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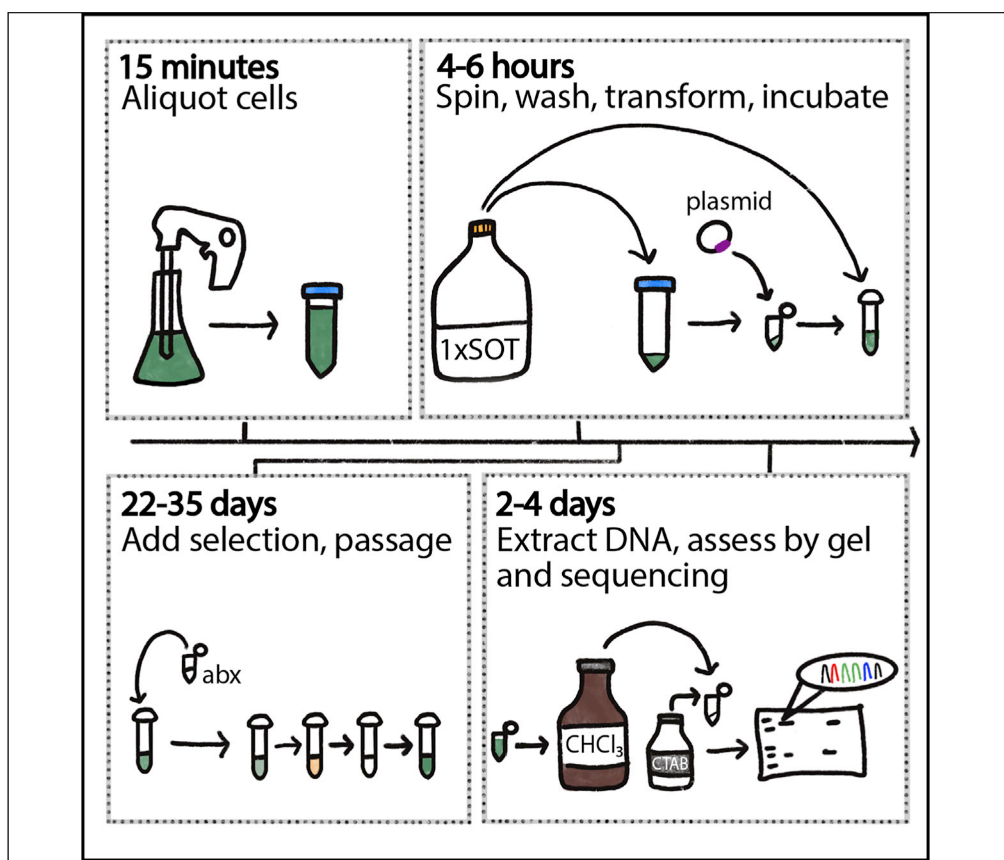
Protocol for the transformation and engineering of edible algae *Arthrospira platensis* to generate heterologous protein-expressing strains



Here, we present a protocol for harnessing the natural transformability of the edible algae *Arthrospira platensis* (common name: spirulina) to generate strains that express heterologous proteins. We describe the preparation of plasmids and the steps to grow *A. platensis*. We then detail the transformation and passage of the strains, followed by genomic DNA extraction and genotyping to assess integration of the gene of interest. This simple transformation protocol can be applied to genome manipulation of edible algae.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Harnessing natural *Arthrospira platensis* competency for plasmid transformation

Robust technique for heterologous protein expression

Steps to extract genomic DNA for integration assessment

Applicable for edible algae genome manipulation

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Protocol for the transformation and engineering of edible algae *Arthrospira platensis* to generate heterologous protein-expressing strains

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SUMMARY

Here, we present a protocol for harnessing the natural transformability of the edible algae *Arthrospira platensis* (common name: spirulina) to generate strains that express heterologous proteins. We describe the preparation of plasmids and the steps to grow *A. platensis*. We then detail the transformation and passage of the strains, followed by genomic DNA extraction and genotyping to assess integration of the gene of interest. This simple transformation protocol can be applied to genome manipulation of edible algae.

For complete details on the use and execution of this protocol, please refer to Jester et al. (2022).[1]

BEFORE YOU BEGIN

Cloning of shuttle plasmid

Timing: 1–3 weeks

This step describes how to design a plasmid for introducing a transgene into a neutral site in the *Arthrospira platensis* genome.

Note: the exact sequences of the genes and promoters listed in this section may require optimization based on which *A. platensis* strain will be used by the experimenters.

Note: Unfortunately, the *A. platensis* strain UTEX LB 1926 that was used by the authors does not have a complete deposited genome available in the NCBI database. The most similar deposited strain is *Arthrospira platensis* strain YZ.[2] Therefore, this protocol uses *A. platensis* strain YZ for NCBI sequence reference, with the nucleotide differences between *Arthrospira platensis* strain YZ and *A. platensis* strain UTEX LB 1926 noted in Table 1.

To design a plasmid for introducing a transgene into a neutral site in the *A. platensis* genome follow the below instructions:

1. Choose a promoter to drive the transgene within the *A. platensis* genome. An example of a robust and validated promoter is the 600 bp upstream region of the cpcB gene (P_*cpc600*).

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Table 1. *A. platensis* strain YZ versus *A. platensis* strain UTEX LB 1926 nucleotide differences in loci of interest

Gene or locus name	Gene or locus length (bp)	*A. platensis* strain YZ (genbank ID: CP013008) reference sequence location	*A. platensis* strain UTEX LB 1926 nucleotide differences from YZ
P_*pilA*	168	1272461 to 1272628	G to A at position 128
P_*cpc600*	600	1737781 to 1737181 (antisense)	No differences
KmR locus	2596	1613741 to 1616336; 1614745 to 1615316: *kmR* site that is replaced with transgene in final strain; p_*KmR* is within 5′ left arm of sequence	G to C at position 411; G to A at position 425
NS1 locus	3213	6182212 to 6179000 (antisense); 6180661 to 6180500: NS1 site that is replaced with transgene in final strain	A to G at position 1735; C to A at position 2874; A to C at position 2895; T to G at position 2909

2. Choose a locus in the genome for your target. The authors recommend the NS1 or *kmR* sites as validated neutral sites within the *A. platensis* genome.
3. Design a homologous recombination construct that includes at least 1,200 base pair length homologous arms on each side of the insert.

   Note: Although 1,200 bp has been validated as a sufficient length for homologous recombination to occur, the authors recommend the use of 2,000 bp homologous arms.

4. Include a selection marker of choice with a constitutive promoter. Two validated selection markers, with promoters:
   a. *pilA* promoter with *aadA* gene (streptomycin/spectinomycin resistance marker).[3]
   b. *kmR* promoter with *kmR* gene (kanamycin resistance); must be inserted into a Δ*kmR* strain.[1]
5. Clone the gene of interest and homologous arms into the shuttle vector backbone of the experimenters' choice.

   Note: To simplify high throughput plasmid generation, the shuttle vectors used by the authors include BsaI restriction sites that are compatible with modular Golden Gate cloning.[4] Maps of backbone plasmids used by the authors are indicated in Figure 1. These plasmids and their nucleotide sequences are available through Addgene. More information about obtaining the plasmids is in the "materials availability" section of the manuscript.

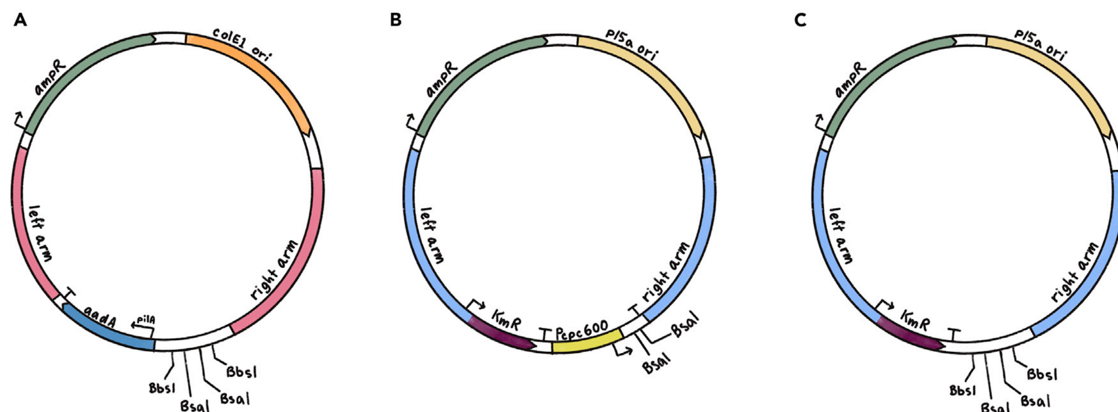

Figure 1. Examples of available backbone plasmids for constructing shuttle vectors for *A. platensis* transformation
(A–C) (A) pDV002, a streptomycin-selectable plasmid targeting the NS1 site in the *A. platensis* genome (B) pDV044, a kanamycin-selectable plasmid targeting the KmR-modified region (as described in Jester et al.[1]) in the *A. platensis* genome. The plasmid contains the validated promoter region *P_cpc600* (C) pDV052, a kanamycin-selectable plasmid targeting the KmR-modified region in the *A. platensis* genome. "Left arm" is the 5′ homologous arm of the region of insertion; "right arm" is the 3′ homologous arm of the region of insertion. BsaI and BbsI are restriction sites available for Golden Gate cloning. "aadA" is the streptomycin marker for *A. platensis*. "KmR" is the kanamycin selection marker for *A. platensis*. "AmpR" is the ampicillin marker for selection in E. coli. "p15a ori" is a low-copy origin of replication in *E. coli* and "colE1 ori" is a high-copy origin of replication in *E. coli*.

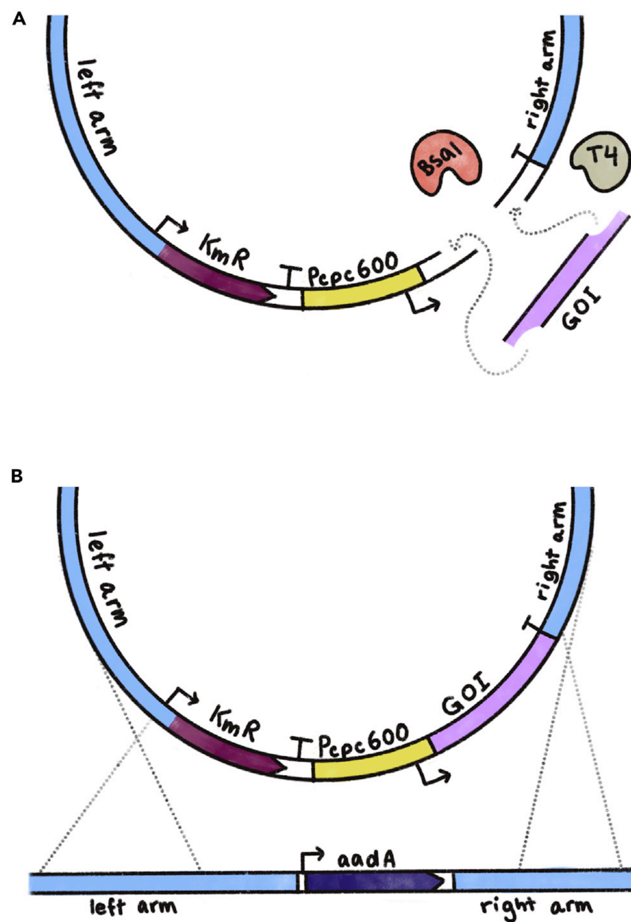

Figure 2. Schematic of shuttle vector cloning using Golden Gate and its subsequent homologous recombination into the *A. platensis* genome

(A) A simple representation of the Golden Gate cloning process. The BsaI restriction enzyme cleaves both the backbone plasmid and the gene of interest ("GOI") PCR-amplified fragment. The compatible sticky ends are then ligated together by the T4 DNA ligase for the generation of the final shuttle vector.

(B) The shuttle vector carrying the GOI is introduced into the *A. platensis* cell, where it is inserted into the targeted locus via homologous recombination. In this diagram, the insertion occurs at the modified KmR site carrying the *aadA* streptomycin resistance gene.

Note: The generalized process of Golden Gate cloning into backbone plasmids is illustrated in Figure 2A. Once verified, the final shuttle vectors can be used for downstream *A. platensis* transformation. The transgene is transferred from the shuttle vector into the *A. platensis* genome via homologous recombination (Figure 2B).

Plasmid preparation

Timing: 1 h

Follow a standard miniprep protocol, such as the Qiagen Miniprep Kit Protocol. Make sure to generate at least 300 ng of plasmid at 50 ng/μL per each desired transformation. Plasmids do not need to be made endotoxin-free.

1×SOT media preparation

Timing: 2 days

The authors use 1×SOT media (Spirulina-Ogawa-Terui Media) to grow *A. platensis*.[5] Prepare this media in advance according to the recipes found in the "materials and equipment" section below.

Genomic DNA extraction solutions preparation

Timing: 2 h

Prepare the solutions required for genomic DNA (gDNA) extraction in advance according to the recipes found in the "materials and equipment" section below.

A. platensis growth in preparation for transformation

Timing: 1–2 weeks

If thawing *A. platensis* from frozen stocks:

6. Thaw the tube by warming the cells in a 37°C water bath.
   a. If the tube contains 1 mL or more of cells, inoculate into a sterile 250 mL Erlenmeyer flask containing 50 mL of antibiotic-free 1×SOT.
   b. If it contains less than 1 mL of cells, inoculate into a sterile 50 mL Erlenmeyer flask containing 15 mL of antibiotic-free 1×SOT.
7. Grow the cells in a lighted incubator with the following settings:
   a. 35°C, 270 RPM orbital shaking.
   b. 100–125 μEi light.
   c. Supplemental CO_2 (about 0.40%).
8. Once the cells reach OD_{750} 0.8–1.2, subculture them at OD_{750} 0.1–0.2 into fresh media with selection (as appropriate) and grow them in an ambient CO_2 incubator with the following settings:
   a. 30°C.
   b. 120 RPM orbital shaking.
   c. 50–100 μEi light.
   d. Ambient CO_2 (about 0.04%).

Note: Grow the cells for 3–5 days, checking OD_{750} every 24 h, until they reach OD_{750} 0.8–1.2.

Note: A. platensis can be continuously grown for many months in an ambient CO_2 shaker set to the above conditions. They need to be passaged every time they reach OD_{750} 0.8–1.2 by dilution to an OD_{750} of 0.1–0.2, which is typically every 4–5 days. Examples of relevant OD_{750} are in Figure 3.

Note: The filamentous nature of the cells can make OD determination difficult. Make sure that the cells are as homogenous and evenly mixed as possible prior to assessment by pipetting the cells gently up and down using a 1,000 μL pipette.

Note: Strains can be grown in any volume from 15 to 50 mL in Erlenmeyer flasks with sufficient aeration. The authors suggest growing 15–20 mL in 50 mL Erlenmeyer flasks and 50 mL in 250 mL flasks (Figure 4). The general rule of thumb for growth volume is: 5 mL at OD_{750} 0.8–1.2 is enough for 6 transformations. Calculate the flask volume and number of flasks needed based on this estimate.

Figure 3. Examples of *A. platensis* diluted to various OD_{750} in a 10 mL glass tube that can help guide the growth of the experimenters' cultures

From left to right: OD_{750} 0.1, 0.2, 1.0 and 2.0. The filamentous nature of the cells can make OD determination difficult.

9. Once the cells reach the appropriate OD_{750} of 0.8–1.2 and appear healthy (no major clumping, no yellowing, no significant overgrowth of companion organisms, as evidenced by foul odor and/or biofilm formation on the inside of the flask), prepare your cells and plasmids for transformation.

   Note: *A. platensis* natural competence is driven by the presence of companion bacteria that co-culture with the algae.[1] These co-culturing bacteria can sometimes overgrow and overtake an unhealthy *A. platensis* culture.

   Note: 300 ng of shuttle plasmid can be aliquoted ahead of time into microcentrifuge tubes and stored at -20 C for up to a month. In that case, microcentrifuge tubes containing appropriately aliquoted DNA should be removed from the -20 C storage and thawed at 18–25 C for an hour prior to transformation.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Arthrospira platensis UTEX LB 1926	Culture Collection of Algae at the University of Texas at Austin	www.utex.org
Chemicals, peptides, and recombinant proteins		
Proteinase K powder (≥30 U/mg)	Thermo Fisher Scientific	Cat# 17916
RNAse A powder, DNAse free	Fisher Scientific	Cat# 50-153-8124
Lysozyme	VWR	Cat# 97062-138
Cetyltrimethylammonium bromide	Fisher Scientific	Cat# ICN19502905
Sodium bicarbonate	VWR	Cat# 144-55-8
Boric acid	Fisher Scientific	Cat# AC423485000
Manganese sulfate monohydrate	Thermo Fisher Scientific	Cat# A17615.36
Zinc sulfate heptahydrate	Thermo Fisher Scientific	Cat# A12915.36
Copper (II) sulfate pentahydrate	VWR	Cat# BDH9312-500G
Sodium molybdate dihydrate	Millipore Sigma	Cat# M1003-100G
Potassium phosphate dibasic	Millipore Sigma	Cat# P8281-100G
Sodium nitrate	Thermo Fisher Scientific	Cat# 014493.30
Potassium sulfate	Thermo Fisher Scientific	Cat# A13975.0I
Sodium chloride	Thermo Fisher Scientific	Cat# 012314.A9
Magnesium sulfate	Thermo Fisher Scientific	Cat# 413485000
Calcium chloride dihydrate	VWR	Cat# BDH9224-1KG

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Iron (II) sulfate heptahydrate	Thermo Fisher Scientific	Cat# A15178.0E
Sodium ethylenediaminetetraacetic acid dihydrate	Millipore Sigma	Cat# ED4SS-100G
Hydrochloric acid 6 N	Millipore Sigma	Cat# XX0628-01
Sodium hydroxide 10 N	Millipore Sigma	Cat # SX0607N-6
Sodium hydroxide pellets	Millipore Sigma	Cat # S5881-500G
Molecular grade water	Fisher Scientific	Cat# AAJ71786K8
Tris base	Fisher Scientific	Cat# BP152-500
Tris HCl	Millipore Sigma	Cat# T5941-100G
SDS (sodium dodecyl sulfate)	Millipore Sigma	Cat# 11667289001
Glycerol	Fisher Scientific	Cat# BP229-1
Ammonium acetate	Fisher Scientific	Cat# AAA1634330
Kanamycin sulfate	Fisher Scientific	Cat# BP906-5
Streptomycin sulfate salt	Millipore Sigma	Cat# S6501-5G
G418 Sulfate, powder	Fisher Scientific	Cat# MT61234RF
Molecular grade isopropanol	Fisher Scientific	Cat# BP2618500
Molecular grade ethanol (200 proof)	Fisher Scientific	Cat# BP2818500
Non-sterile isopropyl alcohol, 70%	Fisher Scientific	Cat# 19-130-713
Sodium acetate 3 M, pH 5.2	Fisher Scientific	Cat# 56-742-2100ML
Linear acrylamide, 5 mg/mL	Fisher Scientific	Cat# NC1781917
Chloroform isoamyl alcohol mixture, 24:1	Fisher Scientific	Cat# 11-101-6907
SapphireAmp Master Mix	Takara	Cat# RR350B
KAPA HiFi PCR Kit	Roche	Cat# KK2101
Critical commercial assays		
Miniprep Kit	Qiagen	Cat# 27106
NEBridge Golden Gate Assembly Kit	NEB	Cat# E1601S
Deposited data		
Arthrospira platensis YZ	Xu et al.[2]	www.ncbi.nlm.nih.gov Taxonomy ID: 1738638
Recombinant DNA		
pDV002	This paper	Addgene 191823
pDV044	This paper	Addgene 191824
pDV052	This paper	Addgene 191825
Other		
Phase-lock tube, heavy	VWR	Cat# 10847-802
Laminar flow hood in good working condition with a UV irradiating sterilization lamp and aspirator	Any brand/model available in the lab	N/A
A light meter to determine light intensity	Any brand/model available in the lab. Example used by authors in "identifier" column	Licor LI-250A light meter
Aliquoting pipetteman	Any brand/model available in the lab. Example used by authors in "identifier" column	Vistalab ali-Q 2 VS with variable aliquoting speed
Standard pipetteman	Any brand/model available in the lab	N/A
Microcentrifuge	Any brand/model available in the lab. Example used by authors in "identifier" column	Eppendorf 5425
Tabletop centrifuge that can accommodate 50 and 15 mL conical tubes	Any brand/model available in the lab. Example used by authors in "identifier" column	Eppendorf 5810R
Standard 1,000, 200 and 20 µL pipettors	Any brand/model available in the lab	N/A
1.5 mL microcentrifuge tube-compatible racks	Any brand/model available in the lab	N/A
14 mL round bottom tube-compatible racks	Any brand/model available in the lab	N/A
Sterile 50- and 250-mL flasks	Any brand/model available in the lab	N/A
Sterile 0.5, 1 and 2 L bottles	Any brand/model available in the lab	N/A
Spray bottle for holding the cleaning solution (70% isopropanol)	Any brand/model available in the lab	N/A

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
An adjustable-light shaker with ambient CO_2 that can accommodate 14 mL plastic round-bottom tubes and flasks	Any brand/model available in the lab. Example used by authors in "identifier" column	New Brunswick Innova 4400 with programmable illumination up to 1,500 μE
An adjustable-light shaker with supplemental CO_2 that can accommodate 14 mL plastic round-bottom tubes and flasks	Any brand/model available in the lab. Example used by authors in "identifier" column	Infors Multitron Pro shaker-incubators with programmable illumination up to 1,500 μE
Thermomixer	Any brand/model available in the lab. Example used by authors in "identifier" column	Eppendorf Thermomixer C with 2.0 mL, 1.5 mL, 50 mL attachments
DNA fluorometer	Any brand/model available in the lab. Example used by authors in "identifier" column	Qubit Flex Fluorometer
DNA fluoremetry kit	Any brand/model available in the lab. Example used by authors in "identifier" column	Qubit 1× dsDNA High Sensitivity (HS) Kit
Vacufuge	Any brand/model available in the lab. Example used by authors in "identifier" column	Eppendorf Vacufuge Plus Concentrator
Sterile 1,000, 200 and 10 μL filtered pipette tips	Any brand/model available in the lab	N/A
Sterile 14 mL plastic round-bottom tubes (ex: Corning Falcon round-bottom polypropylene test tubes)	Any brand/model available in the lab. Example used by authors in "identifier" column	Corning Falcon round-bottom polypropylene test tubes
Sterile 50 mL, 25 mL, 10 mL and 5 mL serological pipettes	Any brand/model available in the lab	N/A
Sterile 1.5 mL clear microcentrifuge tubes (avoid colored plastics for more consistency of light penetration)	Any brand/model available in the lab	N/A
Sterile 50 mL and 15 mL conical tubes	Any brand/model available in the lab	N/A
Sterile 1,000, 200 and 10 μL filtered pipette tips	Any brand/model available in the lab	N/A
Filter sterilization system for bottles, 0.22 μM or smaller	Any brand/model available in the lab	N/A
Positive-displacement pipette, 5 mL	Any brand/model available in the lab	N/A
Kimwipes	Fisher Scientific	Cat#06-666

MATERIALS AND EQUIPMENT

Equipment

Required equipment is listed in the "key resources table, section Other," above. In addition, you will need:

- A space with low light to recover your *A. platensis* transformants (20–60 μEi).
- A sterile vessel for 1×SOT + antibiotic master mix: can be flask, bottle or conical.

Figure 4. A representative photograph of 50 mL of healthy OD_{750} = 1 *A. platensis* culture grown in a 250 mL Erlenmeyer flask

Materials

Required materials are listed in the "key resources table, section Other," above. In addition, you will need to make the following medias and solutions:

A5 solution (1 L):

- Start with about 500 mL deionized water in a glass bottle.
- While stirring, add the following components:

A5 solution		
Reagent	Final concentration	Amount
Deionized water	N/A	About 1 L
H_3BO_3 (boric acid)	46.2 mM	2.86 g
$MnSO_4 \cdot H_2O$ (Manganese sulfate monohydrate)	9 mM	1.52 g
$ZnSO_4 \cdot 7H_2O$ (Zinc sulfate heptahydrate)	0.77 mM	0.222 g
$CuSO_4 \cdot 5H_2O$ (Copper (II) sulfate pentahydrate)	0.315 mM	0.079 g
$Na_2MoO_4 \cdot 2H_2O$ (Sodium molybdate dihydrate)	0.085 mM	0.021 g
Total		1 L
Store at 4 C for up to 2 years.		

- Once dissolved, bring to 1 L volume and filter sterilize into a 1 L sterile bottle.

10×SOT (1 L)

- Start with about 600 mL of deionized water in a 1 L glass bottle.
- While stirring, add the following components:

10×SOT		
Reagent	Final concentration	Amount
Deionized water	N/A	About 990 mL
K_2HPO_4 (Potassium phosphate dibasic)	28.6 mM	5 g
$NaNO_3$ (Sodium nitrate)	294 mM	25 g
K_2SO_4 (Potassium sulfate)	57.3 mM	10 g
NaCl (Sodium chloride)	171 mM	10 g
$MgSO_4$ (Magnesium sulfate)	8.12 mM	0.977 g
$CaCl_2 \cdot 2H_2O$ (Calcium chloride dihydrate)	2.7 mM	0.4 g
$FeSO_4 \cdot 7H_2O$ (Iron (II) sulfate heptahydrate)	0.35 mM	0.1 g
$Na_2EDTA \cdot 2H_2O$ (Sodium ethylenediaminetetraacetic acid dihydrate)	2.15 mM	0.8 g
A5 solution	N/A	10 mL
Total		1 L
Store at 4 C for up to one month.		

- Once mostly dissolved (5–7 min), adjust the solution's pH to 5 using HCl 6 N (hydrochloric acid).
- When the solution becomes clear, transfer to a 1 L graduated cylinder and fill to 1,000 mL with deionized water.
- Transfer back to 1 L bottle and stir.

⚠ CRITICAL: 10× SOT is never autoclaved.

Sodium bicarbonate 0.5 M (1 L)

- Start with about 800 mL deionized water in a 1 L glass bottle.
- While stirring, add 42 g sodium bicarbonate solid.
- Bring to a final volume of 1,000 mL with deionized water.
- Filter-sterilize using a 0.22 µm filter and store at 18–25 C for up to six months.

1×SOT (2 L)

1× SOT used by the authors contains 3 components:

1×SOT		
Reagent	Final concentration	Amount
Deionized water	N/A	1 L
10×SOT	1×	200 mL
0.5 M sodium bicarbonate	0.2 M	800 mL
Total		**2 L**

Store at 18–25 C for up to 3 months.

To prepare 1×SOT, perform the following:

- Day 1:
  - Mix 200 mL of 10× SOT and 1,000 mL deionized water for a final volume of 1.2 L.
  - Autoclave the mixture at 121 C for 45 min on a liquid autoclave cycle.
  - Remove the mixture from the autoclave and let cool overnight (12–16 h).
- Day 2:
  - Combine 1.2 L of the autoclaved and cooled media with 800 mL of sodium bicarbonate 0.5 M in a 2 L container for a final sodium bicarbonate concentration of 0.2 M.
  - Filter-sterilize the 1×SOT using a 0.22 µm filter into a new sterile 2 L bottle and store at 18–25 C for up to 3 months.

The following solutions are required to perform the "A. platensis transformant genotyping" step:

- **NaCl 5 M:** Per 1 L of final solution, weight out 292.2 g of NaCl (sodium chloride) and resuspend in 700 mL of deionized water. Stir to mix. Once the NaCl is resuspended, top up to 1 L. Store at 18–25 C for up to a year.
- **10% CTAB solution**: Per 5 mL final solution, weigh 0.5 g of CTAB (cetyltrimethylammonium bromide) powder and resuspend in 5 mL of molecular grade water in a 50 mL conical tube. To resuspend, heat the conical tube in a heat block at 65 C shaking at 200 rpm for 2 h. Aliquot the solution into 1.5 mL microcentrifuge tubes and store at 18–25 C for up to a year.

  Note: This solution precipitates out after some time at 18–25 C and may require re-heating at 65 C to re-dissolve prior to use.

- **EDTA 0.5 M, pH 8.0**: Per 500 mL of final solution, weigh out 93.05 g of $Na_2EDTA \cdot 2H_2O$. Resuspend in 400 mL deionized water and heat at 70 C until EDTA has dissolved. Add 9 g of solid NaOH (sodium hydroxide) and continue stirring. Once the NaOH has dissolved, continue with pH adjustment using 10 N NaOH solution until the solution reaches pH 8.0. Finally, top up with deionized water until the volume reaches 500 mL. Store at 18–25 C for up to a year.

- **Tris HCl 50 mM, pH 8.0**: per 1 L of final solution, weigh out 2.65 g of Tris base and 4.44 g of Tris HCl. Resuspend in 1 L of deionized water. Use a pH meter to confirm the pH. If pH is not within an acceptable range of 7.95–8.05, adjust as appropriate with dropwise addition of concentrated hydrochloric acid or sodium hydroxide solutions. Store at 18–25 C for up to a year.
- **Tris HCl 10 mM, pH 8.0:** per 1 L of final solution, add 200 mL of 50 mM Tris HCl, pH 8.0 solution to 800 mL of deionized water. Swirl to mix. Adjust pH if necessary. Store at 18–25 C for up to a year.
- **TE buffer (Tris HCl, 10 mM and EDTA, 1 mM, pH 8.0):** Per 1 L of final TE buffer, add 200 mL of Tris 50 mM stock and 2 mL of EDTA 0.5 M stock to 700 mL of deionized grade water. Swirl to mix and top off with deionized grade water up to a final volume of 1 L. Store at 18–25 C for up to a year.
- **70% molecular grade ethanol**: Per 100 mL of final solution, mix 70 mL of molecular grade ethanol with 30 mL of molecular grade water. Swirl to mix and store at -20 C for up to 2 months.
- **20% SDS**: Per 50 mL, weight out 10 g of SDS. Transfer the SDS into a 50 mL conical. Add 15–20 mL of molecular grade water. To resuspend, heat the conical tube in a heat block at 65 C shaking at 200 rpm for 2 h. If after 2 h the solution is not dissolved, add 5–7 mL of molecular grade water, and continue the 65 C agitation for another 30 min. Once the solution is dissolved, top off with molecular grade water up to a final volume of 50 mL. Store at 18–25 C for up to a year.

  Note: SDS powder is an irritant and prone to aerosolization. Handle with care.

- **50 mg/mL lysozyme solution**: Per 10 mL final solution, weigh out 500 mg of lysozyme into a 15 mL conical tube. Top off with Tris HCl 10 mM pH 8.0 up to a final volume of 10 mL. Mix by inversion. Filter-sterilize using a 0.22 μm filter and aliquot into 0.2 mL microcentrifuge tubes at a 100 μL volume per tube. Store at -20 C for up to a year.
- **20 mg/mL proteinase K solution in 50% glycerol**: per 5 mL final solution, weigh out 100 mg of proteinase K powder in a 15 mL conical tube. Add 2.5 mL of Tris HCl, 10 mM, pH 8.0 solution and swirl to mix. Slowly add 2.5 mL of pure glycerol to the solution. Swirl to mix. Aliquot into 0.2 mL microcentrifuge tubes at a 100 μL volume per tube. Store at -20 C for up to a year.

  Note: to accurately pipette pure glycerol the authors recommend using positive displacement pipettes.

- **10 mg/mL RNase A solution**: per 10 mL final solution, weigh out 100 mg of RNase A in a 15 mL conical tube. Top off with 10 mL molecular grade water and swirl to mix. Filter-sterilize using a 0.22 μm filter and aliquot into 0.2 mL microcentrifuge tubes at a 100 μL volume per tube. Store at -20 C for up to a year.
- **Ammonium acetate 7.5 M**: Per 50 mL final solution, weight out 29 g of ammonium acetate powder into a 50 mL conical tube. Top off with molecular grade water up to a final volume of 50 mL. Heat the solution at 65 C in a heat block for 2 h with agitation. Store at 18–25 C for up to a year.

  ⚠ CRITICAL: 7.5 M is a very concentrated ammonium acetate solution, so the volume of powder will be large. Make sure to add water to the powder rather than powder to the water, to avoid over-diluting the solution.

  Note: Ammonium acetate 7.5 M is only required if using the proteinase K-isopropanol precipitation extraction method.

STEP-BY-STEP METHOD DETAILS

Day 1: *A. platensis* transformation

⏲ Timing: 6 h

Day 1 of the *A. platensis* transformation involves the introduction of the shuttle vector into the cells.

Optional: This step is if plasmids were aliquoted ahead of time and frozen. Remove the plasmids from the -20 C and thaw for an hour at 18–25 C prior to transformation. Prior to use, spin briefly to transfer the DNA to the bottom of the microcentrifuge tube and set aside.

1. If plasmids were not aliquoted ahead of time, aliquot them into pre-labeled microcentrifuge tubes. You will need 300 ng of plasmid per transformation at a concentration of 50 ng/μL or higher. Once aliquoted, spin briefly to move the DNA to the bottom of the tubes and set aside.
2. Adequately sterilize the laminar flow hood prior to beginning work.
   a. Perform 15 min of UV irradiation with the sash closed, followed by 15 min of air circulation with the sash open, though the specifics will depend on the model available to the experimenter.
   b. Spray down the working surface of the laminar flow hood with 70% isopropanol or ethanol.
   c. Wipe down all required equipment (pipettes, pipetteman) with 70% isopropanol or ethanol prior to moving them into the hood.
3. Take the healthy growing OD_{750} 0.8–1.2 range *A. platensis* from the lighted incubator and move it into the laminar flow hood.
4. Calculate the volume of growing *A. platensis* required for the number of transformations being performed. The following is a simple way to calculate the needed volume:

$$\frac{(30\ \mu L * [number\ of\ transformations + 1\ negative\ control]) * 25}{1000\ \mu L/mL} = x\ mLs\ of\ culture$$

Example: $\frac{(30\ \mu L * [9+1]) * 25}{1000\ \mu L/mL} = 7.5\ \ mLs\ of\ culture$

5. Once the volume has been calculated, prepare an appropriately sized conical (15 or 50 mL) in the hood.
6. Swirl the culture to resuspend and homogenize the cells, as *A. platensis* tend to settle and clump if left sitting without agitation.
7. Use a pipetteman with an appropriately sized serological pipette to transfer the calculated culture volume to the conical.
8. Centrifuge the cells at 1,600 × *g* for 10 min at 18–25 C.
9. Return the cells to the hood and carefully aspirate the supernatant. It is okay if some supernatant remains. *A. platensis* forms a loose pellet, and it is more important to not lose cells.
10. Add an equivalent volume (ex. 10 mL of 1×SOT to a pellet formed from 10 mL of *A. platensis* culture) of 18–25 C 1×SOT to the *A. platensis* pellet.
11. Resuspend the pellet by gently inverting the tube.
12. Centrifuge the cells at 1,600 × *g* for 5 min at 18–25 C. Carefully aspirate the supernatant, taking care to not disturb the pellet.
13. Use the following formula to calculate the 1×SOT volume for resuspension:

$$30\ \mu L * [number\ of\ transformations + 1\ negative\ control] = total\ \mu L\ needed$$

14. Add 1×SOT and resuspend the cells in the calculated volume of 1×SOT by pipetting up and down gently. You have generated *A. platensis* transformation slurry.
15. Remove 30 μL of the transformation slurry from the conical and add it to your first plasmid containing microcentrifuge tube by pipetting gently into the bottom of the tube.
16. Pipette the slurry-DNA mix up and down gently at least 10 times. Use a new tip for each sample to avoid cross-contamination.
17. Continue with the remaining transformations. Include an empty microcentrifuge tube as a negative control.
18. Once the DNA and slurry mixing is done for all samples, set the microcentrifuge tubes in a space with low light to transform (20–60 μEi). Transformation requires a minimum of 2.5 h but can be done for as long as 5 with no reduction in efficiency.

19. During the transformation period, set up and label the 14 mL round bottom tubes. Aliquot 600 μL antibiotic-free 1×SOT into each tube.
20. Once the transformation period is completed, transfer the slurry-DNA mix into the prelabeled 1×SOT-containing round bottom tubes. Pipette up and down 2–5 times gently to resuspend.
21. Move the tubes into the shaker set to the following conditions: 30 C, 120 RPM orbital shaking, 50–100 μEi light, ambient CO_2 (about 0.04%). Keep the cells, without selection, in the shaker overnight (12–16 h).

Day 2: *A. platensis* transformation

Timing: 1 h

Day 2 of the *A. platensis* transformation involves adding the selection antibiotic to the cells and moving the cells into a high-light, high CO_2 environment.

22. On day 2, prepare the laminar flow hood as before. In addition, set out the antibiotic that will be used for selection.
23. Move the *A. platensis* transformation tubes into the hood. It is normal for the cells to have settled to the bottom of the tube.
24. Calculate the volume of master mix of 1×SOT containing the selection antibiotic needed to be able to add 2.4 mL to each tube.
25. Calculate the volume of antibiotic stock needed in each 2.4 mL aliquot to account for the 0.6 mL volume already present in the tube by multiplying the concentration by 1.25-fold. For example, if you need a final concentration of 100 μg/mL of an antibiotic, you will need 125 μg/mL in the master mix to account for dilution.

Note: the authors have found that starting with a lower concentration of antibiotic and ramping it up throughout the transformation process works better than immediately exposing the cells to the maximum antibiotic concentration. This will require empirical optimization, and it depends on the strain of *A. platensis* and the antibiotics used.

26. Add the appropriate amount of antibiotic to your 1×SOT master mix and swirl to mix.

Note: the authors have successfully used the following concentrations for *A. platensis* selection: 2.5 μg/mL streptomycin (maximum concentration 5 μg/mL), 70 μg/mL kanamycin (maximum concentration 100 μg/mL), 30 μg/mL gentamicin (G418) (maximum concentration 60 μg/mL).

27. Using an aliquoting pipettor, add 2.4 mL to each 14 mL round bottom *A. platensis* tube. Be mindful to add the media slowly to prevent splashing and cross-contamination.
28. Move the tubes into a shaker set to the following conditions: 35 C, 270 RPM orbital shaking, 125 μEi light, supplemental CO_2 (about 0.40%).

Day 3–35: *A. platensis* transformation

Timing: 35 days

29. For the next 35 days, passage the strains twice a week. The strains will go through a death and resurrection phase if undergoing a successful transformation. For each passage:
    a. Remove the tubes from the shaker.
    b. Centrifuge the cells at 1,600 × *g* for 10 min in a tabletop centrifuge.
    c. Aspirate the spent media.
    d. Add 3 mL of fresh 1×SOT with selection antibiotic.

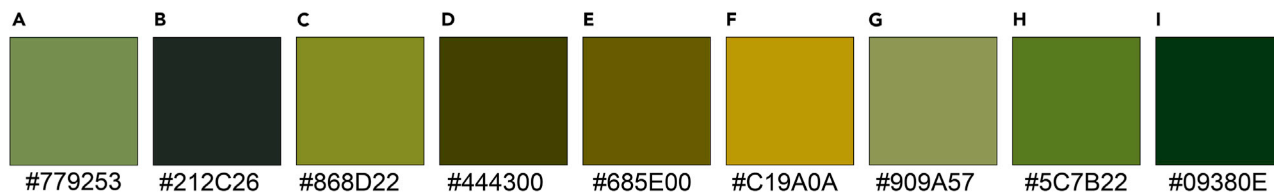

Figure 5. A representative example of the color gradations throughout *A. platensis* transformation with the color hex codes listed below
(A) Day 1.
(B) Day 6.
(C) Day 8.
(D) Day 10.
(E) Day 12.
(F) Day 14.
(G) Day 18.
(H) Day 22.
(I) Day 24.
These are simply representative. The duration and specifics of the color gradations may vary from sample to sample.

e. Place tubes back into the shaker.

Note: the dying and resurrection phase generally follows a pattern of Figure 5. Days 1–5: cells look healthy. Days 6–8: cells become a deep, dark green (does not always happen, some cells proceed to next step directly). Days 8–18: cells become yellow, brown and clumpy. Days 18–21: cells appear to disappear from the tubes. The tubes will look clear, except when spun down you will often see a small, white pellet. (This does not always happen, some cells proceed to next step directly). Days 21–35: the strains will start to emerge with a light green that will at first only be visible by pelleting. This is a successful transformation. If green cells do not emerge by day 35, this means the strains did not successfully transform.

30. Once the strains emerge, the passaging process changes from pelleting the cells to aspirating the cells, diluting the *A. platensis* to an OD_{750} of about 0.1 in 3 mL of media.
    a. At this point, you can begin increasing the antibiotic concentration. Typically, the authors increase the antibiotics as follows:
        i. Kanamycin: 70 μg/mL initial; 80 μg/mL at 1 month post transformation; 100 μg/mL at 2 months post transformation.
        ii. Streptomycin: 2.5 μg/mL initial; 3.5 μg/mL at 1 month post transformation; 5 μg/mL at 2 months post transformation.
        iii. Gentamicin (G418): 30 μg/mL initial; 50 μg/mL at 1 month post transformation; 60 μg/mL at 2 months post transformation.
31. Continue to passage strains every 4–5 days for 3–4 months.
    a. Most strains generated by the authors reach complete segregation (no detectable presence of the original locus, suggesting 100% of the chromosomes carry the transgene of interest) by 4 months.
32. Strains can now be assessed for segregation, expression and any other bioactivities the experimenters are interested in.

A. platensis transformant genotyping

Timing: 1–2 weeks

A. platensis' gDNA (genomic DNA) can be difficult to extract for colony PCR using standard colony PCR protocols such as simple 95 C thermocycler boiling. The DNA may be significantly contaminated with polyphenols and carbohydrates, as is common with many photosynthetic organisms.[6]

Due to this limitation, the authors find that performing one of the following gDNA extractions is necessary for successful and consistent genotyping.

Genomic DNA can be isolated following two methods: the CTAB-chloroform method or the alternate proteinase K–isopropanol precipitation method.

CTAB-chloroform method

This method generates "pure" genomic DNA – the DNA is rid of contaminating carbohydrates and polyphenols and can be used in any desired application. It can be quantified by Nanodrop or another OD_{260} absorbance quantification method.

33. Collect 15–25 mL of healthy, growing cells at OD_{750} 0.8–1.2 (day 3–5 post dilution at 30 C, 120 RPM orbital shaking, 50–100 μEi light, ambient CO_2).
34. Centrifuge at 1,600 × *g* for 10 min and aspirate media.
35. Wash cells with equivalent volume of sterile deionized water, centrifuge at 1,600 × *g* for 10 min and aspirate the water.
36. Freeze the pellet at -80 C for a minimum of 12 h before thawing for lysis steps.

Note: The freeze-thaw step aids lysis of the cells.

37. Allow the pellet to thaw at 18–25 C and resuspend the pellet in 500 μL TE buffer.
38. Add 100 μL of 50 mg/mL lysozyme for a final concentration of 8.3 mg/mL.
39. Incubate at 37 C for 30 min with no shaking in the thermomixer.
40. To the same solution add 12.5 μL proteinase K (20 mg/mL) and 70 μL SDS (20%). The final concentrations are 2% SDS and 366 μg/mL proteinase K.
41. Incubate in the thermomixer at 56 C shaking at 500 rpm overnight (12–16 h).
42. Remove the tubes from the thermomixer and centrifuge the cells at 15,000 × *g* for 30 s to pellet the cell debris.
43. Carefully transfer 500 μL of the supernatant to a new 2.0 mL microcentrifuge tube.

Pause point: The supernatant can be stored at -20 C for 3–6 months. When ready, thaw at 18–25 C and proceed with the next steps.

44. Add 150 μL 5 M NaCl to the supernatant, followed by 65 μL of 10% CTAB solution. Gently mix by inversion.
45. Incubate in the thermomixer at 65 C for 10 min with no shaking.
46. Remove the tubes from the thermomixer and let cool to 18–25 C.
47. Add 715 μL of chloroform-isoamyl alcohol to the sample. Mix by inversion.
48. Place the tubes on ice for 30–60 min to allow for the precipitation of the CTAB: contaminant complexes.
49. While the tubes are sitting on ice, pre-spin the Phase Lock Heavy tubes at 12,000 × *g* for 30 s.
50. Gently transfer the entire contents of the chloroform-DNA tube to the pre-spun Phase Lock tube.
51. Centrifuge the Phase Lock at 12,000 × *g* for 5 min to separate the aqueous and organic phases.
52. While the Phase Lock tubes are spinning, aliquot 420 μL of isopropanol into new 1.5 mL microcentrifuge tubes. Figure 6 is an example of a successfully phase-separated *A. platensis* genomic DNA sample in a phase-lock tube.
53. Gently remove 700 μL of the aqueous layer from the Phase Lock tubes and add to the Isopropanol-containing tubes. Mix by inversion until a string-like white or off-white precipitate is observed. That is the genomic DNA.

Optional: Prior to adding the aqueous layer to isopropanol, add 1.4 μL of linear acrylamide (5 mg/mL) to the 700 μL solution for a final concentration of 10 μg/mL. This will help visualize

Figure 6. A post-spin phase-lock tube containing the chloroform-CTAB-DNA mixture
A clear aqueous layer which contains the purified DNA is visible above a yellow organic layer which contains the non-DNA contaminants.

the DNA pellet during the washing steps if the DNA concentration is low. Linear acrylamide can be added directly to the Phase Lock tube post-spin.

54. Centrifuge the isopropanol-DNA containing tubes at 15,000 × *g* for 10 min at 4 C in a tabletop microcentrifuge.
55. Carefully decant the isopropanol from the tubes, ensuring to not disturb the pellet.
56. Wash the pellet using 1 mL of ice cold 70% ethanol to remove residual salts.
57. Centrifuge at 15,000 × *g* for 10 min at 4 C in a tabletop microcentrifuge.
58. Carefully decant the supernatant to avoid perturbing the DNA pellet.
59. Invert the tube onto a clean Kimwipe and allow the Kimwipe to absorb as much of the ethanol supernatant as possible.
60. Move the tubes into the vacufuge and dry for 5 min on the vacuum-alcohol setting with no heat.
61. Resuspend the DNA pellet in 100 μL of TE buffer.

Optional: RNAse A treatment

For using extracted gDNA for an assay which required the removal of RNA, perform RNAse A treatment:

62. Treat the DNA sample with 1 μL of RNAse A 10 mg/mL (for a final concentration of 100 μg/mL) for 1 h at 37 C in the thermomixer with no shaking.
63. Add 350 μL of TE Buffer and 50 μL of sodium acetate 3 M to the DNA sample. Invert to mix.
64. Add 300 μL of isopropanol to the sample. Invert to mix.
65. Place the sample in the centrifuge for 5 min at 15,000 × *g* and 4 C.
66. Carefully decant the supernatant.
67. Wash with 500 μL ice-cold 70% ethanol.
68. Place the sample in the centrifuge for 5 min at 15,000 × *g* and 4 C.
69. Carefully decant the supernatant.

70. Dry in the vacufuge for 5 min on the vacuum-alcohol setting with no heat.
71. Resuspend the sample in 100 μL TE buffer.

Alternate approach: Proteinase K-isopropanol precipitation method

If short on time, the proteinase K-isopropanol precipitation method is an alternate approach that will generate only "semi-pure" DNA. The DNA is still significantly contaminated with polyphenols, making it unsuitable for highly sensitive applications such as PacBio sequencing.[7] It also cannot be quantified by Nanodrop or other OD_{260} absorbance quantification metrics. It is, however, suitable for most standard PCR amplification and can be quantified by intercalating dye assessments such as gel based or fluorometric quantification (ex: Qubit). To use this alternate approach, follow the CTAB-chloroform method (steps 33–43). At the conclusion of step 43, do the following:

72. Add 250 μL of 7.5 M ammonium acetate to the supernatant. The final ammonium acetate concentration will be 2.5 M in 750 μL of solution.
73. Mix well by inversion.
74. After addition of ammonium acetate, centrifuge samples using a microcentrifuge for 30 s at 15,000 × *g*.
75. Move entire supernatant to a fresh 1.5 mL microcentrifuge tube.
76. Discard away tube containing debris.

Optional: Add 1.5 μL of linear acrylamide (5 mg/mL) to the 750 μL of solution for a final concentration of 10 μg/mL. This will help visualize the DNA pellet during the washing steps if the DNA concentration is low.

77. Mix well by inversion.
78. Add 450 μL of 100% isopropanol to the tube.
79. Mix well by inversion.
80. Now proceed with steps 54–61 as with the CTAB-chloroform protocol.
81. PCR for genotyping. Genotyping PCR can be done using the experimenters' favorite enzyme. The authors typically use SapphireAmp Fast PCR Master Mix (Takara, RR350B) for PCR segregation screening and KAPA HiFi (Roche, KK2101) for high-fidelity PCR for amplicon sequencing.

EXPECTED OUTCOMES

This protocol should allow the experimenters to generate *A. platensis* strains expressing any transgenes of interest. As detailed in Jester et al., the authors were able to achieve a heterologous protein expression at 15% of total *A. platensis* biomass, suggesting that this genomic integration-based expression method is suitable for a variety of applications requiring high levels of protein expression (Figure 7).

LIMITATIONS

Currently there are no robust toolkits available for inducible protein expression in *A. platensis*. Because of this it can be difficult to introduce transgenes into the bacterium's genome that encode lethal or deleterious heterologous proteins using the method described in this paper. This method also lacks a robust counterselection and depends on the natural chromosomal segregation process to generate a homozygous *A. platensis* expressor strain. Although eventually a positively selected gene does typically segregate to homozygosity this process is slower than what could potentially be possible with a counterselection protocol.

TROUBLESHOOTING

Problem 1

No transformants present at conclusion of step 29.

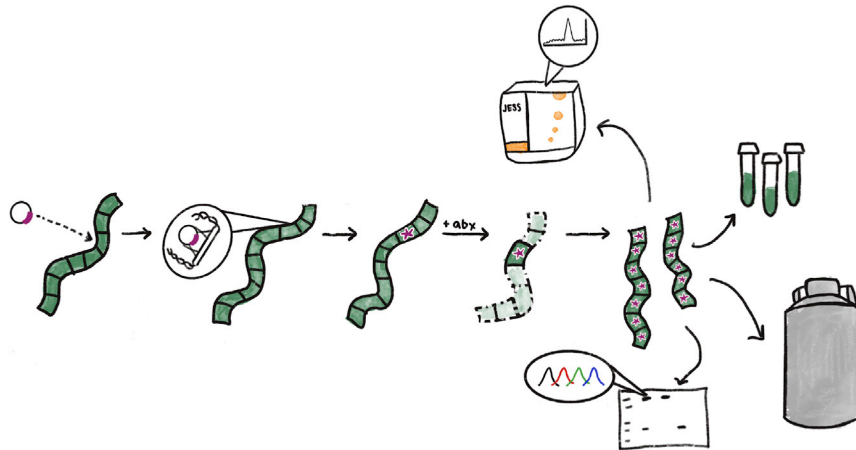

Figure 7. *A. platensis* transformation and assessment process

Strains are transformed with a plasmid carrying a gene of interest flanked by homologous arms for integration into the genome. The cells whose genomes contain the integrated transgene are able to survive antibiotic selection. These surviving strains can then be assessed for segregation, protein expression, frozen and grown for any required purpose.

Potential solution

The authors have found that sometimes strains need to be transformed twice for successful strain generation. Repeat the process with fresh plasmid. If no transformants are visible after a second round of transformation, this suggests that there is an inherent problem with the transgene insertion and the authors suggest a construct re-design.

Problem 2

No gDNA in DNA extraction at the conclusion of step 61. *A. platensis'* DNA is a much smaller fraction of total biomass than in other well-studied bacteria such as *E. coli* and *Bacillus subtilis*,[6] making adequate DNA extraction difficult.

Potential solution

Increase the biomass volume extracted. If using the proteinase K-isopropanol precipitation method, switch to the CTAB-chloroform method, which typically has higher efficiency.

Problem 3

No PCR product during genotyping assessment at the conclusion of step 81. This can be due to low DNA yield, as described above, or due to significant polyphenol and carbohydrate DNA contamination interfering with the PCR.

Potential solution

Potential solutions: dilute the DNA 1:10 and 1:100 to minimize the effect of contaminant carryover into the PCR. If using the proteinase K-isopropanol precipitation method for gDNA extraction, switch to the CTAB-chloroform method which minimizes contamination.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Hannah Tabakh (htabakh@lumen.bio).

Materials availability

The following backbone plasmids have been deposited at Addgene:

Plasmid ID	Plasmid name	Plasmid description
191823	pDV002	Plasmid for the transformation of the cyanobacterium *Arthrospira platensis* at the NS1 locus. No promoter. No terminator. Golden Gate compatible. Selection: in A. platensis: aadA; E. coli: AmpR
191824	pDV044	Plasmid for the transformation of the cyanobacterium *Arthrospira platensis* at the KmR locus. pCPC600 promoter. With terminator. Golden Gate compatible. Selection: in A. platensis: KmR; E. coli: AmpR
191825	pDV052	Plasmid for the transformation of the cyanobacterium *Arthrospira platensis* at the KmR locus. No promoter. No terminator. Golden Gate compatible. Selection: in A. platensis: KmR; E. coli: AmpR

Data and code availability

This study did not generate any datasets.

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AUTHOR CONTRIBUTIONS

Conceptualization, H.T., B.W.J., H.Z., R.K., N.K., R.T., J.R.; Investigation, H.T., H.Z., R.K., N.K., C.S., R.T.; Writing – Original Draft, H.T.; Writing – Review & Editing, B.W.J., H.Z., N.K., J.R.; Funding Acquisition, J.R.; Supervision, J.R.

DECLARATION OF INTERESTS

J.R. is a founder and current employee of Lumen Bioscience, Inc. (Lumen) and owns stock/stock options in Lumen. H.T., B.W.J., H.Z., N.K., and C.S. are current employees or paid advisors of Lumen; all current and former employees own stock/stock options of Lumen. R.T. and R.K. were employees of Lumen at the time of data generation; all current and former employees own stock/stock options of Lumen. Lumen has issued patents (US Patent Nos. 10,131,870, 10,415,012, 10,336,982, 10,415,013) and has pending patent applications (U.S. Patent Application No. 16/570,520 and International Patent Application No. PCT/US2022/013529) relating to *Arthrospira platensis* transformation methods.

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