactories[8,9,10].

The main approaches for introducing DNA into cyanobacteria are transformation, conjugation and electroporation. The vectors transferred to cyanobacteria by transformation or electroporation are "suicide" vectors (i.e., integrative vectors that facilitate homologous recombination), while self-replicating vectors can be transferred to cyanobacteria by transformation, conjugation or electroporation. For the former, a protocol is available for engineering model species amenable to natural transformation[11]. More recently, a modular cloning (MoClo) toolkit for cyanobacteria called CyanoGate has been developed that employs a standardized Golden Gate vector assembly method for engineering using natural transformation, electroporation or conjugation[12].

Golden Gate-type assembly techniques have become increasingly popular in recent years, and assembly standards and part libraries are now available for a variety of organisms[13,14,15,16,17]. Golden Gate uses type IIS restriction enzymes (e.g., BsaI, BpiI, BsmBI, BtgZI and AarI) and a suit of acceptors and unique overhangs to facilitate directional hierarchical assembly of multiple sequences in a "one pot" assembly reaction. Type IIS restriction enzymes recognize a unique asymmetric sequence and cut a defined distance from their recognition sites to generate a staggered, "sticky end" cut (typically a 4 nucleotide [NT] overhang), which can be subsequently exploited to drive ordered DNA assembly reactions[15,18]. This has facilitated the development of large libraries of modular Level 0 parts (e.g., promoters, open reading frames and terminators) defined by a common syntax, such as the PhytoBricks standard[19]. Level 0 parts can then be readily assembled into Level 1 expression cassettes, following which more complex higher order assemblies (e.g., multigene expression constructs) can be built in an acceptor vector of choice[12,15]. A key advantage of Golden Gate-type assembly techniques is their amenability to automation at high-throughput facilities, such as DNA foundries[20,21], which can allow for the testing of complex experimental designs that cannot easily be achieved by manual labor.

CyanoGate builds on the established Plant MoClo system[12,15]. To incorporate a new part into CyanoGate, the part sequence must first be domesticated, i.e., "illegal" recognition sites for BsaI and BpiI must be removed. In the case of a part coding for an open reading frame (i.e., a coding sequence, CDS), recognition sites can be disrupted by generating synonymous mutations in the sequence (i.e., changing a codon to an alternative that encodes for the same amino acid residue). This can be achieved by a variety of approaches, ranging from DNA synthesis

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to polymerase chain reaction (PCR) amplification-based strategies such as Gibson assembly[22]. Depending on the expression host being used, care should be taken to avoid the introduction of rare codons that could inhibit the efficiency of translation[23]. Removing recognition sites in promoter and terminator sequences is typically a riskier endeavor, as modifications may affect function and the part might not perform as expected. For example, changes to putative transcription factor binding sites or the ribosome binding site within a promoter could alter strength and responsiveness to induction/repression. Likewise, modifications to key terminator structural features (e.g., the GC rich stem, loop and poly-U tail) may change termination efficiency and effect gene expression[24,25]. Although several online resources are available to predict the activity of promoter and terminator sequences, and inform whether a proposed mutation will impact performance[26,27], these tools are often poor predictors of performance in cyanobacteria[28,29,30]. As such, in vivo characterization of modified parts is still recommended to confirm activity. To assist with the cloning of recalcitrant sequences, CyanoGate includes a low copy cloning acceptor vector based on the BioBrick vector pSB4K5[12,16,31]. Furthermore, a "Design and Build" portal is available through the Edinburgh Genome Foundry to help with vector design (dab.genomefoundry.org). Lastly, and most importantly, CyanoGate includes two Level T acceptor vector designs (equivalent to Level 2 acceptor vectors)[15] for introducing DNA into cyanobacteria using suicide vectors, or broad host-range vectors capable of self-replication in several cyanobacterial species[32,33,34].

Here we will focus on describing a protocol for generating Level T self-replicating vectors and the genetic modification of *Synechocystis* PCC 6803 and *Synechococcus elongatus* UTEX 2973 (*Synechocystis* PCC 6803 and *S. elongatus* UTEX 2973 hereafter) by conjugation (also known as tri-parental mating). Conjugal transfer of DNA between bacterial cells is a well described process and has been previously used for engineering cyanobacterial species, in particular those that are not naturally competent, such as *S. elongatus* UTEX 2973[35,36,37,38,39,40,41]. In brief, cyanobacterial cultures are incubated with an *E. coli* strain carrying the vector to be transferred (the "cargo" vector) and vectors (either in the same *E. coli* strain or in additional strains) to enable conjugation ("mobilizer" and "helper" vectors). Four key conditions are required for conjugal transfer to occur: 1) direct contact between cells involved in DNA transfer, 2) the cargo vector must be compatible with the conjugation system (i.e., it must contain a suitable origin of transfer (*oriT*), also known as a *bom* (basis of mobility) site), 3) a DNA nicking protein (e.g., encoded by the *mob* gene) that nicks DNA at the *oriT* to initiate single-stranded transfer of the DNA into the cyanobacterium must be present and expressed from either the cargo or helper vectors, and 4) the transferred DNA must not be destroyed in the recipient cyanobacterium (i.e., must be resistant to degradation by, for example, restriction endonuclease activity)[35,42]. For the cargo vector to persist, the origin of replication must be compatible with the recipient cyanobacterium to allow for self-replication and proliferation into daughter cells post division. To aid with conditions 3 and 4, several helper vectors are available through Addgene and other commercial sources that encode for *mob* as well as several methylases to protect from native endonucleases in the host cyanobacterium[43]. In this protocol, conjugation was facilitated by an MC1061 *E. coli* strain carrying mobilizer and helper vectors pRK24 (www.addgeneorg/51950) and pRL528 (www.addgene.org/58495), respectively. Care must be taken when choosing the vectors to be used for conjugal transfer. For example, in the CyanoGate kit the self-replicating cargo vector pPMQAK1-T encodes for a Mob protein[12]. However, pSEVA421-T does not[44], and as such, *mob* must be expressed from a suitable helper vector. The vectors used should also be appropriate to the target organism. For example, efficient conjugal transfer in *Anabaena* sp. PCC 7120 requires a helper vector that protects the mobilizer vector against digestion (e.g., pRL623, which encodes for the three methylases AvaiM, Eco47iiM and Ecot22iM)[45,46].

In this protocol we further outline how to characterize the performance of parts (i.e., promoters) with a fluorescent marker using a plate reader or a flow cytometer. Flow cytometers are able to measure fluorescence on a single cell basis for a large population. Furthermore, flow cytometers allow users to "gate" the acquired data and remove background noise (e.g., from particulate matter in the culture or contamination). In contrast, plate readers acquire an aggregate fluorescence measurement of a given volume of culture, typically in several replicate wells. Key advantages of plate readers over cytometers include the lower cost, higher availability and typically no requirement for specialist software for downstream data analyses. The main drawbacks of plate readers are the relatively lower sensitivity compared to cytometers and potential issues with the optical density of measured cultures. For comparative analyses, plate reader samples must be normalised for each well (e.g., to a measurement of culture density, typically taken as the absorbance at the optical density at 750 nm [OD_{750}]), which can lead to inaccuracies for samples that are too dense and/or not well mixed (e.g., when prone to aggregation or flocculation).

As an overview, here we demonstrate in detail the principles of generating Level 0 parts, followed by hierarchical assembly using the CyanoGate kit and cloning into a vector suitable for conjugal transfer. We then demonstrate the conjugal transfer process, selection of axenic transconjugant strains expressing a fluorescent marker, and subsequent acquisition of fluorescence data using a flow cytometer or a plate reader.

Protocol

1. Vector assembly using the Plant MoClo and CyanoGate toolkits

NOTE: Before proceeding with vector assembly, it is strongly recommended that users familiarize themselves with the vector level structures of the Plant and CyanoGate MoClo systems[12,15].

1. Construction of Level 0 parts
   NOTE: Level 0 parts can be synthesized as complete vectors or as linear sequences for assembly with Level 0 acceptors (e.g., gBlocks, IDT). Alternatively, sequences can be amplified from a source template (e.g., a vector or purified genomic DNA). Here, how to generate a new Level 0 part from an amplified product is described. An overview of the Golden Gate assembly process from Level 0 to Level T is shown in **Figure 1**.
   1. Design the primers.
      1. Decide what Level 0 module to assemble and identify the appropriate 5′ and 3′ overhangs (**Table 1**)[12,15]. Check the DNA sequence to clone for the presence of BpiI or BsaI restriction sites.
         NOTE: A sequence containing one of these sites must be domesticated by modifying one or more NTs in the restriction site sequence. A strategy for doing this using Golden Gate assembly is outlined in **Figure 2**.
      2. To amplify a DNA sequence, design an appropriate forward and reverse primer pair. For the forward primer, select 18−30 bp complementary to the 5′ end of the DNA template sequence. For the reverse primer, select 18−30 bp reverse complementary to the 3′ end of the DNA template sequence.

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NOTE: Primers with melting temperatures (T_m) of 58−62 °C typically give the most consistent amplification results (**Figure 1A**).

3. Add the following to the 5′ end of the forward primer: 1) a random string of 4−6 NTs at the 5′ end of the BpiI site, 2) the BpiI restriction site (GAAGAC), 3) two random NTs, and 4) the 5′ overhang selected in step 1.1.1.1. Add the following to the 5′ end of the reverse primer: 1) a random string of 4−6 NTs at the 5′ end of the BpiI site, 2) the BpiI restriction site (GAAGAC), 3) two random NTs, and 4) the 3′ overhang selected in step 1.1.1.1. When finalized, order the primer pairs.
NOTE: See **Figure 1A** for an example of a forward and reverse primer pair.

2. Amplify a DNA sequence from genomic DNA.
    1. Extract genomic DNA as described in section 5. Amplify products by PCR using a high-fidelity DNA polymerase (**Table of Materials**).
    NOTE: As an example, set up PCR reactions (20−50 µL) according to manufacturer's instructions. Use ~100 ng of genomic DNA per reaction. Use a thermal cycling program consisting of an initial denaturation step of 98 °C for 30 s, followed by no more than 25 cycles of denaturation at 98 °C for 10 s, primer annealing at 58 °C for 15 s and product extension at 72 °C for 30 s (modify the latter depending on the size of the product/type of DNA polymerase used), followed by a final extension step of 72 °C for 2 min.
    2. If the PCR product is to be gel purified, run the entire PCR reaction on an agarose gel as described in section 6. Cut the band of interest out of the agarose gel and purify it using a gel extraction kit (**Table of Materials**).
    3. Alternative to step 1.1.2.2, if the PCR product is to be used without gel purification, verify the band size by running an aliquot of the PCR reaction sample (~5 µL) on an agarose gel. If the gel shows only the appropriate band and no evidence of primer dimers, purify the PCR product using a DNA purification kit (**Table of Materials**).
    4. Elute purified DNA in a small volume of deionized water (e.g., 10 µL) to obtain a high DNA concentration (>20 ng/µL is typically sufficient).

3. Assemble the amplified DNA product (or products, see **Figure 2**) in Level 0. Prepare a 20 µL reaction mix with BpiI (**Figure 1B**) and set up the thermal cycler program as described in **Table 2**. Proceed to *E. coli* transformation using 5 µL of the assembled Level 0 reaction mix (as described in section 2).

2. Construction of Level 1 assemblies
    1. Decide what Level 0 parts to assemble (**Figure 1C** and **Table 1**). Choose an appropriate Level 1 acceptor vector[15].
    NOTE: At this stage it is important to know what the final vector design will be in Level T, as this will impact the choice of Level 1 acceptor vector. Level 1 position 1 (Forward) acceptor vector (pICH47732) can be used as a default if the goal is to have a single Level 1 assembly (e.g., a gene expression cassette) in Level T. However, if two or more Level 1 assemblies are to be assembled in Level T, the position and direction of each Level 1 assembly must be considered. Up to seven Level 1 assemblies can be assembled in a Level T acceptor vector by using Level 1 acceptor vectors with appropriate positions[12].
    2. Assemble the Level 0 parts in Level 1. Prepare a 20 µL reaction mix with BsaI and set up the thermal cycler program as described in **Table 2**. Proceed to *E. coli* transformation using 5 µL of the assembled Level 1 reaction mix (as described in section 2).

3. Construction of Level T assemblies
    1. Decide what Level 1 assemblies to assemble (**Figure 1D**). Choose an appropriate Level T acceptor vector.
    NOTE: pUC19A-T (ampicillin resistance) and pUC19S-T (spectinomycin resistance) are high-copy number integrative vectors that are not able to replicate in cyanobacteria and are primarily used for genomic integration (i.e., knock-in or knock-out of genes) via homologous recombination[12]. Delivery of integrative vectors can proceed by natural transformation in amenable cyanobacterial species[11]. pPMQAK1-T is a broad host range, replicative vector that is delivered by conjugal transfer (section 3).
    2. Choose an appropriate End-Link to ligate the 3′ end of the final Level 1 assembly to the Level T backbone[15].
    NOTE: The End-Link required is the same number as the position of the final part. For example, a Level T vector with only one Level 1 position 1 (forward or reverse) part will require End-Link 1 (pICH50872) for ligation into the Level T backbone.
    3. Assemble one or more Level 1 assemblies in Level T. Prepare a reaction mix with BpiI and the required End-Link vector and set up the thermal cycler program as described in **Table 2**. Proceed to *E. coli* transformation using 5 µL of the assembled Level T reaction mix (as described in section 2).

2. *E. coli* transformation and vector purification

1. *E. coli* transformation (day 1)
    1. Defrost an aliquot (~25 µL) of chemically competent *E. coli* cells (**Table of Materials**) and gently pipette into a 1.5 mL tube on ice. Add 5 µL of the assembly mix (Level 0, 1 or T) and incubate the tube on ice for a further 30−60 min.
    2. Heat-shock cells by incubating the tube in a water bath at 42 °C for 30 s, then place the tube back on ice for 2 min. Add room temperature (RT) super optimal broth with catabolite repression (S.O.C.) medium (250 µL) to the tube. Incubate the tube at 37 °C for 1 h at 225 rpm in a shaking incubator.
    3. Plate 40 µL of the culture onto an LB agar plate containing the appropriate final concentration of antibiotics (100 µg/mL for spectinomycin dihydrochloride pentahydrate [Level 0], 100 µg/mL of carbenicillin disodium [Level 1], or 50 µg/mL of kanamycin sulphate [Level T]), 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) and 40 µg/mL of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) for blue-white screening. Incubate the plate overnight at 37 °C.
    NOTE: The amount of culture plated can be varied depending on the efficiency of the *E. coli* competent cells and the ligation reaction. Plate a larger volume if <10 colonies are observed after overnight incubation.

2. Selection of white colonies and preparation of liquid cultures (day 2)
NOTE: Depending on the efficiencies of the assembly reaction and subsequent transformation, LB agar plates may contain no colonies, blue colonies or white colonies (**Figure 3**). Blue colonies are indicative of acceptor vectors that have not undergone restriction (i.e., a functional copy of *lacZ* is still present). White colonies indicate that the *lacZ* expression cassette has been lost and replaced by a part/assembly.
    1. Optionally, validate that white colonies contain the expected vector by performing PCR as described in section 7.

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2. Pick single white colonies (or PCR verified colonies) with a 10 µL tip and transfer to a 15 mL centrifuge tube containing LB medium (5 mL) and appropriate antibiotic concentrations (step 2.1.3). Incubate the tubes at 37 °C overnight at 225 rpm in a shaking incubator.

3. Plasmid vector purification (day 3)
   1. Optionally, prepare a glycerol stock of the overnight *E. coli* culture for long-term cryostorage of vectors. Add 500 µL of bacterial culture to 500 µL of 50% (v/v) glycerol in an appropriate 1.5−2.0 mL tube for cryostorage at -80 °C. Mix gently by inverting 5−10x. Flash-freeze samples in liquid nitrogen and store in a -80 °C freezer.
   2. Spin down cultures in 15 mL centrifuge tubes at 3,000 x *g* for 5−10 min. Discard the supernatant without disturbing the cell pellet. Purify the vector using a plasmid purification kit (**Table of Materials**). Elute purified vector in 35 µL of deionized water.
      NOTE: Use lower elution volumes to further increase the vector concentration. The same eluent can be put through a purification column twice for increased yields.
   3. Measure the concentration of the vector in the eluent using a spectrophotometer (**Table of Materials**).
      NOTE: High copy-number vectors in *E. coli*, such as pUC19, typically give yields of 50−300 ng/µL. Low copy-number vectors, such as pPMQAK1-T, typically give yields of 15−60 ng/µL.

4. Vector validation
   NOTE: Vectors can be verified by restriction digestion (step 2.4.1) and/or sequencing (step 2.4.2).
   1. Restrict 0.5−1 µg of vector with an appropriate restriction enzyme(s) and verify the expected band sizes as described in section 6 (**Figure 4**).
      NOTE: Incorrect band sizes typically indicate erroneous assembly, in which case more white colonies can be screened or assembly can be repeated. BsaI and BpiI can be used to validate the correct size of the insert for Level 0 and Level 1 assemblies, respectively. BsaI or BpiI can be used in conjunction with an additional, compatible restriction enzyme that cuts within the insert and/or the vector backbone to produce a distinct set of well-separated bands following digestion.
   2. Sequence the vector by Sanger sequencing using an appropriate primer upstream of the assembled region using commercial sequencing facility (**Table 3**).
      NOTE: All new Level 0 parts should be sequenced to confirm the expected sequence identity. Sequence validation of Level 1 and T vectors is not typically required if assembled from previously sequenced level 0 parts.

3. Generation of mutants by conjugation

NOTE: Here, a protocol for conjugal transfer of a self-replicating cargo vector into *Synechocystis* PCC 6803 or *S. elongatus* UTEX 2973[11,47] is described. This protocol is applicable to other model species (e.g., *S. elongatus* PCC 7942 and *Synechococcus* sp. PCC 7002). All work with cyanobacteria (and associated buffer preparations) should be done under sterile conditions in a laminar flow hood.

1. Growth of the cyanobacterial culture (day 1)
   1. Prepare BG11 medium according to Lea-Smith et al.[11], and agar plates with LB-BG11 and BG11+Kan50 (section 8).
   2. Set up a fresh culture of *Synechocystis* PCC 6803 or *S. elongatus* UTEX 2973 by inoculating a 100 mL conical flask of fresh BG11 medium (50 mL) with cells sourced from an axenic BG11 agar plate. Grow *Synechocystis* PCC 6803 cultures at 30 °C, 100 µmol photons $m^{-2}s^{-1}$ at 100 rpm and grow *S. elongatus* UTEX 2973 at 40 °C, 300 µmol photons $m^{-2}s^{-1}$ at 100 rpm. Grow cultures until OD_{750} = 0.5−1.5 (typically 1−2 days).
      NOTE: *S. elongatus* UTEX 2973 cultures can be grown at 40 °C in high light intensities (e.g., 2000 µmol photons $m^{-2}s^{-1}$)[48].

2. Growth of helper and cargo *E. coli* strains (day 2)
   1. Inoculate LB medium containing ampicillin (final concentration 100 µg/mL) and chloramphenicol (final concentration 25 µg/mL) with a MC1061 *E. coli* strain containing vectors pRK24 and pRL528 (i.e., the helper strain) and grow at 37 °C overnight at 225 rpm in a shaking incubator. Grow up a sufficient volume of helper strain culture, assuming 1 mL of culture is required per conjugation.
   2. Inoculate LB medium (5 mL) containing appropriate antibiotics with the *E. coli* culture carrying the cargo vector (i.e., a Level T vector). Grow the culture at 37 °C overnight at 225 rpm in a shaking incubator.

3. Conjugal transfer (tri-parental mating) (day 3)
   1. Prepare the *E. coli* helper and cargo strains. Centrifuge the helper and the cargo *E. coli* overnight cultures at 3,000 x *g* for 10 min at room temperature. Discard the supernatant without disturbing the cell pellet.
   2. Wash the pellet by adding fresh LB medium without antibiotics. Use the same volume as the initial culture. Resuspend the pellet by gently pipetting up and down. Do not vortex the culture. Repeat this step 3x to remove residual antibiotics from the overnight culture.
   3. Centrifuge the resuspended culture (as in step 3.3.1), discard the supernatant and resuspend in half the volume of LB medium of the initial culture volume (e.g., 2.5 mL if the overnight culture was 5 mL). Combine 450 µL of the helper strain with 450 µL of the cargo strain in a 2 mL tube and set aside (leave at RT) until step 3.3.6.
   4. Prepare the cyanobacterial culture. For each conjugation reaction, use 1 mL of cyanobacterial culture (OD_{750} = 0.5−1.5).
   5. Centrifuge the required total volume of cyanobacterial culture at 1,500 x *g* for 10 min at RT, then discard the supernatant carefully without disturbing the cell pellet. Wash the pellet by adding fresh BG11 medium of the same initial volume. Resuspend the pellet by gently pipetting up and down, do not vortex the culture. Repeat this step 3x and set the washed culture aside.
   6. Add an aliquot of washed cyanobacterial culture (900 µL) to the combined *E. coli* strains (helper and cargo) (900 µL) in a 2 mL tube. Mix the cultures by gently pipetting up and down. Do not vortex. Incubate the mixture at RT for 30 min for *Synechocystis* PCC 6803 or 2 h for *S. elongatus* UTEX 2973.
   7. Centrifuge the mixture at 1,500 x *g* for 10 min at RT. Remove 1.6 mL of the supernatant. Resuspend the pellet in the remaining ~200 µL of supernatant. Place one 0.45 µm membrane filter on an LB-BG11 agar plate lacking antibiotics (section 8). Carefully spread 200 µL of the *E. coli*/cyanobacterial culture mix on the membrane with a sterile spreader or a sterile bended tip and seal the plate with paraffin film.

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8. Incubate the LB-BG11 plate with the membrane for 24 h. Maintain membranes with *Synechocystis* PCC 6803 cultures at 30 °C, 100 µmol photons $m^{-2}s^{-1}$. Maintain membranes with *S. elongatus* UTEX 2973 cultures at 40 °C in 150 µmol photons $m^{-2}s^{-1}$.

4. Membrane transfer
   1. After 24 h, carefully transfer the membrane using flame-sterilized forceps to a fresh BG11 agar plate containing appropriate antibiotics (section 8) to select for the cargo vector. Seal the plate with paraffin film.
   2. Incubate the BG11 agar plate under appropriate growth conditions, as described above for *Synechocystis* PCC 6803 or *S. elongatus* UTEX 2973, until colonies appear.
      NOTE: Colonies typically appear after 7−14 days for *Synechocystis* PCC 6803 and 3−7 days for *S. elongatus* UTEX 2973.

5. Selection of conjugants
   NOTE: Only cyanobacterial colonies carrying the cargo vector will be able to grow on the membrane (**Figure 5**).
   1. Using a heat sterile loop, select at least two individual colonies from the membrane and streak onto a new BG11 agar plate containing appropriate antibiotics (**Figure 5C**).
      NOTE: Freshly streaked colonies may still be contaminated with *E. coli* carried over from conjugation (i.e., if small white colonies are evident on the plate), so two or three additional rounds of re-streaking onto fresh BG11 agar plates typically are needed to obtain an axenic cyanobacterial culture.
   2. Confirm absence of *E. coli* contamination by inoculating a streak of cyanobacterial culture into a 15 mL centrifuge tube containing 5 mL of LB medium and incubating at 37 °C overnight at 225 rpm in a shaking incubator. Following a sufficient growth period (~7 days), pick individual axenic colonies to set up liquid cultures for long-term cryostorage or subsequent experimentation.

6. Cryostorage of cyanobacterial strains
   1. Grow a cyanobacterial liquid culture in BG11 (as described in section 3.1) until OD_{750} = 1.5−3.0. Centrifuge 10 mL of culture for 10 min at 1,500 x *g*, remove the supernatant and resuspend the cells in 5 mL of fresh BG11 medium.
   2. Add 3.5 mL of autoclave sterilized 50% (v/v) glycerol for a final glycerol concentration of ~20% (v/v)[49]. This approach works well for *Synechocystis* PCC 6803. Alternatively, add 5 mL of filter sterilized BG11 containing 16% (v/v) dimethyl sulfoxide (DMSO) for a final DMSO concentration of ~8% (v/v)[50]. This approach is recommended for most strains, including *S. elongatus* UTEX 2973.
      CAUTION: DMSO is toxic and should be handled with appropriate protection.
   3. Mix gently by inverting 5−10x. Subaliquot ~1 mL of culture into separate cryostorage compatible 1.5 mL screw-cap tubes (**Table of Materials**). Place tubes in a -80 °C freezer for cryostorage. Do not flash freeze in liquid nitrogen.
      NOTE: At least three stocks per strain are recommended.
   4. For recovery, remove a tube from the -80 °C freezer and thaw the culture in a 35 °C water bath while gently mixing. Add the thawed culture to 50 mL of fresh BG11 medium and grow as a liquid culture (as described in section 3.1).
      NOTE: Alternatively, the culture can be streaked and grown on a fresh BG11 agar plate. Transgenic cultures carrying selection markers must be revived initially on BG11 agar plates without antibiotics and then restreaked onto BG11 agar plates with appropriate antibiotics.

4. Promoter characterization

NOTE: Here a standard approach is described for analyzing the strength of a promoter part by measuring the expression levels of a fluorescent marker (eYFP) following a 72 h growth period using either a plate reader or a flow cytometer[12].

1. Culture growth
   1. Set up seed cultures by inoculating 10 mL of BG11 medium containing appropriate antibiotics with a single colony of the transgenic cyanobacterial strain carrying the fluorescent marker expression cassette. Also prepare seed cultures for appropriate negative control strains (e.g., a wild type strain and/or a transgenic strain carrying the same vector backbone but lacking the fluorescent marker expression cassette).
      NOTE: At least four biological replicates are recommended.
   2. Grow the seed cultures for 48 h or until OD_{750} = 1−1.5 under growth conditions appropriate for the species strain.
   3. To track promoter expression over time, first measure the OD_{750} of each seed culture. Calculate the dilution requirements to bring each culture to a starting OD_{750} = 0.2. Set up diluted experimental culture samples (2 mL total volume) in a flat-bottom 24-well plate (**Table of Materials**).
   4. Incubate the plate in a shaking incubator with white LED lights (**Table of Materials**) under appropriate growth conditions. Measure culture growth density (OD_{750}) and enhanced yellow fluorescent protein (eYFP) fluorescence using either a plate reader (section 4.2) or a flow cytometer (section 4.3).
      NOTE: *Synechocystis* PCC 6803 and *S. elongatus* UTEX 2973 cultures can be grown as in step 3.1.2. It is highly recommended that the plate be maintained under a high humidity (95%) to avoid evaporation of the culture samples.

2. Plate reader
   1. Briefly mix the cultures in the 24-well plate (step 4.1.4) with gentle pipetting. Transfer a sub-sample of each culture to a black flat-bottom 96-well plate (**Table of Materials**). Dilute if necessary (100 µL final volume). Avoid the formation of bubbles as this can interfere with measurement accuracy.
      NOTE: It is recommended that all measurements be performed on samples in an OD_{750} range of 0.2−1.0. As the density of the cultures in the 24-well will increase over time, the following dilutions are recommended based on the expected increases in standard growth conditions: no dilution at 0 h, 1:4 at 24 h, 1:10 at 48 h and 1:10 at 72 h. So for example, at 24 h harvest 25 µL of culture and mix with 75 µL of BG11 medium.
   2. Include two blank wells in the 96-well plate (i.e., 100 µL of BG11 medium). Put the 96-well plate into a plate reader (**Table of Materials**). Shake the plate for 60 s at 500 rpm using the orbital shaker in the plate reader to mix the wells.
      NOTE: Cyanobacterial cultures can aggregate and/or flocculate, so good mixing is critical prior to reading for accurate measurements.
   3. Measure OD_{750} and eYFP fluorescence with excitation/emission wavelengths at 485 nm/520 nm.

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4. Subtract the average of the OD_{750} measurements of the two blank wells from the OD_{750} measurement of each sample well containing cyanobacteria culture.
5. Normalize the fluorescence values of each culture sample by dividing the eYFP fluorescence measurement (step 4.2.3) by the adjusted OD_{750} of the culture (step 4.2.4). Then, subtract the average normalized eYFP fluorescence value (eYFP fluorescence/OD_{750}) of the biological replicates of an appropriate negative control strain from the transgenic strains carrying the eYFP expression cassette.
NOTE: Cyanobacteria naturally fluoresce due the presence of pigments, such as chlorophyll and phycobiliproteins.
6. Plot culture growth over time (**Figure 6A**) and the average normalized eYFP fluorescence values of each experimental culture at the desired time points (e.g., 72 h; **Figure 6B**).

3. Flow cytometer
    1. Choose a compatible plate for the flow cytometer liquid handling system. For example, use a round-bottom 96-well plate (**Table of Materials**) with the flow cytometer (**Table of Materials**) used in this protocol.
    2. Briefly mix the cultures in the 24-well plate (step 4.1.4) with gentle pipetting. Dilute cultures to OD_{750} = 0.1−0.2 to avoid nozzle blockages in the liquid handling system. Add an appropriate volume of culture sample to the 96-well plate and bring to a final volume of 250 µL with filter-sterilized 1x phosphate-buffered saline (PBS). Include a blank well for the medium solution on the plate containing 60 µL of BG11 and 190 µL of 1x PBS.
    NOTE: This volume is recommended in case there is a need to re-run samples. Volumes higher than 250 µL are not recommended as the maximum volume of each well is 300 µL.
    3. Once the flow cytometer is ready for use, place the 96-well plate with culture samples in the liquid handling station. Set up the software protocol for the flow cytometer to collect the measurements of 10,000 individual events (e.g., cells). Measure eYFP fluorescence with excitation/emission wavelengths of 488 nm/515−545 nm. First measure and check the reading from the blank well (**Figure 7A**), then run the samples.
    4. Gate the population of cyanobacteria cells within the forward and side scatter data sets, excluding regions common with the blank reading (**Figure 7B**). Subtract the average eYFP fluorescence values of the biological replicates of an appropriate negative control strain from the transgenic strains carrying the eYFP expression cassette (**Figure 7C,D**). Plot the average of the median fluorescence values per cell for each experimental culture at the desired time points (e.g., 72 h; **Figure 7E**).

5. Genomic DNA extraction from cyanobacteria

NOTE: The protocol below uses a commercial DNA extraction kit (**Table of Materials**).

1. Grow a cyanobacterial liquid culture in BG11 (as described in section 3.1) until OD_{750} = 1.5−3.0. Spin down 10 mL of culture at 3,000 x *g* for 10 min and discard the supernatant. Freeze the pellet by incubating the tubes at -20 °C for 30 min.
2. Add 400 µL of lysis buffer (buffer AP1) and 400 µL of ribonuclease solution (RNase A), and 50% (w/v) of glass beads (0.5 mm diameter). Disrupt samples using a bead mill (**Table of Materials**) at 30 Hz (i.e., equivalent to 1,800 oscillations/min) for 6 min.
3. Spin the sample at 17,000 x *g* for 5 min and carefully transfer the supernatant into a new tube and discard the pellet. Proceed according to the manufacturer's instructions (**Table of Materials**).

6. Agarose gel electrophoresis

1. Cast a 1% (w/v) agarose gel containing 0.02% (v/v) ethidium bromide. Load samples and an appropriate DNA ladder reference.
2. Run the samples for 50 min at 125 V. Check for band separation on an ultraviolet (UV) transilluminator.
NOTE: The running time and agarose gel percentage can be modified to suit the expected band size. For example, a higher percentage agarose gel and longer running time may improve the band resolution and separation of DNA products <500 bp.

7. Colony PCR

1. Set up a PCR reaction mix using a standard kit (**Table of Materials**) and an appropriate combination of primers (e.g., primers that flank the assembly region or are specific to sequences within the assembly region (**Table 3**). Pipette 10 µL into a PCR tube.
2. Gently touch the top of a single white colony with a sterile toothpick or 10 µL pipette tip and inoculate a PCR tube containing PCR reaction mix. Take care to mark the colony and match with the specific PCR tube. Gently stir the reaction mix to ensure *E. coli* cells are shed into the solution.
3. Amplify products by PCR. Use a program consisting of an initial denaturation step of 95 °C for 60 s, 30 rounds of 95 °C for 15 s, 58 °C for 15 s (few degrees below the T_m values of the primers) , 72 °C for 30 s (30 s/kb of insert), followed by a final extension step of 72 °C for 5 min.

8. Preparation of BG11 medium and plates

1. Prepare stock solutions of 100x BG11 medium, iron (ammonium ferric citrate), trace elements, phosphate (K_2HPO_4), Na_2CO_3 and TES buffer according to Lea-Smith et al.[11]. Autoclave phosphate and Na_2CO_3 stocks. Use 0.2 µm filters to filter sterilize the TES buffer (pH 8.2) and $NaHCO_3$ stock solutions.
2. Prepare 1 L of BG11 medium. Mix 10 mL of 100x BG11, 1 mL of trace elements and 1 mL of iron stock and autoclave the solution with 976 mL of water. Once the solution has cooled down to RT, add 1 mL of phosphate stock, 1 mL of Na_2CO_3 stock and 10 mL of $NaHCO_3$, and adjust to pH 7.6−7.8 with 1 M HCl.
3. LB-BG11 agar plates (1.5% [w/v])
    1. Combine 700 mL of deionized water and 15 g of agar in a glass flask. In a second flask, add 186 mL of water, 10 mL of 100x BG11, 1 mL of trace elements and 1 mL of iron stock. Autoclave both solutions.

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2. Once the solutions have cooled down to around 60 °C, combine them and add 1 mL of phosphate stock, 1 mL of Na_2CO_3 stock, 10 mL of $NaHCO_3$ stock and 50 mL of LB sterile medium, which should give a final volume of 1 L. Cast Petri dishes with 25 mL of LB-BG11 agar medium.

4. BG11+Kan50 agar plates (1.5% [w/v])
    1. Combine 700 mL of deionized water and 15 g of agar in a glass flask. In a second flask, add 3 g of sodium thiosulphate ($Na_2S_2O_3$), 226 mL of water, 10 mL of 100x BG11 stock, 1 mL of trace elements and 1 mL of iron stock. Autoclave both solutions.
    2. Once the solutions have cooled down to around 60 °C, combine them and add 1 mL of phosphate stock, 1 mL of Na_2CO_3 stock, 10 mL of TES buffer stock, and 10 mL of $NaHCO_3$ stock, which should give a final volume of 1 L. Add kanamycin sulphate to a final concentration of 50 µg/mL and cast Petri dishes with 35 mL of medium.

Representative Results

To demonstrate the Golden Gate assembly workflow, an expression cassette was assembled in the Level 1 position 1 (Forward) acceptor vector (pICH47732) containing the following Level 0 parts: the promoter of the C-phycocyanin operon P_{cpc560} (pC0.005), the coding sequence for eYFP (pC0.008) and the double terminator T_{rrnB} (pC0.082)[12]. Following transformation of the assembly reaction, successful assemblies were identified using standard blue-white screening of *E. coli* colonies (**Figure 3**). The eYFP expression cassette in the Level 1 vector and the End-Link 1 vector (pICH50872) were then assembled into a Level T acceptor vector (pPMQAK1-T) to give the vector *cpcBA*-eYFP (**Figure 4A**). The assembled *cpcBA*-eYFP vector was verified by restriction digestion (**Figure 4B**).

Successful conjugal transfer of *cpcBA*-eYFP or the empty pPMQAK1-T vector (i.e., a negative control lacking the eYFP expression cassette) resulted in the growth of up to several hundred colonies on the membrane for *Synechocystis* PCC 6803 and *S. elongatus* UTEX 2973 after 7−14 days and 3−7 days, respectively (**Figure 5**). Individual colonies were picked and streaked onto fresh BG11+Kan50 agar plates; 2−3 re-streaks were required to generate axenic cultures.

As expected for the strong P_{cpc560} promoter[51], the values for normalised eYFP fluorescence from the plate reader and eYFP fluorescence per cell from the flow cytometer were high compared to the negative control (**Figure 6** and **Figure 7**). Fluorescence values were higher in *S. elongatus* UTEX 2973 than in *Synechocystis* PCC 6803[12].

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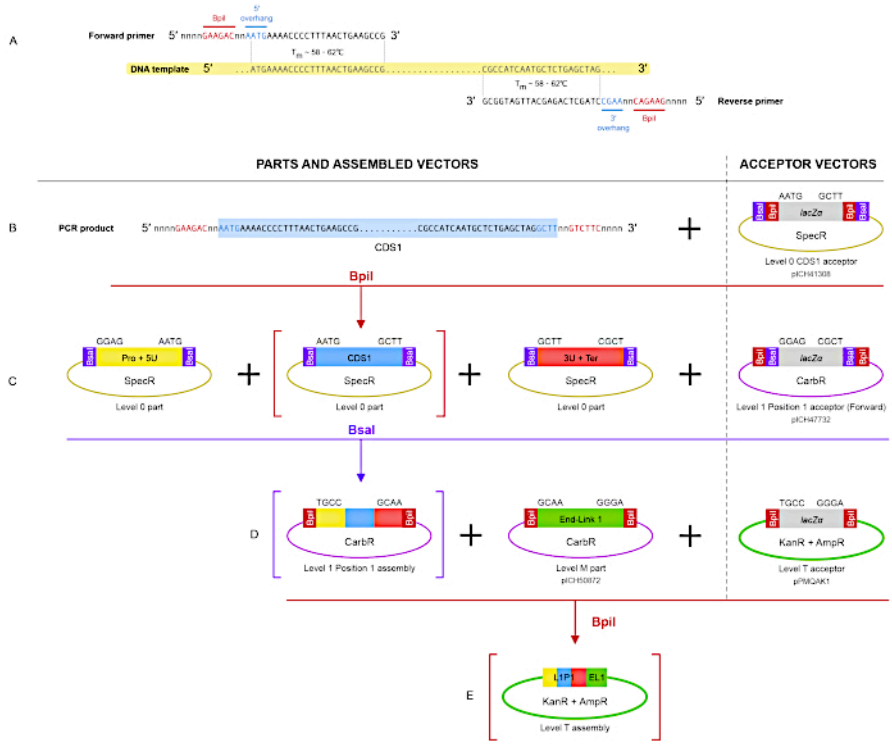

Figure 1: Overview of the Golden Gate assembly process in CyanoGate. Assembly of a gene expression cassette is shown, starting from amplification of a sequence of interest from template DNA to assembly in a Level T vector (parts are not drawn to scale). (**A**) Design of forward and reverse primers for amplification of a CDS1 part from template DNA (e.g., genomic or plasmid DNA). The locations of the BpiI restriction sites and overhangs required for insertion into the acceptor vector (i.e., 5′ and 3′ of the template sequence) are highlighted in red and blue, respectively. The letter "n" denotes that any NT can be used at this position. The annealing regions of the primers with the DNA template and their recommended melting temperatures (T_m) are indicated. (**B**) Level 0 assembly of the PCR product into the Level 0 CDS1 acceptor vector pICH41308. The sequence that will be excised by BpiI and ligated into the acceptor vector is highlighted in blue. (**C**) Level 1 assembly of a gene expression cassette containing three Level 0 parts (Pro + 5U, CDS1 and 3U + Ter) into the Level 1 position 1 (forward) acceptor vector pICH47732 using BsaI. (**D**) Level T assembly of the Level 1 assembly and End-Link 1 (pICH50872, called "Level M End-link 1" in Engler et al.[15]) into a Level T acceptor vector (e.g., pPMQAK1-T) using BpiI. (**E**) The final assembled Level T vector. Abbreviations: AmpR, ampicillin resistance cassette; CarbR, carbenicillin resistance cassette; CDS1, coding sequence in the Level 0 syntax[15]; EL1, End-Link 1 part; KanR, kanamycin resistance cassette; *lacZα*, β-galactosidase expression cassette; SpecR, spectinomycin resistance cassette. Please click here to view a larger version of this figure.

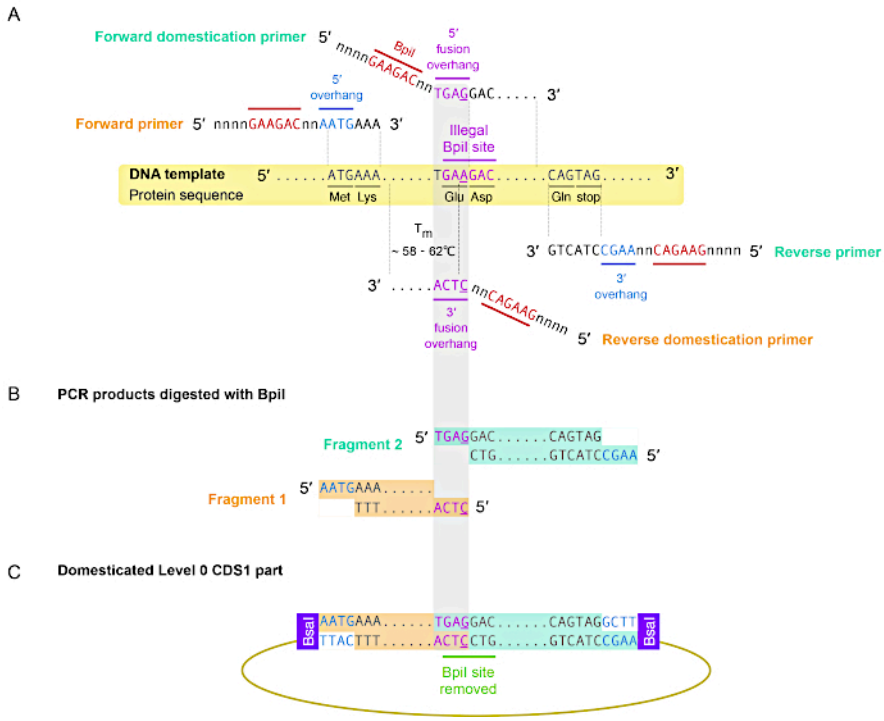

Figure 2: A PCR-based domestication strategy for removal of an illegal type IIS restriction site. (**A**) Schematic diagram showing two primer pairs (in green and orange, respectively) for modifying a BpiI site (GAAGAC) to GAGGAC in a protein coding DNA sequence intended for assembly into the CDS1 Level 0 acceptor vector (pICH41308). Note that modification preserves the codon for glutamic acid (Glu) (i.e., GAA to GAG). Although the BpiI site is shown in frame with the start codon, this approach will work even if the site is not in frame (i.e., as long as the site is disrupted, and the protein sequence preserved). The locations of the BpiI restriction sites and overhangs in the primers are highlighted in red and blue, respectively. The DNA template and the translated protein sequence is highlighted in yellow. The annealing regions of the primers with the DNA template and their recommended melting temperatures (T_m) are indicated. The orange pair is used to amplify the 5′ end of the sequence with overhangs AATG and TGAA (fragment 1), while the green pair is used to amplify the 3′ end with overhangs TGAA and GCTT (fragment 2). Before ordering the primers, the fidelity of the TGAA fusion overhang for Fragment 1 and 2 was carefully checked[52]. Poorly designed fusion overhangs can lead to assembly failure (i.e., no colonies following transformation; **Figure 5**) or false positives (e.g., truncated or erroneous assemblies). The latter can be resolved be screening a larger number of white colonies to identify a correctly assembled construct. (**B**) Amplicons of fragments 1 and 2 after restriction with BpiI during Golden Gate assembly. (**C**) The domesticated sequence assembled into the Level 0 CDS1 acceptor vector. Please click here to view a larger version of this figure.

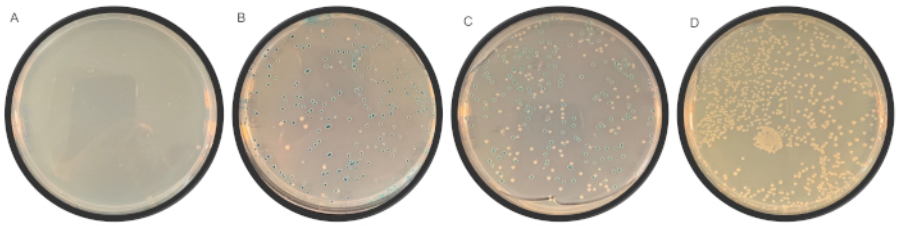

Figure 3: Blue-white colony screening of *E. coli* transformants following Golden Gate assembly. The plates shown contain LB agar (1% [w/v]) supplemented with X-Gal, IPTG and antibiotics at appropriate concentrations. (**A**) No colonies, suggesting a failed assembly reaction and/or *E. coli* transformation. (**B**) Mostly blue colonies, indicating a successful assembly, but that the efficiency of restriction enzyme used in the assembly reaction was low. (**C**) Mostly white colonies, indicative of a typical, successful assembly reaction. (**D**) No blue colonies, indicating very efficient assembly. Please click here to view a larger version of this figure.

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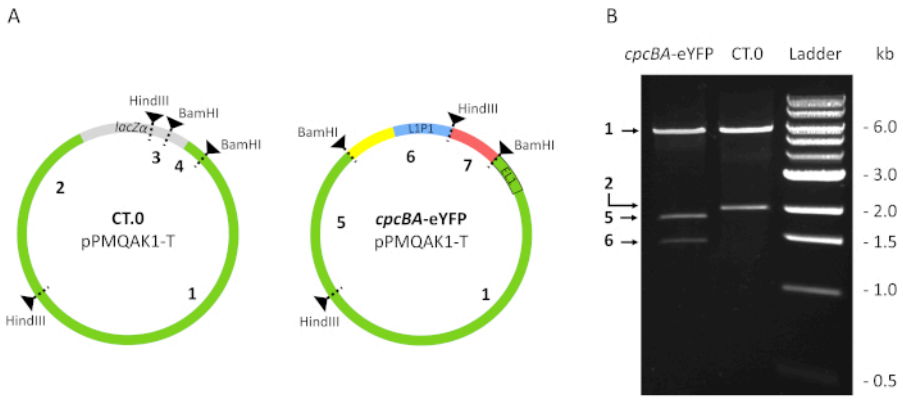

Figure 4: Verification of an assembled Level T vector by restriction digestion. The vectors were digested with HindIII and BamHI. (**A**) Sequence map of empty pPMQAK1-T acceptor vector (CT.0) and Level T assembly (*cpcBA*-eYFP) showing components of the eYFP expression cassette (P_{cpc560}-eYFP-T_{rrnB}). The positions of the restriction sites for HindIII and BamHI are indicated. Following double digestion, the predicted sizes of the DNA fragments are indicated: (1) 5,847 bp, (2) 2,004 bp, (3) 30 bp, (4) 374 bp, (5) 1,820 bp, (6) 1,289 bp, and (7) 156 bp. (**B**) An agarose gel (0.8% [w/v]) run at 125 V for 60 min loaded with the digested Level T assembly (*cpcBA*-eYFP) showing bands 1, 5 and 6, the digested empty pPMQAK1-T acceptor vector (CT.0) showing bands 1 and 2 and a DNA ladder (**Table of Materials**). Note that bands 3, 4 and 7 were too small to visualize on the gel. Please click here to view a larger version of this figure.

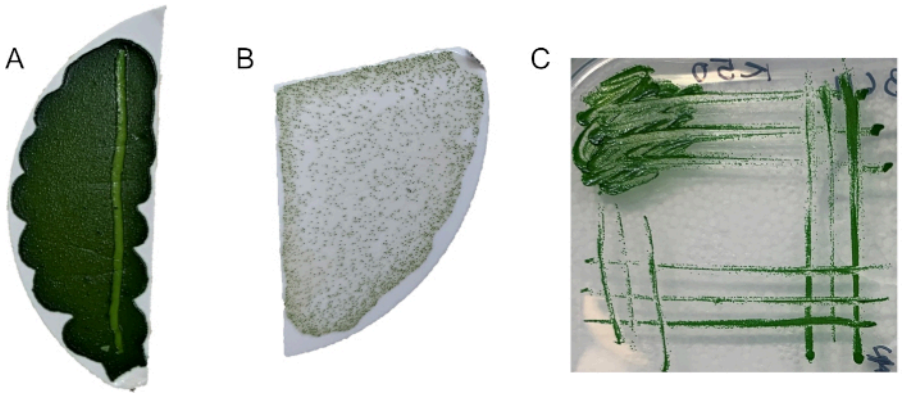

Figure 5: Growth of transgenic *Synechocystis* PCC 6803 colonies following successful conjugation. Examples of membranes following incubation on BG11+Kan50 agar plates are shown. (**A**) Overgrowth following very efficient conjugation-the *Synechocystis* PCC 6803 colonies have developed into a lawn with no individual colonies. (**B**) A good conjugation efficiency showing several hundred individual colonies after 12 days. (**C**) Growth of an axenic strain after 14 days following several rounds of re-streaking onto a fresh BG11+Kan50 agar plate. Absence of bacterial contamination indicated that the *Synechocystis* PCC 6803 transconjugant was axenic. Please click here to view a larger version of this figure.

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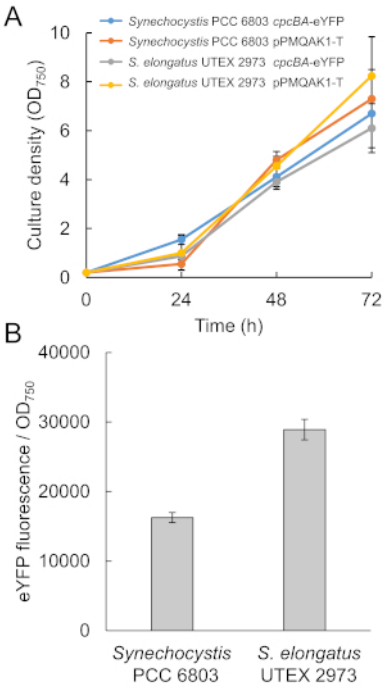

Figure 6: Representative growth data and normalized eYFP fluorescence values using a plate reader. (**A**) Growth comparison of strains carrying *cpcBA*-eYFP or the empty pPMQAK1-T vector (CT.0, negative control) in *Synechocystis* PCC 6803 and *S. elongatus* UTEX 2973. Values are the means ± SE from four biological replicates. *Synechocystis* PCC 6803 and *S. elongatus* UTEX 2973 were cultured for 72 h at 30 °C with continuous light (100 µmol photons $m^{-2}s^{-1}$) and 40 °C with 300 µmol photons $m^{-2}s^{-1}$, respectively. (**B**) Normalized eYFP fluorescence values for *Synechocystis PCC* 6803 or *S. elongatus* UTEX 2973 conjugated with *cpcBA*-eYFP at 72 h. Values are the means ± SE from four biological replicates. Please click here to view a larger version of this figure.

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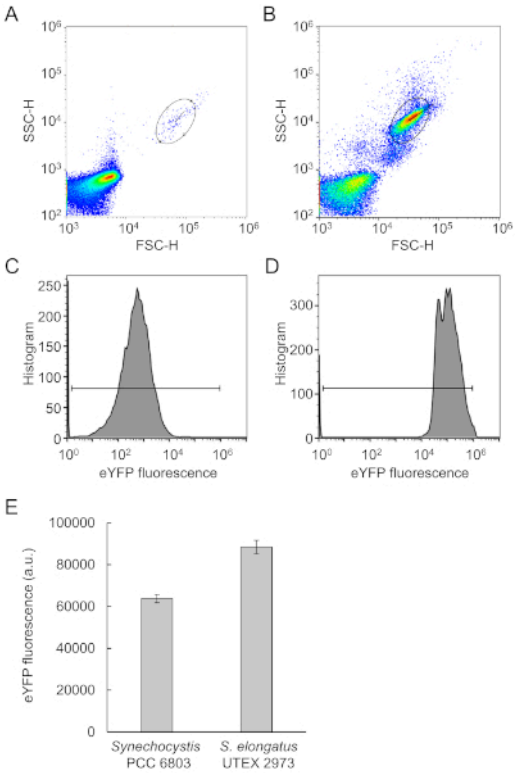

Figure 7: Representative eYFP fluorescence values using a flow cytometer. (**A**) Forward (FSC-H) and side (SSC-H) scatter plot from the "blank" medium solution (BG11 and PBS). (**B**) Scatter plot for a *Synechocystis PCC* 6803 sample (right). The circle indicates the selected region gating the cyanobacteria population from the remainder of the sample signal. (**C**) Histogram of the gated region for a strain carrying the empty pPMQAK1-T vector (CT.0, negative control). (**D**) Histogram of the gated region for a strain carrying *cpcBA*-eYFP. (**E**) eYFP fluorescence values per cell in *Synechocystis* PCC 6803 and *S. elongatus* UTEX 2973 at 72 h. Fluorescence from the negative control has been subtracted. Values are the means ± SE from four biological replicates. Please click here to view a larger version of this figure.

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No.	Vector ID	Name	Description	5' overhang	3' overhang
1	pICH41233	Pro	Promoter	GGAG	TACT
2	pICH41295	Pro + 5U	Promoter and 5′ untranslated region	GGAG	AATG
3	pAGM1251	Pro + 5U (f)	Promoter and 5′ untranslated sequence for N terminal fusions	GGAG	CCAT
4	pICH41246	5U	5′ untranslated region	TACT	CCAT
5	pAGM1263	5U (f)	5′ untranslated sequence for N terminal fusions	TACT	CCAT
6	pICH41246	5U + NT1	5′ untranslated region and N terminal coding region	TACT	CCAT
7	pAGM1276	NT1	N terminal tag or localisation signal	CCAT	AATG
8	pICH41258	SP	Signal peptide	AATG	AGGT
9	pICH41258	NT2	N terminal tag or localisation signal	AATG	AGGT
10	pAGM1287	CDS1 ns	Coding region without stop codon	AATG	TTCG
11	pICH41308	CDS1 stop	Coding region with stop codon	AATG	GCTT
12	pAGM1299	CDS2 ns	Coding region - without start and stop codon	AGGT	TTCG
13	pICH41264	CDS2 stop	Coding region - without start and with stop codon	AGGT	GCTT
14	pAGM1301	CT	C terminal tag or localization signal	TTCG	GCTT
15	pICH53388	3U	3′ untranslated region	GCTT	GGTA
16	pICH53399	Ter	Terminator	GGTA	CGCT
17	pICH41276	3U + Ter	3′ untranslated region and terminator	GCTT	CGCT
18	pICH41331	CGM	Acceptor for complete gene cassettes	GGAG	CGCT
19	pCA0.002	Pro (low copy)	Promoter, low copy number acceptor (pSC101 ori)	GGAG	TACT
20	pCA0.001	Pro TSS	Promoter truncated to the transcription start site	GGAG	TAGC
21	*Direct to Level 1*	srRNA	Small regulatory RNA (for translational silencing)	TAGC	GTTT
22	*Direct to Level 1*	sgRNA	Single guide RNA (for CRISPRi)	TAGC	GTTT
23	pICH41295	UP FLANK	Flanking sequence upstream of target homologous recombination site	GGAG	AATG
24	pICH41276	DOWN FLANK	Flanking sequence downstream of	GCTT	CGCT

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			target homologous recombination site		

Table 1: A list of available Level 0 acceptor vectors and overhangs. Vectors 1−18 are from the Plant MoClo kit[15]. Vectors 19−22 are from the CyanoGate kit[12]. For srRNA and sgRNA parts, synthesized sequences or PCR products are assembled directly into Level 1 acceptor vectors. Vectors 23−24 are from the Plant MoClo kit that have been re-purposed for transformation by homologous recombination using the CyanoGate kit.

BpiI assembly components (Level 0, T)		**BsaI assembly components (Level 1)**	
50−100 ng of acceptor vector		50−100 ng of acceptor vector	
For each vector/part to insert, use a 2:1 ratio of insert: acceptor vector.		For each vector/part to insert, use a 2:1 ratio of insert: acceptor vector.	
2 µL 10 mM ATP (**Table of Materials**)		2 µL 10 mM ATP (**Table of Materials**)	
2 µL buffer G (buffer for BpiI/BsaI)		2 µL buffer G (buffer for BpiI/BsaI)	
2 µL BSA (10x) (**Table of Materials**)		2 µL BSA (10x) (**Table of Materials**)	
10 units BpiI (1 µL10 U/µL BpiI, **Table of Materials**)		10 units BsaI (1 µL 10 U/µL BsaI, **Table of Materials**)	
Bring to 20 µL with dH_2O.		Bring to 20 µL with dH_2O.	
200 units T4 DNA ligase (1 µL 200 U/µL, **Table of Materials**)		200 units T4 DNA ligase (1 µL 200 U/µL, **Table of Materials**)	
Thermocycler protocol (Level 0, T)		**Thermocycler protocol (Level 1)**	
37 °C for 10 min	cycle x 5	37 °C for 10 min	cycle x 5
16 °C for 10 min		16 °C for 10 min	
37 °C for 20 min		37 °C for 20 min	
65 °C for 10 min		65 °C for 10 min	
16 °C (hold)		16 °C (hold)	

Table 2: Protocols for Golden Gate assemblies in Levels 0, 1 and T. Assembly in Level 0 and Level T acceptor vectors uses restriction enzyme BpiI (left). Assembly in Level 1 acceptor vectors uses restriction enzyme BsaI (right). This table has been adapted from Vasudevan et al.[12].

Primer No.	**Sequence (5'-3')**	**Length (bp)**	**Description**
L0T forward	GTCTCATGAGCGGATACATA TTTGAATG	28	For amplification from Level 0 and Level T
L1 reverse	GAACCCTGTGGTTGGCATGC ACATAC	26	For amplification from Level 1
L1 forward	CTGGTGGCAGGATATATTGT GGTG	24	For amplification from Level 1
L0T reverse	TTGAGTGAGCTGATACCGCT	20	For amplification from Level 0 and Level T

Table 3: List of primers for PCR validation or sequencing of Level 0, 1 and T vectors.

Discussion

Golden Gate assembly has several advantages compared to other vector assembly methods, particularly in terms of scalability[20,21]. Nevertheless, setting up the Golden Gate system in a lab requires time to develop a familiarity with the various parts and acceptor vector libraries and overall assembly processes. Careful planning is often needed for more complex assemblies or when performing a large number of complex assemblies in parallel (e.g., making a suite of Level T vectors containing multiple gene expression cassettes). We recommend first listing all the gene expression cassette combinations required and then mapping the workflow from Level 0 to Level T in silico. During this process, users should consider the Level 1 "Dummy" parts available in the Plant MoClo kit that allow for the assembly of non-sequential Level 1 vectors in Level T (e.g., Level 1 position 1 and position 3 vectors can be assembled together with "Dummy" part Level 1 position 2), which can reduce the overall number of assembly reactions and cloning steps required[15].

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DNA synthesis is typically the simplest method for building new Level 0 parts. However, when cloning is required (e.g., from plasmids or genomic DNA), optimizing the design of the primers used for amplification is important for maximizing the efficiency of subsequent Level 0 vector assembly. The two most critical steps in primer design are: 1) checking that the correct overhangs are included and are in the appropriate orientation for the forward and reverse primers (**Figure 1** and **Table 1**), and 2) ensuring that the length of the primer sequence that anneals to the template is sufficiently long (18−30 bp) and that the T_m value for this sequence (ideally 58−62°C) is similar for the primer pair (**Figure 1A**). If a sequence requires domestication, several strategies are available. For short sequences (e.g., <200 bp), a pair of long forward and reverse primers can be designed in which the 3′ ends anneal to each other (i.e., an overlap of >20 bp) and form a double stranded sequence following amplification. For longer sequences, separate fragments of the sequence can be amplified that remove illegal restriction sites and then assembled using a Golden Gate assembly approach (**Figure 2**). If assembly efficiency with PCR products is poor, individual fragments of a Level 0 sequence can be cloned into the Level 1 universal acceptor vector (pAGM1311), validated, and then assembled together into the appropriate Level 0 acceptor vector[15]. A 2:1 insert:acceptor vector molar ratio is recommended for efficient Golden Gate assembly. However, for assemblies of only 2-3 vectors (e.g., two Level 0 parts and a Level 1 acceptor vector), combining ~100 ng of each regularly typically results in successful assemblies. The efficiency of assemblies does tend to decrease as the number of vectors used per reaction increases, resulting in a reduction in total numbers of white colonies following transformation (**Figure 3**).

Prior to conjugation, validation of finalized Level T vectors by restriction digest and PCR is recommended. Conjugal DNA transfer is a well stablished technique for cyanobacterial strains, including those that are not naturally transformable[41,45]. Important steps in the conjugation protocol include: 1) careful handling of the helper *E. coli* strain following overnight growth (e.g., avoid vortexing)[35], 2) taking care to completely remove traces of the antibiotics used to grow helper and cargo *E. coli* strains, 3) an appropriate incubation period for the mixture of cargo and helper strains and cyanobacteria (e.g., a longer incubation period was critical for *S. elongatus* UTEX 2973), and 4) the initial transfer period of the cell mixture on membranes in LB-BG11 agar plates lacking antibiotics for 24 h.

Isolated cyanobacterial colonies should develop on the membrane within two weeks for *Synechocystis* PCC 6803 and *S. elongatus* UTEX 2973, otherwise it is likely that conjugation has failed. Several modifications to the protocol could then be tested, including 1) using a cyanobacterial culture with a higher starting density (e.g., OD_{750} = 1.5−2); 2) increasing the incubation period before transfer to the membrane; and 3) extending the initial incubation period on the membrane from 24 h to 48 h (i.e., to allow more time for the expression of the antibiotic resistance gene on the transferred vector). If conjugation still fails, alternative methods such as electroporation could be tried[53]. Confirming a transgenic cyanobacterial strain is axenic is important prior to further experimentation. Finally, it is good practice to confirm the size of the heterologous vector in the transgenic cyanobacterial strain. The latter requires DNA extraction (section 5), transformation into *E. coli* and selection (section 2.1), and vector validation (section 2.4).

The outlined promoter characterization protocol uses small culture volumes (i.e., 2 mL) as a means of achieving a high throughput screening methodology. Larger volumes could be used depending on the photobioreactor space available, which would help to mitigate culture evaporation issues. If high throughput screening with small culture volumes is required, it is essential to have high humidity within the growth chamber to inhibit evaporation. Evaporation during a growth experiment can be detrimental to the accuracy and validity of sample measurements. Do check culture volumes during and after the experiment to confirm how much evaporation has occurred.

For plate reader measurements, it is important to measure cultures at low densities, ideally OD_{750} < 1, to ensure the acquisition of reliable and reproducible growth and fluorescence data. A linear relationship between cell number and OD_{750} is observed only within a specific range[54]. To establish this range, we recommend performing a serial dilution (e.g., from OD_{750} = 0.1−1.0) using a known transformant where eYFP fluorescence has been confirmed. Plotting absolute fluorescence against normalized fluorescence (eYFP fluorescence/OD_{750}) will help to identify the linear working range of culture densities. Several plate readers include a "gain" feature to modify the sensitivity of the fluorescence detector. In this case, the gain value should be set to an appropriate level before beginning the experiment and not changed between different experimental runs or the data will not be directly comparable.

Although the operation of different flow cytometers will vary between manufacturers, it is important to take a blank reading of the medium solution to facilitate the identification and gating of the target cyanobacterial population from any background signal in the medium (**Figure 7A,B**). Following this, subtraction of the fluorescence value of the negative control sample (e.g., a wild type strain) will help to remove native autofluorescence (**Figure 7C,D**). The photomultiplier tube (PMT) voltage parameter in a flow cytometer has a similar function to the gain in a plate reader, i.e., increasing or decreasing the sensitivity of the detector to the intensity of the fluorescence signal. As with the plate reader, PMT voltage should be set to an appropriate level before beginning the experiment[55]. Once set, the PMT voltage value should be maintained between different experimental runs or the data will not be directly comparable.

Disclosures

The authors have nothing to disclose.

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