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# Genetic Modification of Cyanobacteria by Conjugation Using the Cyanogate Modular Cloning Toolkit

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**TITLE:**

# Genetic Modification of Cyanobacteria by Conjugation Using the Cyanogate Modular Cloning Toolkit

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**KEYWORDS:**

flow cytometry, fluorescence, Golden Gate, plate reader, *Synechocystis* sp. PCC 6803, *Synechococcus elongatus* UTEX 2973, toolkit, transient expression

**SUMMARY:**

Here, we present a protocol describing how to i) assemble a self-replicating vector using the CyanoGate modular cloning toolkit, ii) introduce the vector into a cyanobacterial host by conjugation, and iii) characterize transgenic cyanobacteria strains using a plate reader or flow cytometry.

**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.

**INTRODUCTION:**

Cyanobacteria are autotrophic bacteria that can be used for the biosynthesis of a wide variety of natural and heterologous high value metabolic products[1-6]. 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[8-10].

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[13-17]. 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 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[28-30]. 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[32-34].

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[35-41]. 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].

### 1.1. 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**.

#### 1.1.1. Design the primers.

1.1.1.1. 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**.

1.1.1.2. 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.

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

1.1.1.3. 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.

1.1.2. Amplify a DNA sequence from genomic DNA.

1.1.2.1. 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.

1.1.2.2. 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**).

1.1.2.3. 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**).

1.1.2.4. 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).

1.1.3. 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).

1.2. Construction of Level 1 assemblies

1.2.1. 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].

1.2.2. 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).

1.3. Construction of Level T assemblies

1.3.1. 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).

1.3.2. 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.

1.3.3. 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**

2.1. *E. coli* transformation (day 1)

2.1.1. 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.

2.1.2. 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.

2.1.3. 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.

2.2. 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.

2.2.1. Optionally, validate that white colonies contain the expected vector by performing PCR as described in section 7.

2.2.2 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.

2.3. Plasmid vector purification (day 3)

2.3.1. 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.

2.3.2. 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.

2.3.3 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.

2.4. Vector validation

NOTE: Vectors can be verified by restriction digestion (step 2.4.1) and/or sequencing (step 2.4.2).

2.4.1. 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.

2.4.2. 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.

### 3.1. Growth of the cyanobacterial culture (day 1)

3.1.1. Prepare BG11 medium according to Lea-Smith et al.[11], and agar plates with LB-BG11 and BG11+Kan50 (section 8).

3.1.2. 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].

### 3.2. Growth of helper and cargo *E. coli* strains (day 2)

3.2.1. 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.

3.2.2. 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.

### 3.3. Conjugal transfer (tri-parental mating) (day 3)

3.3.1. 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 4 °C. Discard the supernatant without disturbing the cell pellet.

3.3.2 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.

3.3.3. 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.

3.3.4. Prepare the cyanobacterial culture. For each conjugation reaction, use 1 mL of cyanobacterial culture ($OD_{750}$ = 0.5–1.5).

3.3.5. 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.

3.3.6. 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.

3.3.7. 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.

3.3.8. 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 $m^{-2}s^{-1}$.

3.4. Membrane transfer

3.4.1. 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.

3.4.2. 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.

3.5. Selection of conjugants

NOTE: Only cyanobacterial colonies carrying the cargo vector will be able to grow on the membrane (**Figure 5**).

3.5.1. 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.

3.5.1. 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.

3.6. Cryostorage of cyanobacterial strains

3.6.1. 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.

3.6.2. 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.

3.6.3. 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.

3.6.4. 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].

4.1. Culture growth

4.1.1. 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.

4.1.2. Grow the seed cultures for 48 h or until $OD_{750}$ = 1−1.5 under growth conditions appropriate for the species strain.

4.1.3. 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**).

4.1.4. 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.

4.2. Plate reader

4.2.1. 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.

4.2.2. 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.

4.2.3. Measure $OD_{750}$ and eYFP fluorescence with excitation/emission wavelengths at 485 nm/520 nm.

4.2.4. 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.

4.2.5. 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.

4.2.6. 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**).

4.3. Flow cytometer

4.3.1. 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.

4.3.2. 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.

4.3.3. 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.

4.3.4. 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**).

5.1. 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.

5.2. 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.

5.3. 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**

6.1. Cast a 1% (w/v) agarose gel containing 0.02% (v/v) ethidium bromide. Load samples and an appropriate DNA ladder reference.

6.2. 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**

7.1. 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.

7.2. 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.

7.3. 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**

8.1. 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.

8.2. 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.

8.3. LB-BG11 agar plates (1.5% [w/v])

8.3.1. 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.

8.3.2. 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.

8.4. BG11+Kan50 agar plates (1.5% [w/v])

8.4.1. 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.

8.4.2. 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].

**FIGURE AND TABLE LEGENDS:**

**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.

**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.

**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.

**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.

**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.

**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.

**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.

**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.

**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].

**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].

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.

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**DISCLOSURES:**

The authors have nothing to disclose.

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Figure 1

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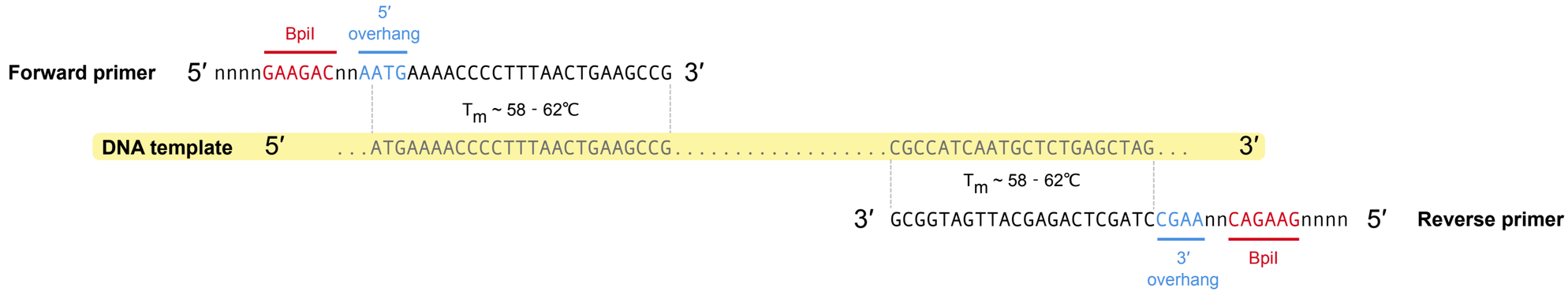


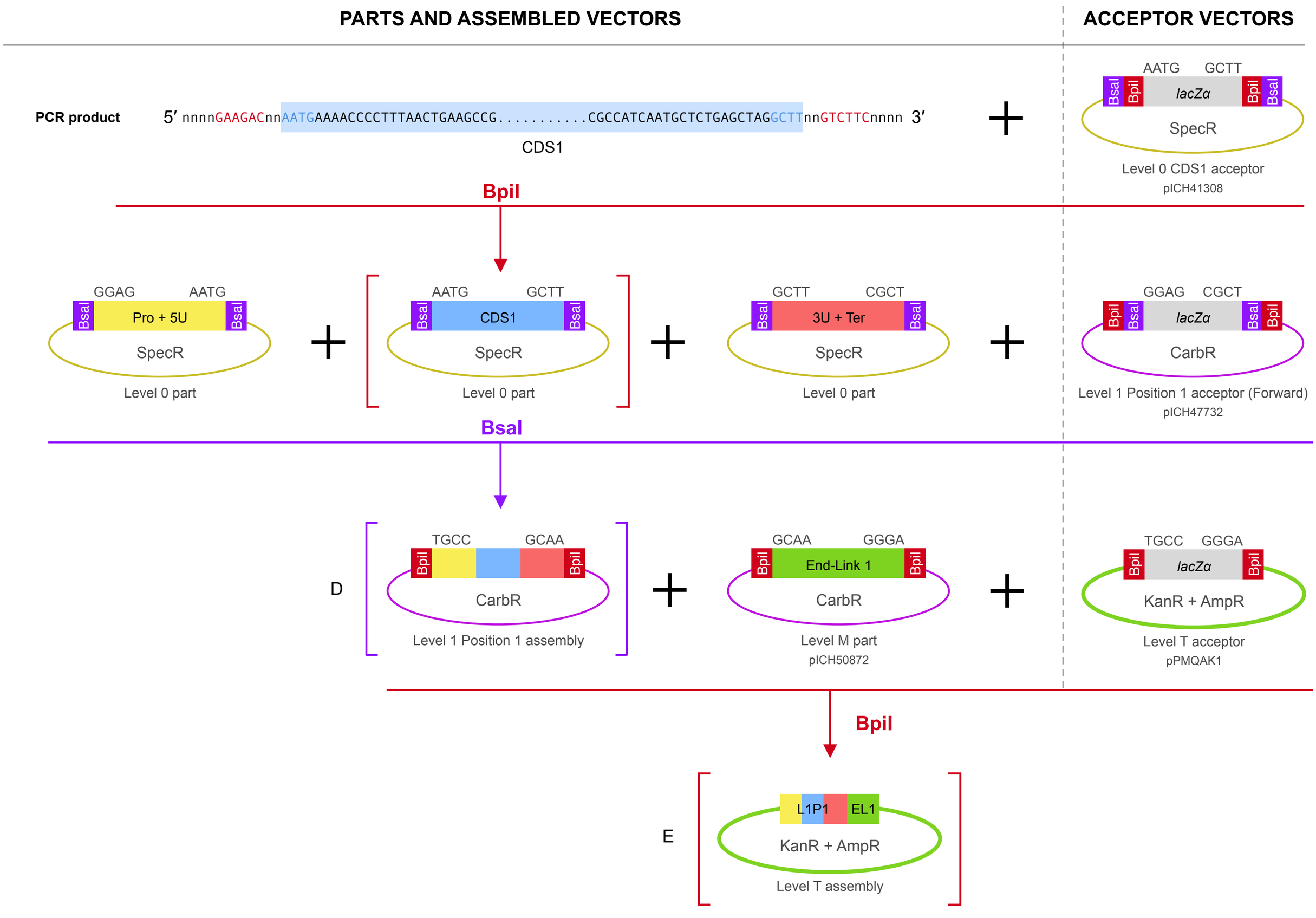

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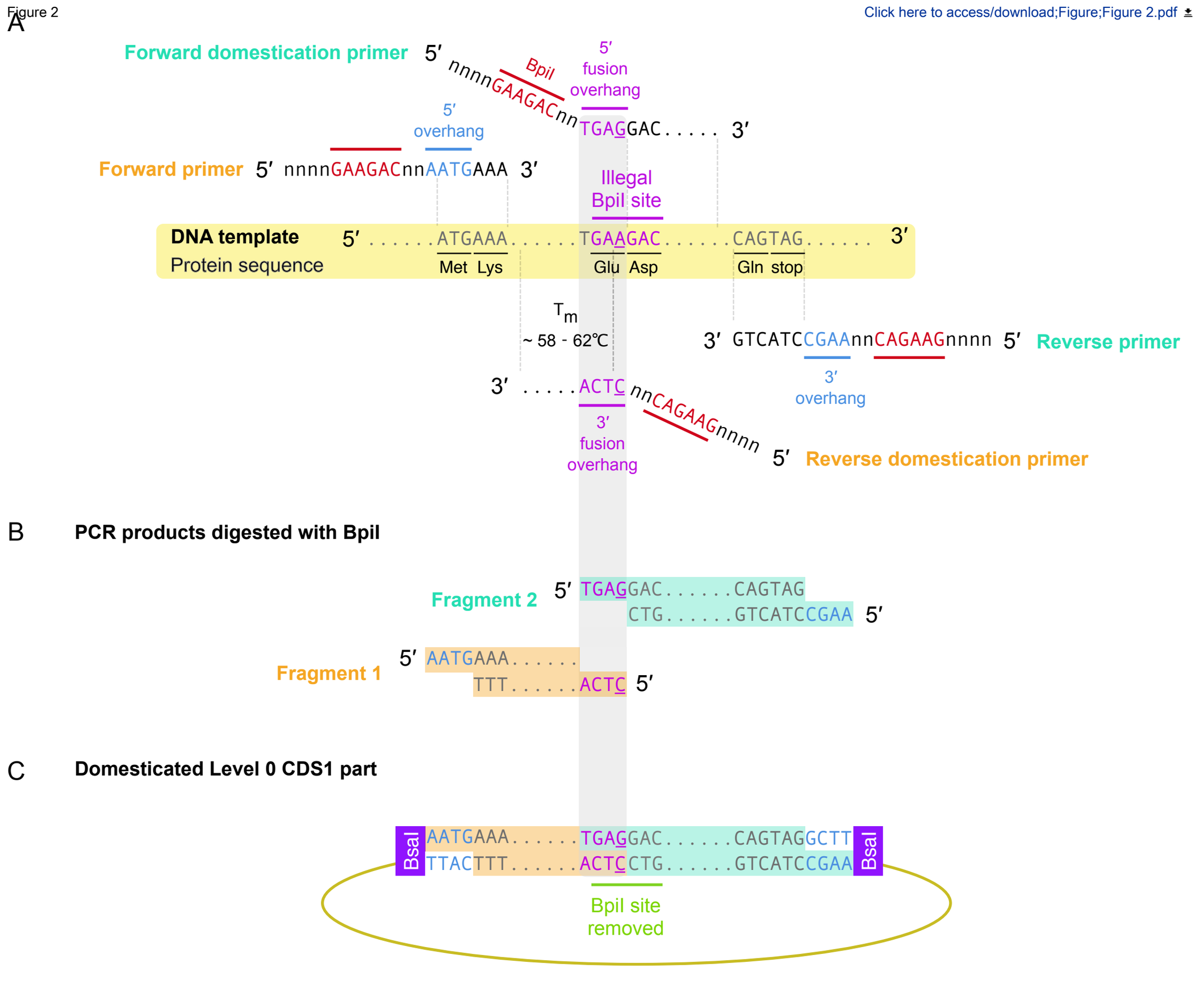


Figure 3



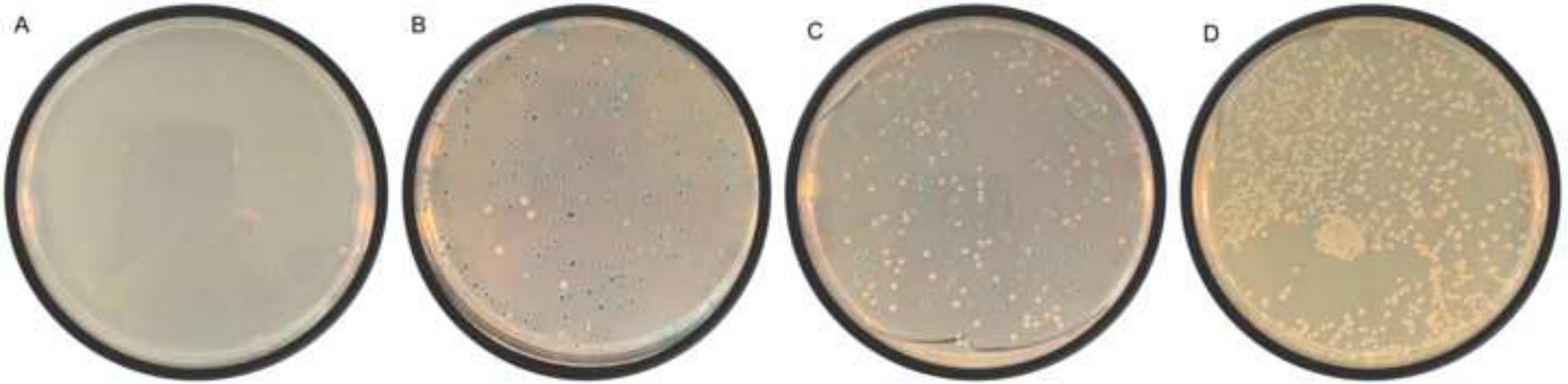


Figure 4



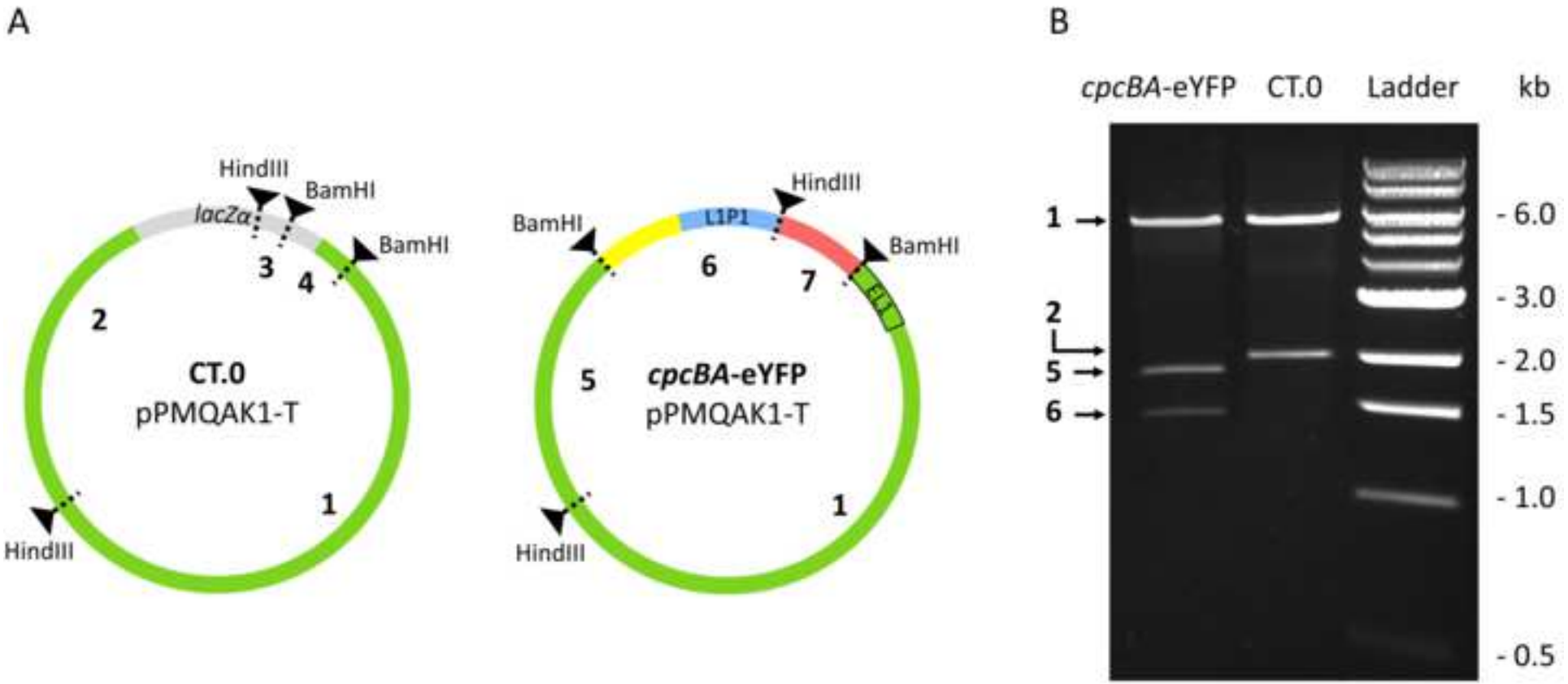


Figure 5



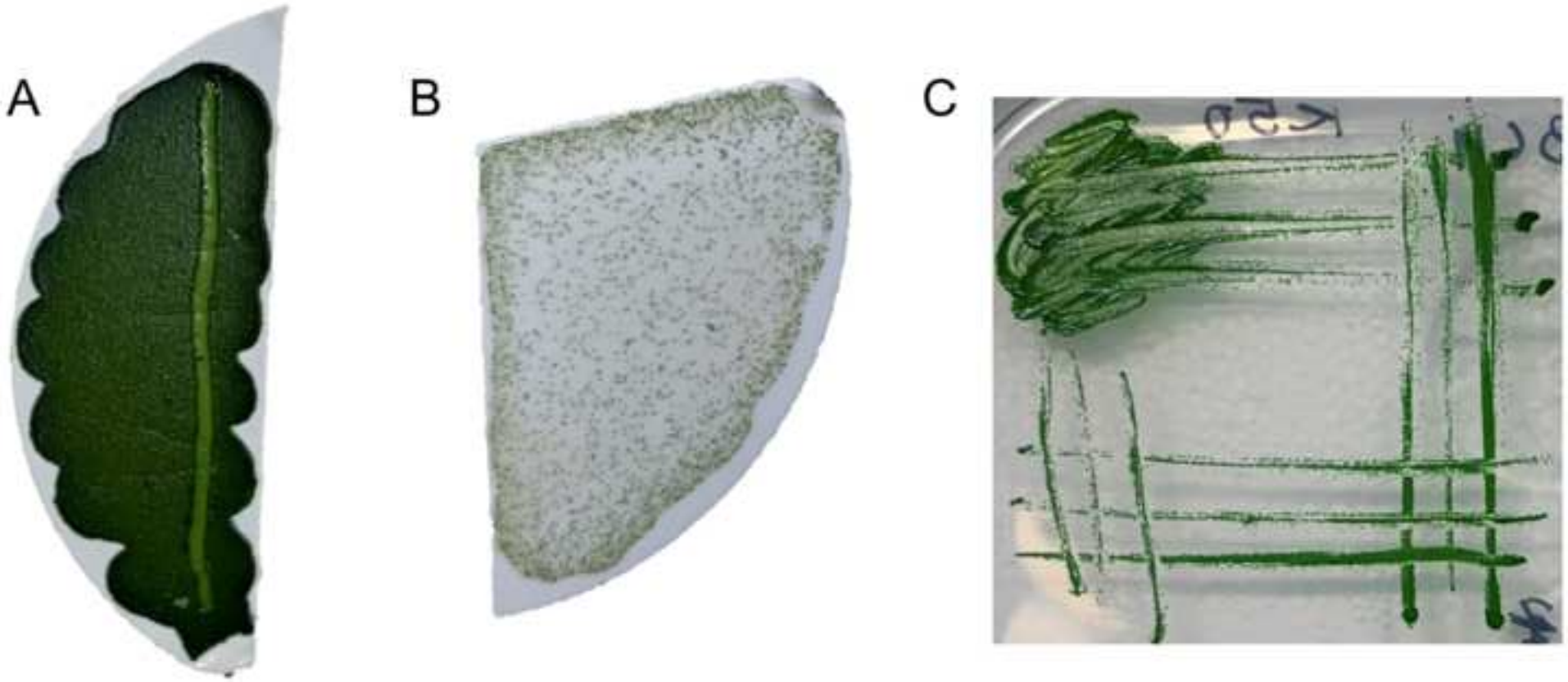
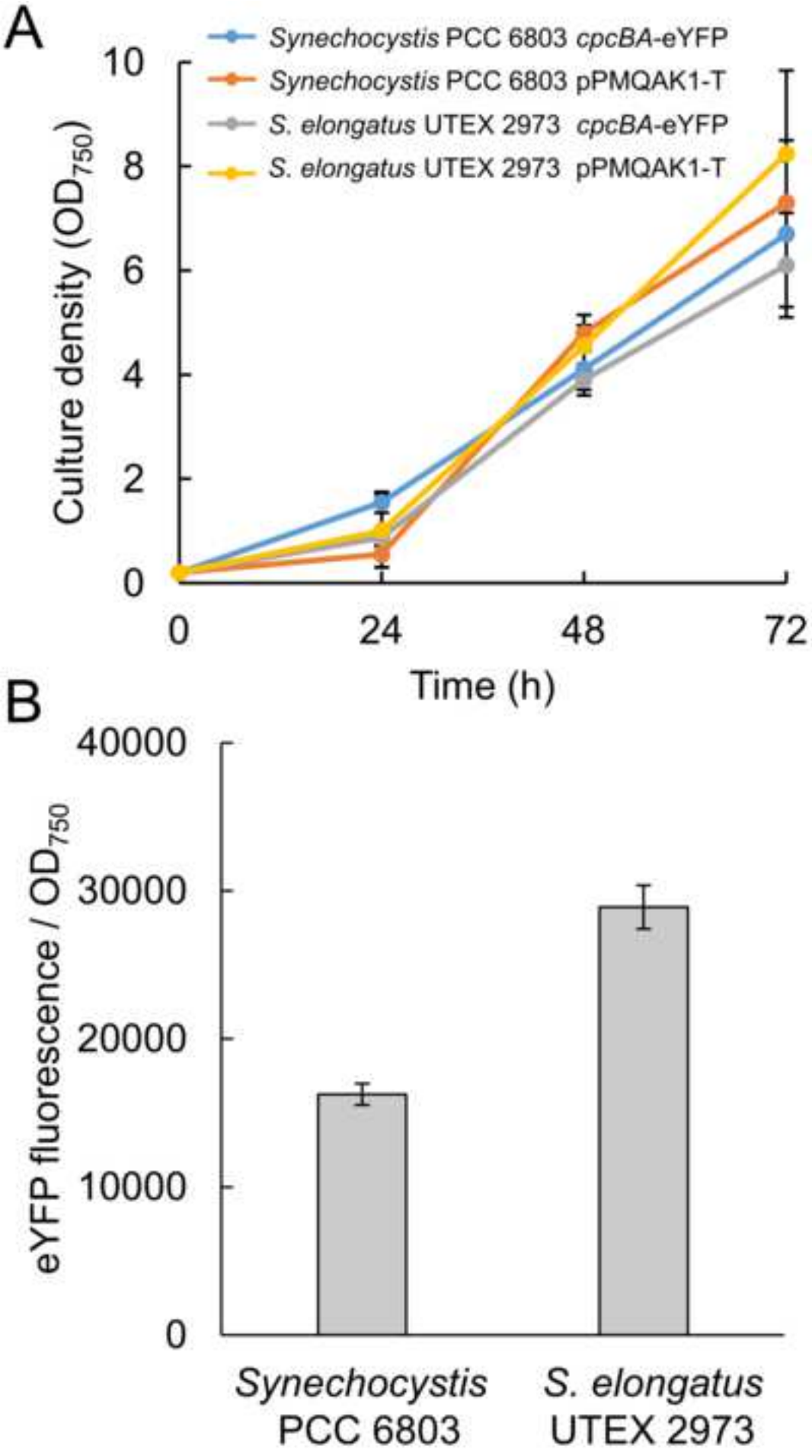


Figure 6





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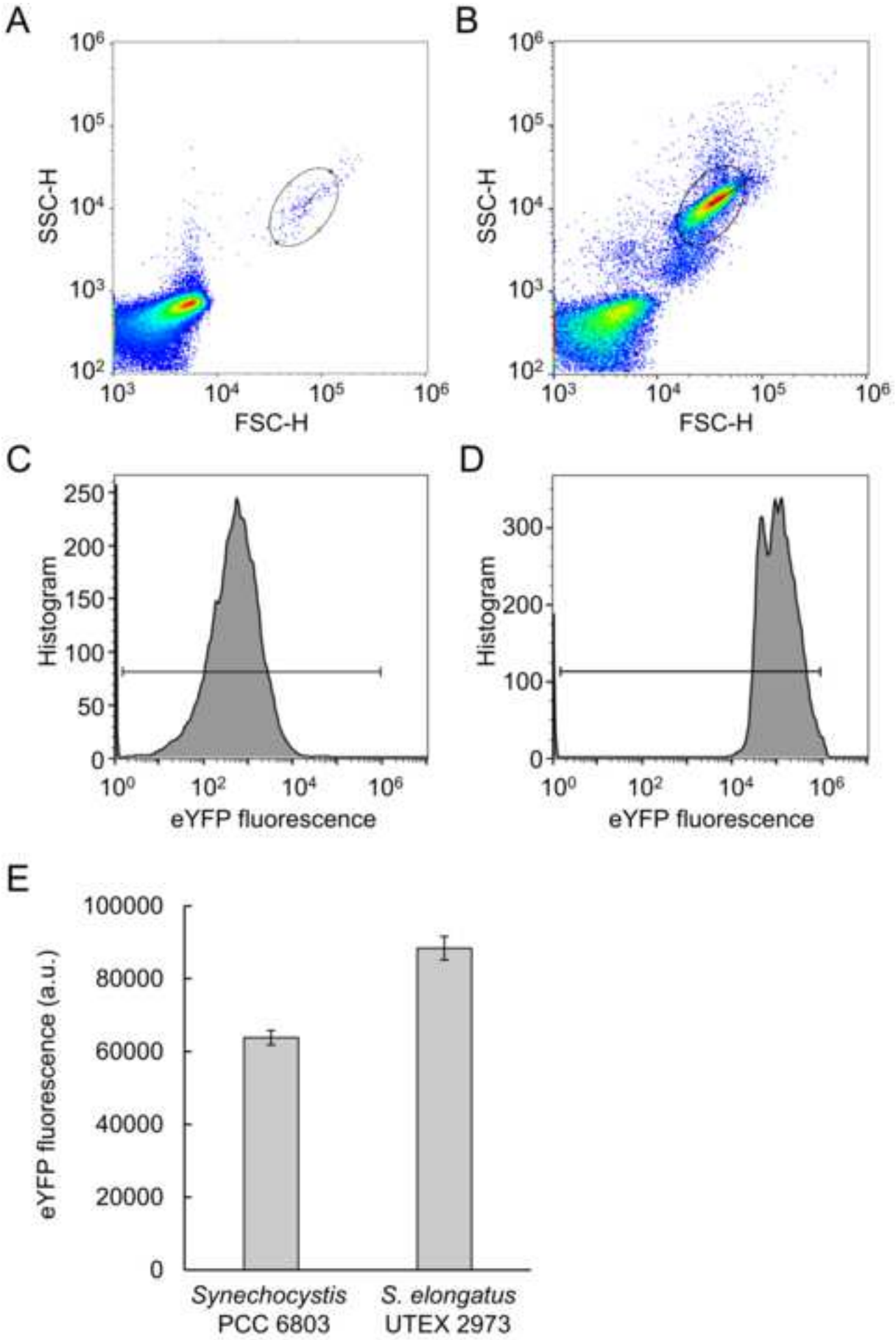

Table 1



| No. | Vector ID | Name |
|---|---|---|
| 1 | pICH41233 | Pro |
| 2 | pICH41295 | Pro + 5U |
| 3 | pAGM1251 | Pro + 5U (f) |
| 4 | pICH41246 | 5U |
| 5 | pAGM1263 | 5U (f) |
| 6 | pICH41246 | 5U + NT1 |
| 7 | pAGM1276 | NT1 |
| 8 | pICH41258 | SP |
| 9 | pICH41258 | NT2 |
| 10 | pAGM1287 | CDS1 ns |
| 11 | pICH41308 | CDS1 stop |
| 12 | pAGM1299 | CDS2 ns |
| 13 | pICH41264 | CDS2 stop |
| 14 | pAGM1301 | CT |
| 15 | pICH53388 | 3U |
| 16 | pICH53399 | Ter |
| 17 | pICH41276 | 3U + Ter |
| 18 | pICH41331 | CGM |
| 19 | pCA0.002 | Pro (low copy) |
| 20 | pCA0.001 | Pro TSS |
| 21 | *Direct to Level 1* | srRNA |
| 22 | *Direct to Level 1* | sgRNA |
| 23 | pICH41295 | UP FLANK |
| 24 | pICH41276 | DOWN FLANK |

| Description | 5' overhang |
|---|---|
| Promoter | GGAG |
| Promoter and 5ˈ untranslated region | GGAG |
| Promoter and 5ˈ untranslated sequence for N terminal fusions | GGAG |
| 5ˈ untranslated region | TACT |
| 5’ untranslated sequence for N terminal fusions | TACT |
| 5ˈ untranslated region and N terminal coding region | TACT |
| N terminal tag or localisation signal | CCAT |
| Signal peptide | AATG |
| N terminal tag or localisation signal | AATG |
| Coding region without stop codon | AATG |
| Coding region with stop codon | AATG |
| Coding region - without start and stop codon | AGGT |
| Coding region - without start and with stop codon | AGGT |
| C terminal tag or localization signal | TTCG |
| 3ˈ untranslated region | GCTT |
| Terminator | GGTA |
| 3ˈ untranslated region and terminator | GCTT |
| Acceptor for complete gene cassettes | GGAG |
| Promoter, low copy number acceptor (pSC101 ori) | GGAG |
| Promoter truncated to the transcription start site | GGAG |
| Small regulatory RNA (for translational silencing) | TAGC |
| Single guide RNA (for CRISPRi) | TAGC |
| Flanking sequence upstream of target homologous recombination site | GGAG |
| Flanking sequence downstream of target homologous recombination site | GCTT |

| 3' overhang |
| --- |
| TACT |
| AATG |
| CCAT |
| CCAT |
| CCAT |
| CCAT |
| AATG |
| AGGT |
| AGGT |
| TTCG |
| GCTT |
| TTCG |
| GCTT |
| GCTT |
| GGTA |
| CGCT |
| CGCT |
| CGCT |
| TACT |
| TAGC |
| GTTT |
| GTTT |
| AATG |
| CGCT |

Table 2 Click here to access/download;Table;Table 2.xlsx

| **BpiI assembly components (Level 0, T)** | **BsaI assembly comp** |
|---|---|
| 50–100 ng of acceptor vector<br>For each vector/part to insert, use a 2:1 ratio of insert: acceptor vector. | 50–100 ng of accepto<br>For each vector/part |
| 2 µL 10 mM ATP (**Table of Materials**) | 2 µL 10 mM ATP (**Tab** |
| 2 µL buffer G (buffer for BpiI/BsaI) | 2 µL buffer G (buffer |
| 2 µL BSA (10x) (**Table of Materials**) | 2 µL BSA (10x) (**Table** |
| 10 units BpiI (1 µL10 U/µL BpiI, **Table of Materials**) | 10 units BsaI (1 µL 10 |
| Bring to 20 µL with $dH_2O$. | Bring to 20 µL with d |
| 200 units T4 DNA ligase (1 µL 200 U/µL, **Table of Materials**) | 200 units T4 DNA liga |

| **Thermocycler protocol (Level 0, T)** | | **Thermocycler protoc** |
|---|---|---|
| 37 °C for 10 min<br>16 °C for 10 min | cycle x 5 | 37 °C for 10 min<br>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) |

**ponents (Level 1)**

or vector
to insert, use a 2:1 ratio of insert: acceptor vector.

**ble of Materials**)
for BpiI/BsaI)
**e of Materials**)

) U/µL BsaI, **Table of Materials**)

$H_2O$.

ase (1 µL 200 U/µL, **Table of Materials**)

**col (Level 1)**

cycle x 5

Table 3



| Primer No. | Sequence (5'-3') | Length (bp) |
|---|---|---|
| L0T forward | GTCTCATGAGCGGATACATATTTGAATG | 28 |
| L1 reverse | GAACCCTGTGGTTGGCATGCACATAC | 26 |
| L1 forward | CTGGTGGCAGGATATATTGTGGTG | 24 |
| L0T reverse | TTGAGTGAGCTGATACCGCT | 20 |

| Description |
| --- |
| For amplification from Level 0 and Level T |
| For amplification from Level 1 |
| For amplification from Level 1 |
| For amplification from Level 0 and Level T |

| Name of Material/Equipment | Company | Catalog Number |
|---|---|---|
| 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) | Thermo Fisher Scientific | R0404 |
| Adenosine 5’-triphosphate (ATP) disodium salt | Sigma-Aldrich | A2383 |
| Agar (microbiology tested) | Sigma-Aldrich | A1296-500g |
| Agarose | Bioline | BIO-41026 |
| Attune NxT Flow Cytometer | Thermo Fisher Scientific | - |
| Bovine Serum Albumin (BSA) | Sigma-Aldrich | A2153 |
| BpiI (BbsI) | Thermo Fisher Scientific | ER1011 |
| BsaI (Eco31I) | Thermo Fisher Scientific | ER0291 |
| Carbenicillin disodium | VWR International | A1491.0005 |
| Corning Costar TC-Treated flat-bottom 24 well plates | Sigma-Aldrich | CLS3527 |
| Dimethyl Sulfoxide (DMSO) | Thermo Fisher Scientific | BP231-100 |
| DNeasy Plant Mini Kit | Qiagen | 69104 |
| FLUOstar Omega Microplate reader | BMG Labtech | - |
| GeneJET Plasmid Miniprep Kit | Thermo Fisher Scientific | K0503 |
| Glass beads (0.5 mm diameter) | BioSpec Products | 11079105 |
| Glycerol | Thermo Fisher Scientific | 10021083 |
| Isopropyl-beta-D-thiogalactopyranoside (IPTG) | Thermo Fisher Scientific | 10356553 |
| Kanamycin sulphate (Gibco) | Thermo Fisher Scientific | 11815-024 |
| Membrane filters (0.45 µm) | MF-Millipore | HAWP02500 |
| Microplates, 96-well, flat-bottom (Chimney Well) µCLEAR | Greiner Bio-One | 655096 |
| Monarch DNA Gel Extraction Kit | New England Biolabs | T1020S |
| Monarch PCR DNA Cleanup Kit | New England Biolabs | T1030 |
| Multitron Pro incubator with LEDs | Infors HT | - |
| MyTaq DNA Polymerase | Bioline | BIO-21108 |
| NanoDrop One | Thermo Fisher Scientific | ND-ONE-W |
| One Shot TOP10 chemically competent *E. coli* | Thermo Fisher Scientific | C404010 |
| Phosphate buffer saline (PBS) solution (10X concentrate) | VWR International | K813 |
| Q5 High-Fidelity DNA Polymerase | New England Biolabs | M0491S |
| Quick-Load 1 kb DNA Ladder | New England Biolabs | N0468S |
| Screw-cap tubes (1.5 ml) | Starstedt | 72.692.210 |
| Spectinomycin dihydrochloride pentahydrate | VWR International | 61820.06 |
| Sterilin Clear Microtiter round-bottom 96-well plates | Thermo Fisher Scientific | 612U96 |

| | | |
|---|---|---|
| T4 DNA ligase | Thermo Fisher Scientific | EL0011 |
| TissueLyser II | Qiagen | 85300 |

**Comments/Description**
Used in 2.1.3.
Used in Table 2.
Used in 8.3.
Used in 6.
Used in 4.3.1.
Used in Table 2.
Used in Table 2.
Used in Table 2.
Used in 2.1.3.
Used in 4.1.3.
Used in 3.6.2.
DNA extraction kit. Used in 5.
Used in 4.2.2.
Plasmid purification kit. Used in step 2.3.2.
Used in 5.2.
Used in 2.3.1, 3.6.2.
Used in 2.1.3.
Used in 2.1.3.
Used in 3.3.7
Used in 4.2.1.
Used in 1.1.2.2.
DNA purification kit. Used in 1.1.2.3.
Shaking incubator with white LED lights. Used in 4.1.4.
Used in 7.1.
Used in 2.3.3.
Used in 2.1.1.
Used in 4.3.2.
Used in 1.1.2.1.
Used in Figure 4.
Used in 3.6.3
Used in 2.1.3.
Used in 4.3.1.

Used in Table 2.
Bead mill. Used in 5.2.

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Dear Editor,

The authors would like to thank the reviewers very much for the significant effort and detail put into their comments and feedback. We have addressed the editorial and reviewer comments, and have made substantial changes to the text. We feel that the manuscript is now much improved and we hope suitable for publication. We have provided the main text file both in finalised format and a format with track changes to compare to the previous draft. Our responses are as follows:

**Editorial comments:**

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
- We have carefully proofread the manuscript.

2. Please add a Summary section before the Abstract section to clearly describe the protocol and its applications in complete sentences between 10–50 words: "Here, we present a protocol to …"
- We have added the following Summary section: Here, we present a protocol describing how to i) assemble a self-replicating vector using the CyanoGate modular cloning system, ii) introduce the vector into cyanobacterial host by conjugation and iii) characterise transgenic strains using a plate reader or flow cytometry.

3. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc. Please use the micro symbol µ instead of u and abbreviate liters to L (L, mL, µL) to avoid confusion.
- We have searched the document and corrected as requested.

4. Please include a space between all numbers and the corresponding unit: 15 mL, 5 g, 7 cm, 37 °C, 60 s, 24 h, etc.
- We have searched the document spacing errors and corrected.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Q5®, New England BioLabs, Monarch®, One Shot TOP10, Thermo Fisher Scientific, Eppendorf, GeneJET, Corning® Costar®, Sigma-Aldrich, µClear®, Greiner Bio-One, Quick-Load®, etc.
- We have removed symbols, commercial product names and company names from the text wherever possible and cited "Table of Materials" instead.

6. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. Step 1 followed by 1.1, followed by 1.1.1, etc. Each step should include 1–2 actions and contain 2–3 sentences. Use subheadings and substeps for clarity if there are discrete stages in the protocol. Please refrain from using bullets, dashes, or indentations.
We have reformatted the numbering system as requested. As for the recommendation below, each step contains a maximum of 4 sentences.

7. Please revise the Protocol text to avoid the use of personal pronouns (e.g., I, you, your, we, our) or colloquial phrases.
- We removed personal pronouns and rephrased where necessary.

8. Please revise the Protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “NOTE.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.
- We have removed non-imperative tense phrases such as “could be,” “should be,” and “would be” from the main protocol text.

9. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.
- We have combined steps as such.

10. Please include single line spacing between each numbered step or note in the protocol.
- We have included single line spacing as requested.

11. After you have made all the recommended changes to your protocol section (listed above), please re-evaluate your protocol length. There is a 10-page upper limit (with proper formatting). There is a 2.75-page upper limit on the amount of content we can film for a single video article. Please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.
- We have reduced the length of the protocol to within the 10-page limit.

12. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.
- We have highlighted text that we believe will provide a cohesive narrative with a logical flow to show the processes of CyanoGate MoClo vector assembly and cyanobacterial conjugation.

13. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
- As have included all relevant details in the highlights.

14. Please obtain explicit copyright permission to reuse any figures/table from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”
- We have contacted Plant Physiology for permissions and have been told they will reply shortly. Only Table 2 contains similar information to a Supplementary Figure in the associated Plant Physiology publication.

15. Please upload each Table individually to your Editorial Manager account as an .xlsx file. Avoid any coloring or formatting in the tables.
- We have generated separate .xlsx files for each table and will upload. Colours have been removed and legends updated accordingly.

16. Table of Materials: Please remove any ™/®/© symbols.
- We have removed these symbols from the Table of Materials.

17. For in-text references, the corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text (before punctuation but after closed parenthesis). The references should be numbered in order of appearance.
- We have modified the referencing format as required.

18. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please do not abbreviate journal titles. See the example below:
Bedford, C.D., Harris, R.N., Howd, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2-[(hydroxyimino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).
- We have re-checked the references and corrected the formatting.

**Reviewers' comments:**
**Reviewer #1:**
Manuscript Summary:
In the manuscript "Genetic modification of cyanobacteria by conjugation using the modular cloning CyanoGate toolkit", Gale et al. described a detail protocol of using the recent published CyanoGate cloning suite to genetically engineer cyanobacteria via conjugation. The protocol was further exemplified to compare the promoter activities using eYFP as a reporter, measured by both plate reader and flow cytometer. The CyanoGate cloning suite is shown to be very efficient to assemble DNA parts, hence it facilitates genetic modifications in cyanobacteria. This protocol is indeed a timely contribution to the much promising concept of developing cyanobacteria as photosynthetic cell factories, and interesting for both students and researchers in this community. The manuscript is well written, and all the protocols are clearly presented. I only have a few minor comments.

Major Concerns:
None

Minor Concerns:
1. In line 654, what is the pH of the TES buffer stock?
- We have added “pH 8.2”.

2. In line 812, "Fig. 5" should be "Fig.3".
- Thank you for picking up this error - we have changed to “Fig. 3”.

3. In line 848, "plant readers" should be "plate readers".
- Thank you for picking up this error - we have changed to “plate readers”.

**Reviewer #2:**
Manuscript Summary:
Reviewer report
In Gale et al., the authors describe a protocol to build plasmids using CyanoGate, a standardized modular cloning (MoClo) system. The method was designed for the assembly of self-replicating or integration vectors, which can be transferred to cyanobacterial strains by conjugation. The

functionality of this system was demonstrated by conjugation experiments in Synechocystis sp. PCC6803 and Synechococcus elongatus UTEX 2973. Using recombinatorial MoClo cloning, the authors report two high throughput methodologies to characterize the performance of genetic parts based on the use of a plate reader and flow cytometry. The description of the protocol is solid, and it provides useful information to use this strategy to build vectors and use them to genetic engineer cyanobacterial strains. We provide some comments below that are mostly editorial in nature.

Major concern
Some terms related to the MoClo system are used very frequently (especially Level 0, 1, 2, T vectors) but not explicitly defined in the Introduction. A bit more explicit definition of these terms is strongly recommended. Increasing the detail in the Figure 1 scheme in order to more fully describe the general overview of the hierarchical cloning system, especially showing the different levels, would be useful to understand how the system to build the plasmids works - especially for someone who is just learning about the CyanoGate concepts and techniques.
The recent publication outlines the CyanoGate MoClo system in detail. Rather than repeating that information, we have added the following to the beginning of the protocol: “Before proceeding with vector assembly, we strongly recommend that users familiarize themselves with the vector level structures of the Plant and CyanoGate MoClo systems (Engler et al., 2014; Vasudevan et al., 2019).”

Minor concerns
- It would be possible (but is not required) to reduce the length of some of the discussion of the other less directly relevant topics in the Introduction in order to make room for an expansion of discussion of the MoClo system suggested above if the word count is a concern. The Introduction is currently a bit longer than may be strictly necessary for this type of methodology paper.
- We agree that the Introduction and Discussion are a bit lengthy and have reduced the lengths of these sections.

- Lines 24-25. This sentence does not provide any new information to the abstract and may be removed.
- We agree and have removed “Conjugation is a useful technique for introducing DNA into cyanobacteria.”

- Line 43. It may be desirable to replace the ";" a period and separate this statement into two sentences.
- We agree and have replaced with a full stop.

- Lines 46-49. The main approaches for introducing DNA into cyanobacteria are transformation, conjugation and electroporation. The vectors transferred to cyanobacteria by transformation and electroporation are suicide vectors (they integrate in the genome via homologous recombination), while self-replicating vectors can be transferred to cyanobacteria by transformation, conjugation and electroporation. There is a slight confusion in the use of terminology in this sentence that could be made more precise.
- Thank you for this clear statement. We have replaced the sentence on line 46-49 with a slightly modified version: “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.”

- Lines 63, 187, 189, 192, 194. The abbreviation for nucleotide is most frequently in lower case: that is, "nt", instead of "NT".

- We humbly disagree, as we have found that both are used in the literature. We also feel that NTs is more immediately understandable for readers than nts. Either way, we are happy to defer to the editors preference for this.

- Lines 91-93. In the experience of these reviewers, promoter/RBS calculators that have been developed for E. coli are poorly predictive of their performance in cyanobacteria. Perhaps you may wish to comment on your perception and/or publications regarding the accuracy of these tools for cyanobacterial predictions?
Thank you for this suggestion, we have added "… these tools are often poor predictors of performance in cyanobacteria (Heidorn et al., 2011; Englund et al., 2016; Thiel et al., 2018)."

- Line 96. What oriT does pSB4K5 contain? Is it compatible with other cyanobacterial strains?
- pSB4K5 contains the pSC101 ori. It is not used for cyanobacterial transformation, but rather a lower level component of CyanoGate to assist with the building of the final level T vectors for introducing DNA into cyanobacteria

- Line 108. You may wish to indicate here that Synechococcus elongatus UTEX 2973 is not naturally competent and the DNA can only introduce to the cells by conjugation or electroporation.
- Thank you for this suggestion, we have added to the end of the following sentence "engineering cyanobacterial species, in particular those that are not naturally competent, such as *S. elongatus* UTEX 2973".

- Line 116 and throughout: Please double-check the standard in denoting plasmid features such as the origin of transfer (oriT) and bom sites. We routinely see these features denoted in italics. Similarly, the standard for the abbreviations that is (i.e.,) and for example (e.g.,) is to put the abbreviation in italics and use a comma immediately afterwards.
- We have italicised all references to "oriT" and "*bom*". We do disagree that italics and a comma are standard for i.e. and e.g. – this is typically publication specific. We are happy to defer to the editor as to the journal preference.

- Line 127. What is the genotype of MC1061 E. coli strain? Is there any special reason to choose this strain?
- MC1061 is a common *E. coli* strain used for transformation that was contributed from the University of Copenhagen (as stated in the Acknowledgements) with both mobilizer and helper vectors. We don't believe there was a special reason for using this.

- Line 134. "the mobilizer vector" should be added after "protects", since you do not indicate what vector is being protected by the methylases.
- We have added "the mobilizer vector".

- Line 141. Consider deleting "(e.g. 10,000 cells)"
- We have removed.

- Lines 147-148. Consider if you mean that plate readers have low "sensitivity" compared to cytometers (in contrast to lower "accuracy").
- We agree – sensitivity would be more appropriate – we have changed as such.

- Lines 187 and 192. Take care with the wording "in front of" in this context, as it is subjective in this description. Describing the features in context of 5' or 3' position may be more precise.
- We have changed "in front of" to "at the 5' end of".

- Lines 221-222. The Monarch DNA extraction gel kit is not included in the table of materials. Additionally, it may be worth a brief mention that even primer dimers can be problematic in this instance.
- We have added the Monarch DNA Gel Extraction Kit to the Table of Materials. We have added "However, if the gel shows only the appropriate band and no evidence of primer dimers,…".

- Line 265. Typo: Ligate the 3' end "of" your final….
- We have added "of".

- Line 289. You may wish to add "using glass beads" and its catalog number and company after "the culture", since glass beads are listed in the table of materials and they are not explicitly used in any part of the protocol.
- Glass beads were only used for extracted DNA from cyanobacterial cultures – we reference these in step 5.

- Line 393. "remove" instead of "removed"
- We have changed "removed" to "remove".

- Line 398. How long do you incubate at room temperature the helper and the cargo strain together?
- We have clarified in the text "…and set aside (leave at room temperature) until step 3.3.6."

- Line 421. All the steps with cyanobacteria should be performed in a laminar flow hood. If you mention this at the beginning of the protocol, it would not be necessary to indicate this at every step where cyanobacteria are used. Currently, it is inconsistently mentioned.
- Thank you for this suggestion. We have removed reference to laminar flow hoods and stated in the introduction of step 3. Generation of mutants by conjugation: "All work with cyanobacteria should be done under sterile conditions in a laminar flow hood."

- Lines 421-422. HAFT 0.45 µm membrane filters (Milipore) are not included in the table of materials.
- We have corrected the text to "0.45 µm membrane filter (MF-Millipore)" and added membrane filters (0.45 µm) to the Table of Materials.

- Lines 448-452. Another way to text if the colonies are axenic is inoculating a little amount of biomass of cyanobacterial transconjugants in a tube with a small volume of LB. Sometimes, the small colonies of E. coli are not evident in the plates.
- Thank you for this tip. We have added the following to the protocol:" Confirm absence of *E. coli* contamination by inoculating a streak of cyanobacterial culture into a 15 mL centrifuge tube containing 5 mL LB medium and incubating the tube at 37 °C overnight at 225 rpm in a shaking incubator."

- Lines 453-455. It is also recommended to recover the plasmid from cyanobacterial transconjugants and check by digestion. Plasmids could contain fragments that are not from cyanobacteria, they are not methylated in the E. coli strain (they do not contain HIP1 sequence recognize for methylases) and it could be digested.
- In line with our response below to line 817, we have added details on recovery and validation of the plasmid in the discussion.

- Line 475. "freezer" instead of "freeze"
- We have changed "freeze" to "freezer".

- Line 585. The DNA extraction kit (DNeasy Plant Mini kit, QIAGEN) is not included in the table of materials.
- We have added the DNeasy Plant Mini Kit to the Table of Materials.

- Line 593. The TissueLyser II (Qiagen) is not included in the table of materials.
- We have added the TissueLyser II to the Table of Materials.

- Line 601. The catalog and the company of agarose should be included in this line, since agarose is listed in the table of materials and it is not used in any part of the protocol.
- We have added the manufacturer here "(Bioline)".

- Lines 626-630. It could be possible to reproduce the recipe for BG11 medium, since it is not very complicated and it could likely be summarized in two lines, avoiding having to check a reference to find it.
- We disagree, as a whole page table is dedicated to the BG11 components in Lea-Smith et al. (2016). We feel that it would not simplify interpretation for potential users if these components were summarized in a short paragraph. We could add that table to our protocol? Perhaps the editor can advise if this would be feasible?

- Line 637. Introduce a space between "1" and "M".
- We have added a space.

- Line 640. What kind of agar did you use to prepare the BG11 plates (e.g. noble agar, bactoagar, etc)?
- We use microbiology test Agar (A1296-500g, Sigma-Aldrich). We have added these details to the text and the Table of Materials.

- Line 668, 670, 728, 733, 746, 752, 761, figure 4 and figure 6A. "cpcBA" should be in italics.
- We have changed "cpcBA" to "*cpcBA*" throughout.

- Lines 745-753. Why do you use SE instead of SD?
- Either can be used. We prefer to use standard error (SE) as it takes into account both the standard deviation (SD) and the sample size.

- Line 703. The discussion about preserving the codon for glutamic acid assumes that the BpiI site is in-frame with the start codon. May wish to clarify for precision, or eliminate this statement.
- We have added to the legend "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)."

- Line 817. Two critical steps that you should add are: 1) checking of the colonies are axenic, 2) checking the correct size of the plasmids inside the transconjugants, especially if they are big size.
- Although these steps are important they are not specific to the conjugation protocol per se. Thus we have added them to the end of this paragraph: "Confirming a transgenic cyanobacterial strain is axenic is important before 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 (see step 5), transformation into *E. coli* and selection (see step 2.1), and vector validation (see step 2.4)."

- Line 848. There is a typo, instead of "plant" is "plate".
- We have corrected this typo.

- Table 2. In the steps for the thermocycler protocol for Level 1, you should indicate as units for minutes "min" not "minutes", as you have in the protocol for Level 0, T.
- We have changed to min throughout the manuscript.

- Table 2. The company and the catalog number of ATP and BSA should be indicated in these tables (or removed), since they are listed in the table of materials and they are not used in any part of the protocol.
- We have added reference to the manufacturer in Table 2.

- Table of materials. The screw-cap tubes are not used in any part of the protocol. Please, remove from the table or include them in any part of the protocol.
- We have added 1.5 mL screw-cap tubes (Starstedt) to section “Cryostorage of cyanobacterial strains.”

General comments
- It may simplify the document to use the same nomenclature for all cyanobacterial strains. For example, S. elongatus UTEX 2973 and Synechocystis PCC 6803, instead of UTEX 2973 and Synechocystis.
- We have renamed these species *Synechocystis* PCC 6803 or *S. elongatus* UTEX 2973 throughout.

- You use two different nomenclatures within the text for the unit of seconds.
- We have corrected this.

- Along the text, you use two different nomenclatures for temperature. The correct one is that one that you have a space between the number and the degree symbol.
- We have corrected this.

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