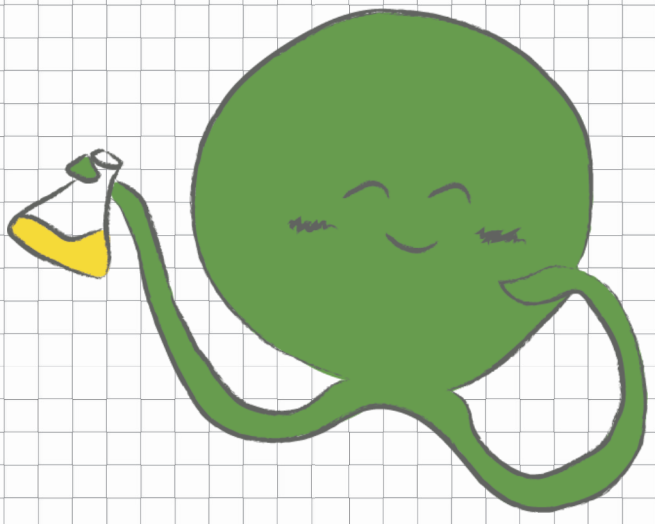

THE CHLAMY GUIDE

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SUMMARY

INTRODUCTION

The green algae *Chlamydomonas* has been used as a model organism for various research areas such as photosynthesis. *Chlamydomonas reinhardtii* has a cell wall, a pyrenoid and an eyespot that senses light.
It has the ability to grow photoautotrophically when exposed to light as well as to grow in darkness when an organic carbon source is available.
Due to the development of the modular cloning system and DNA transformation methods it is an easy to use chassis for synthetic biology. A major drawback is the inability of homologous recombination, as a result transforming DNA is inserted randomly into the genome. As an eukaryote it has a short doubling time of only eight hours, is able to modify proteins post-translationally and allows modification of the chloroplast, mitochondrial and nuclear genomes.
Thus *Chlamy* is perfectly suitable for protein expression and secretion. In addition, it is a non-toxic S1 organism which meets the safety standards of iGEM.

HOW TO CULTURE CHLAMY

In order to perform various tests with *Chlamy*, you have to cultivate them. Therefore, the characteristics of the green algae should be taken into account. It can grow photo autotrophically or in the dark relaying in the carbon source. The liquid cultures in the flask are placed on the shaker which is set to 80-120 rpm for the required mixing, at 25 °C and 80-120 µE. However, not only liquid cultures but also cultures on plates are required, which should be stored under the light as well.

By all the following steps it is necessary to work under sterile conditions.

1. Preparation of liquid and solid media

In the case of liquid media, a distinction must be made depending on the experiment. For any general use you should use TAP medium, if you want to make experiments in the bioreactor you need HMP medium.

TAP medium: The culture medium TAP can be used for both, liquid cultures and plates. However, it should be noted that the selection pressure can be increased by adding an antibiotic to the medium. Thereby, only cultures with the appropriate antibiotic resistance can survive.

HMP medium: The minimal medium HMP can be used for more specific experiments. It is especially suitable for bioreactor experiments. If the medium is used in a flask, it must be noted that it should be filled up to the half of the lowest line. In this way more light reaches the surfaces

2. Inoculation

For a lot of experiments, you need liquid cultures. Therefore, you want to work under sterile conditions when inoculating. To obtain a culture from plate in liquid medium you need to fill the TAP medium to the lowest line of an Erlenmeyer flask. Afterwards the plate has to be opened sterile to take a bit of the colony with a glass rod (alternatively take a pipette tip). Be cautious: do not mix the colonies, do not damage the plate and do not take all of the colony.

3. Set cell count

Chlamy needs approximately 8h for division. Depending on the thickness of the inoculation, all flasks have to be adjusted to the same cell number to obtain comparable results. After two days of growing we usually adjusted the cultures to 2.10^5 cells per ml (depending on which experiments you want to perform). For doing so you first have to measure the number of cells. Afterwards you can use the following formula to calculate the volume you have to add to TAP medium:
(debit/credit)*final volume=volume to add

4. Dilute cultures

Once the culture reaches $\sim 1.5*10^7$, it is in the stationary phase. If you want to use the culture for further experiments you have to consider that it has to be diluted (Otherwise the cells start clumping and are dying). It's the same procedure as described above.

5. Transfer of colonies

In order to prevent the cultures on the plates from dying, they have to be transferred every three weeks. This process describes the transfer of colonies from old plates to new one (Use only one glass rod per colony to prevent contamination).

6. From liquid culture to plate

However, it is also possible to go from liquid culture to plate. This has to be decided according to the cell density in the flask. The higher the cell density of the culture, the less has to be dripped on the plate by using the pipette.

CLONING STRATEGY FOR THE PRODUCTION OF YOUR CONSTRUCT

1. MoClo kit for *Chlamydomonas*

a. Type IIS enzyme

To use the MoClo kit for *Chlamydomonas reinhardtii* you will need to know what are Type IIS restriction enzymes because they are key point of MoClo strategy, which is based on the Golden Gate Cloning. Restriction enzymes are endonucleases which can be classified in four categories: Type I, Type II, Type III and Type IV (NEB. 2019).

Type II enzymes are the best known because they are commonly used in labs. They have a defined cleavage site and cut double stranded DNA (dsDNA) inside or close to the recognition sequence so as to produce fragments. The most common Type II enzymes (such as BamHI or EcoRI) cut dsDNA inside the palindromic recognition sequence. This means that, upon ligation with another compatible fragment, the final construct contains the reconstituted recognition site.

Type IIS enzymes recognize continuous and asymmetric sequences, and, contrary to other Type II enzymes, cleave outside of their recognition sequence to one side. Thus, while performing Golden Gate cloning, the design is done so that the part of the fragment containing the recognition sites is released and in thus absent of the final product after assembly with another compatible fragment (Engler et al. 2008). This process is (quasi-)scarless and allows directional assembly, which allows Golden Gate cloning.

b. Principle of the strategy

If you want to create a new transgenic strain of *Chlamydomonas* for your project, you will need to modify its genome. To do so, the MoClo (Modular Cloning), based on the Golden Gate cloning, is one of the best options as its standard is widely used and a kit of parts is availabe (Crozet et al. 2018). MoClo principle is to assemble standardized basic gene parts, cloned in donor plasmids, into any synthetic gene, which then can be assembled into complex multigenic DNA in only two steps (Weber et al. 2011). The cloning steps must be performed in *E. coli* before using it to transform *Chlamydomonas*.

To build your biobrick, you must remove the recognition motives of two Type IIS enzymes: BsaI and BpiI (a.k.a. BbsI) from your basic parts sequence (Engler et al. 2008). This step is named domestication. SapI restriction sites must also be removed because this site is not allowed in iGEM competition To clone the part of interest into the dedicated MoClo plasmid, the BbsI restriction sites must be added on both ends. You must be careful to match the correct fusion sites (the 4 nt in 5'-overhang flanking your dsDNA fragment) according to the position of your part within the standard (Patron et al., 2015, New Phytol.)

Basic parts are then cloned into a vector called level 0 via BpiI restriction coupled to a ligation step. The vector is based on pUC19 and contains a spectinomycin resistance gene. It also contains BsaI and BpiI restriction sites and lacZ cassette which is removed with BpiI restriction during the cloning process, allowing blue/white selection after transformation in E. coli (Weber et al. 2011). Basic parts with their vector are then called level 0 or parts, and they compose the library of gene parts that are used to build synthetic genes (or level 1).

BsaI is used to build synthetic genes. Level 0 parts are excised through this enzyme from the level 0 vector to be assembled in a level 1 vector and form a transcription unit (TU). This level 1 vector contains a lacZ cassette and an ampicillin resistance gene instead of spectinomycin to differentiate it from level 0 modules (counter selection). Parts are assembled in the correct order in one step thanks to the element-specific fusion sites located at the edges (Weber et al. 2011). To note, the level 1 plasmids are binary vectors. If you wish only to transform *Chlamydomonas* with one synthetic gene (for instance an antibiotic resistance gene), this plasmid can be used directly.

Transcriptional units can be further assembled in a level M vector, according to the same logic than at the previous step, but using BpiI, and will constitute a multigenic construct. Level M vectors contains a lacZ cassette and a spectinomycin resistance gene (Weber et al. 2011). These multigenic constructs are then used to transform *Chlamydomonas* cells and obtain your new strains.

The MoClo strategy is appropriated for iGEM projects as it presents numerous advantages, such as its technical simplicity, its swiftness, its efficiency (Engler et al. 2008), and its modularity which enable rapid characterization of genetic parts by testing different contexts with various combinations of parts (Crozet et al. 2018). Furthermore, as described in the part 1.a., the MoClo used Type IIS restriction enzymes which means that there is (almost) no trace left of the restriction site after the ligation of the diverse genetic elements. Finally, a toolkit containing optimized parts is available for *Chlamydomonas*, which makes design and building easier and avoids domestication step.

c. Overview of the kit and its components (Promoters, tags etc.)

"Standardisation is the key to efficient building." (Crozet et al. 2018). This is why the MoClo kit for *Chlamydomonas* was built of 119 optimized genetic parts, including 67 unique genetic elements, which enable rapid building of engineered cells. Each part is flanked by BsaI recognition sites and standardized fusion sites that allow cloning via BsaI into a set of adapted vectors based on pUC19 backbone (Weber et al. 2011). These parts have been domesticated and optimized in order to match the codons bias of *Chlamydomonas*.

MoClo kit for *Chlamydomonas* include various genetic elements available at different positions (Crozet et al. 2018):

- Seven constitutive or inducible promoters
  The MoClo kit inducible promoters that are activated by various mechanisms. For example, P_{NIT1} is induced by switching the nitrogen source from nitrate to ammonium, while P_{METE} is repressed by vitamin B12.
- Seven promoters coupled to their 5'UTR
- Six 5'UTR, corresponding to the promoters
- One intron
- Nine signal and targeting peptides
  They are used to target the protein of interest to specific cellular compartment.
- Eight immunological or purification tags
  They allow immunodetection or purification through standardized methods.
- Twelve reporter genes
  They are proteins with easily detectable activity or presence, which makes further analyses easier. The kit contains, inter alia, Luciferases and Fluorescent Proteins.
- Five antibiotic resistance genes
  They are used as selection markers and also can be used as reporter genes. The kit contains, inter alia, Kanamycin and Spectinomycin resistance genes.
- Six 3'UTR/terminators

- One 2A peptide from the foot and mouth virus

This peptide allows the expression of at least 2 independent proteins from a single transcriptional unit.

- One amiRNA backbone, allowing to express a designed artificial miRNA to repress specific gene.
- One CrTH14 riboswitch (5' UTR)

It is controlled by vitamin B1 and regulate gene expression by interfering with translation.

All these elements can then easily be assembled into modules and devices to transform your *Chlamydomonas* cells and create new strains.

2. Designing your construct for the MoClo strategy

a. Codon optimization for coding sequences

This step is only necessary if the part you want to integrate into the MoClo kit is a coding sequence as *C. reinhardtii* has a very specific codon usage.

Step 1

Copy and paste the DNA coding sequence from NCBI into Serial Cloner (the free software we used) and reverse translate the DNA sequence into an amino acid sequence.

Step 2

Use a reverse translation tool that takes into account codon usage of *C. reinhardtii* to obtain a new DNA sequence codon optimized for *C. reinhardtii* from the amino acid sequence. These tools can be the integrated one of serial cloner or any other DNA manipulation software, or online tools such as https://toolkit.tuebingen.mpg.de/tools/backtrans.

b. Designing a part compatible to the MoClo standard

Step 0 (specific to CDS parts)

Remove the STOP codon at the end of the amino acid sequence if it is a coding sequence not finishing in B5 position (in B5, a STOP is mandatory, see Table 1).

Step 1 - Domestication

Remove all recognition motives of BpiI (GAAGAC), BsaI (GGTCTC), and SapI (GCTCTTC).

If they are in a CDS, introduce a silent mutation using the *Chlamydomonas reinhardtii* genetic code (included in the software in the "Manage codon usage table"). If they are in a regulatory sequence, check for natural variants of the same sequence without these motives, and/or analyze your sequence carefully to assess the putative impact of this point mutation on the function of your part of interest (for instance, if it is a promoter, check if there is a known motif).

Step 2 – Standardizing

Add the corresponding fusion sites on both 5' and 3' ends depending on which position you want your part to be used (see Figure 1 of Crozet et al. 2018 for the fusion site sequences of each position, or Patron et al, 2015, New Phytol.).
If needed, make sure to add Guanine(s) after the 5' fusion site (one G creating an ATG if the part is a CDS starting in B2) and/or before the 3' fusion site to keep the correct reading frame for parts that are going to be translated (Table 1).

Table 1. Rules for designing MoClo-compatible CDS keeping the ORF.

Position	Add after 5' fusion site	Add before 3' fusion site	Note
Starting in B2	G (giving ATG thus Met)	G (giving GGN NNN thus Gly Xxx. For B3, GGA AATG thus Gly Met)	B2 can be an ORF start if a G is added in 5'
B3 or B4		G (giving GGN NNN thus Gly Xxx)	Remove any stop prior to add these GG
B5		None, just add a Stop codon (TAA is the most common one)	

Step 3 – Restriction sites

Add BpiI restriction sites: 5'-ttGAAGACtt.....ttGTCTTCtt-3'. The part is now ready to be synthetized and cloned into the corresponding level 0.

Note: we did not used the universal level 0 plasmid (Patron et al, 2015, New Phytol) but the rules are the same that the ones cited here, only the actual fusion sites used to generate the level 0 backbone are different. You have to add: 5'-ttGAAGACttCTCAnnnn.....nnnn CGAGaaGTCTTCaa-3'. The nnnn are the fusion sites of standardized positions (i.e. AATG for B3 in 5').

3. Cloning experiments

The cloning strategy of the MoClo Kit consists in doing several cycles of: golden gate reaction, *E. coli* transformation with the reaction product, Plasmid extraction of recombinant clones (white and SpecR colonies) and quality control digestion to ensure the correct assembly of the different modules.

This allows the integration of the linear part into a level 0 plasmid after the first cycle, the assembly of a transcriptional unit in a level 1 plasmid from level 0 donor parts after the second cycle and the assembly of transcriptional(s) unit(s) together in a level M plasmid from level 1 donor plasmids after the third cycle. This final level M plasmid is the one used for the transformation of *C. reinhardtii* as it should contain the selection gene needed to select the recombinant *C. reinhardtii* clones.

a. Digestion/Ligation (Golden Gate) reaction

We used an excel sheet provided on the supplementary material from *"Birth of a Photosynthetic Chassis: A MoClo Toolkit Enabling Synthetic Biology in the Microalga Chlamydomonas reinhardtii. Crozet P, et al. ACS Synth Biol. 2018"*

This excel sheet calculates the quantity and volume needed for the donor parts and the receiver backbone.

1. Prepare the following master mix: multiply these volumes by the amount of reactions you have to do plus one.

Reaction mix	Volume per tube (µL)
BsaI-HFv2 (level 1) or BbsI-HF (level 0 or M)	1
T4 Ligase	1
Buffer NEB CutSmart	2
ATP 10 mM (buffered with 0,1 M Tris-HCl pH7.9)	2
Final Volume	6

2. Add the 6 µL of master mix into the tube containing the donor/receiver plasmids.
3. Incubate the tubes in a thermocycler with the following parameters

Cycles	x 3				
Temperature (°C)	37	16	37	80	16
Time (min)	10	10	10	20	Indefinitely

b. Transformation of E.coli

Once the golden gate reaction is done, you can use it to transform competent *E. coli* so that you can isolate and amplify the resulting plasmids.
For this step any *E. coli* transformation protocols. As an example, here is the one we used with ready-to-use chemo-competent cells:

1. Add 4 µL of the golden gate reaction DNA to 25 µL of JM109 bacteria (Promega) and mix gently by pipetting up and down.

Remember to include an empty vector condition as a negative control.

2. Incubate 10 min on ice, then 55 sec at 42°C, then back on ice for 2 min.

This step applies a heat shock which will help the integration of the plasmid into the bacteria by weakening its cell wall.

3. Add 700 µL of LB and incubate 1h at 37°C.

This step allows for the recovery of the transformed bacteria, which will have the time to express the selection gene. Not letting the bacteria recover will result in their deaths as they are weakened and they did not have the time to express the selection cassette.

4. Centrifuge 2 min at 4,000 g.
5. Discard the supernatant (keep only about 100 µL) and resuspend gently the pellet.
6. Spread the bacteria on a LB + X-gal (50 µg/mL) +appropriate antibiotic (Spec or Amp) Petri dish and incubate at 37°C overnight.

The antibiotic needed depends on the selection cassette that the receiver plasmid holds, Spectinomycin for level 0 and M backbones, Ampicillin for level 1. The X-gal allows for a white/blue screen. Blue colonies holds a native receiver plasmid whereas white ones holds a recombinant plasmid.

c. Plasmid extraction

After obtaining white colonies (recombinant clones), pick them and subculture them in a liquid LB + X-gal (50 µg/mL) + Antibiotic medium at 37°C overnight.
The following day, the culture medium should be cloudy. Use a plasmid extraction kit and follow its instructions to extract the plasmid. You can also refer to (Maniatis et al. Molecular Cloning: a laboratory manual).

d. Quality control digestion

To make sure that your purified plasmid contains the correct insert, you must perform a quality control digestion.

The receiver plasmid used do not possess any specific digestion sites designed specifically for a quality control so in order to perform one, you will have to adapt the restriction enzymes used every time you want to perform one on a newly assembled plasmid.

Generally, the ideal choice for the restriction enzyme would be one that has two restriction sites, one on the backbone and one on the insert. You can also use BsaI for level 0 and M, or BbsI for level 1 quality control. Do not forget to adapt the buffer to the enzymes you use.

As for the protocol, any classical digestion protocol should work as there is no specificity in our case, here is the one we used:

1. Prepare the following master mix:

Reaction mix	Volume (µL)
Buffer NEB CutSmart (10x)	1
BsaI-HFv2 or BbsI-HF	0,5
V to aliquot per tube	1,5

2. Add 100 ng of purified plasmid DNA.
3. Add water to reach 10 µL of final reaction volume.
4. Incubate at least 1 hour at 37°C
5. DNA agarose gel electrophoresis (1%):
   - Dissolve 0,5 g of agarose powder in 50 mL of TAE buffer 0,5X by heating the solution in the microwave.
   CARREFUL: the agarose will be quite warm, wear protections.
   - After cooling the agarose, add 20 µL of Ethidium bromide (EtBr) (giving a final concentration of 0.2 mg/L).

- Cast the solution in an electrophoresis mould, put the comb and let it solidify at room temperature.
- Prepare the samples:
  DNA samples: 10 µL of DNA + 2 µL of Loading Dye 6X
  Ladder: Depends on the DNA Ladder used.
- Load the ladder and the samples in the wells.
- Run the gel for 25 min at 100V.

TRANSFORMING CHLAMYDOMONAS REIHARDTII

After successfully cloning your construct, the next step is to integrate it into *Chlamydomonas*. To this end, there are two methods of transformation: the electroporation and the glass beads methods.

1. Plasmid preparation

As *Chlamydomonas* does not maintain plasmids, we are transforming them with linearized DNA fragments which will randomly integrate into the genomic DNA, thanks to Non-Homologuous End-Joining. Digestion of the transgene is not required but will increase the transformation efficiency (circular plasmid<linear plasmid<linear transgene). We digested the level M plasmid with BsaI to obtain a linear transgene (dsDNA).

1. Digestion of the level M plasmid:

- Prepare a master mix:

Reaction mix	Volume per reaction (µL)
Buffer NEB CutSmart (10x)	1
BsaI-HF	0,3
Vf/tube	1,3

- Add DNA 1 µg of DNA.
- Add water to reach 10 µL per reaction.
- Incubate at 37°C for 3h.

2. Electrophoresis of the digestion:

Prepare an agarose gel 0,8% in TAE buffer:

- Dissolve 0,16 g of agarose powder in 20 mL of TAE buffer 0,5X by heating the solution in the microwave.
- Add 7 µL of Ethidium bromide (EtBr).
- Cast the solution on an electrophoresis gel mould, put the comb and let it solidify at room temperature.
- Prepare the samples by mixing them with a gel loading dye according to its manufacturer's instructions.
- Load the sample and a DNA ladder.
- Run the gel for 25 min at 100V.

3. DNA extraction from the gel:

Cut the agarose gel under blue light in to isolate the insert. Extract the linear DNA using a gel extraction such as the Ozyme ZYMOPUREand PCR clean-up Kit.

4. Determination of DNA concentration:

Measure the concentration of the extracted DNA measured using a NanoDrop such as the NanoDrop 2000 spectrophotometer from Thermo Fisher Scientific.

2. Electroporation method

Detailed protocol

Materials needed:

- Sterile 250 mL culture flasks
- Sterile inoculating loops
- Sterile 50 mL centrifugation tubes
- Centrifuge that can hold 50 mL tubes
- TAP (Tris-Acetate-Phosphate) medium
- Sucrose
- 0.4 electroporation cuvette (Bio-Rad)
- Gene Pulser XCell Electroporator (Bio-Rad)

CAUTION: You should always work in a sterile environment!

DAY 0: Preparation

Before being able to transform *Chlamydomonas* with your construct of interest, two things needs to be done: grow a culture of *Chlamydomonas* and digest your level M plasmid to linearize it (described previously).

Chlamydomonas culture:

To do the transformation, we need a culture of Chlamydomonas that is in the exponential growth phase.

In a sterile 250 mL culture flask inoculate Chlamydomonas in 50 mL of TAP liquid medium by scraping cells of a plate with an inoculation loop.

Incubate at 25°C in constant shaking and with light (100 $\mu mol.ms^{-2}.s^{-1}$) until cells reach the exponential or early stationary phase (typically 3-4 days of growth), at around $2\text{-}5.10^{6}$ cells per mL.

DAY 1: Transform Chlamydomonas

1. Aliquot 50 mL of culture in 50 mL falcon tubes (1 tube = 2 transformations).
2. Centrifuge the tubes for 5 minutes at 2500 g, room temperature.
3. Discard the supernatant.
4. Resuspend each pellet with 500 µL of TAP + 60 mM Sucrose (i.e. concentrating it 100X).
5. Aliquot 250 µL of cells into a 0.4 cm gapped cuvette.
6. Add 400 µg of DNA into the cuvette.
7. Incubate the cuvettes for 15 minutes in ice.

This step allows the DNA to adsorb on the surface of the cells, enhancing the probability of its incorporation when the cell wall is being fragilized by electroporation.

8. Wipe the cuvette to remove water or ice.
9. Place each cuvette in the electroporation machine and use 800V (2000V/cm), 25 µF with no shunt resistance and a time pulse of 9 – 12 ms.

You should check and note down the value of the time pulse, which is in general of 9 to 12 ms. If the transformation fails, this time might indicate why. If it is too long, the DNA might no be pure enough.

10. Transfer the cells in 10 mL of TAP + 60 mM Sucrose.
11. Let the cells recover under constant light (100 µmol photon.m^{-2}.s^{-1}) overnight (at least 16h) under shaking before inoculating into a selection plate.

Cells are incubated in a nutrient rich medium with no antibiotic to allow for their recovery and for the expression of the selection marker as a direct incubation into a selection medium will most likely kill the cells, as they are weakened and haven't had the time to express the selection gene.

DAY 2: Plate the cells

1. Harvest the cells by centrifugation at 3000 g for 3 minutes at room temperature.
2. Resuspend the pellet with 500 µL of TAP liquid medium.
3. Plate into selective TAP agar (1.5%) plate. Spread the liquid evenly by doing circular motions with an inoculating loop all over the plate.
4. Let the plate dry under the hood under dim light for 5-10 min, with the light off.

The drying step under dim light is crucial as Chlamydomonas swims in the direction of the light. This will prevent uneven spreads where all the cells have swam to a particular direction instead of staying spread.

Colonies should be visible after 5-7 days!

3. Glass beads protocol

If you want an organism to produce your desired protein, you first have to transfer the corresponding gene into it. To do that, follow the steps of the protocol underneath.

a. Preparing the transformation

Prepare some 2 ml reaction tubes by putting in a tip of a spatula of glass beads and wrap airtight with tinfoil. Autoclave the reaction tubes and dry them for 2 days at 50°C. Do not close the lids of the tubes until they are dry. Then close them and remove the tinfoil. One day before the transformation dilute the algae to a concentration of 2.10^6 cells/ml and use 100 ml for each transformation as well as the control. Two hours before the transformation, run a linearization of the construct. Choose an enzyme that cuts only once and in the backbone. Run a control gel of the linearization as described above. 30 minutes before the transformation sterilize a 1000 µl and 20µl pipette by pipetting up and down 70% EtOH without a tip.

b. The actual transformation (glass beads protocol)

Work sterile. Measure the concentration of the algae and use 1.10^8 cells per transformation. Centrifuge them down in a 50 ml falcon for 5 min. at 4000 g. Discard the supernatant and resuspend the pellet in 330 µl per 1.10^8 cells. For resuspension use the remaining supernatant that was not successfully discarded. Fill up the lid of a glass bottle with TAP-medium and adjust the volume of your pipette to 330 µl. Take up the resuspended pellet and fill the remaining volume of your pipette tip with the TAP-medium from the lid, then put everything back in the falcon and resuspend again. For each transformation fill 330µl of the chlamys with 1.10^8 cells into a 2 ml tube with glass beads and add 10 µl of the linearized construct. For the control use TAP-medium. Do not touch the reaction tube on the inner side of the lid. Vortex the cells for 15 sec. (UVM4 strain) or 25 sec. (clip strain) by holding two reaction tubes against each other at an angle of 90°. Plate out the cells carefully onto a TAP-agar-plate containing spectinomycin and try not to transfer any glass beads onto your plate. Spread the mixture evenly with a bended tip of a pipette. Only plate out every area on the plate once with your bended pipette tip to prevent the cells from dying. Let the plate dry and then close it with a stripe of parafilm. Keep the plates in the dark overnight and then place them with the lid downside for 8-10 day under light.

4. Selection of positive transformants

If you see single colonies on your plate you can start selecting them. Therefore, use a TAP-agar plate and label it with 1 to 30. Work under the clean bench to ensure that the colonies are picked sterile. Pay attention to only picking single colonies. Use sterile toothpicks or the yellow pipette tips to take up the colonies. Pick a colony in one swipe and turn your picker to avoid destroying your cells. Place the colony on your labelled TAP-Plate. When finished, close the plate with parafilm and place it under light for 3-4 days. Transfer the colonies on a fresh plate every three weeks.

MEASUREMENT IN *CHLAMYDOMONAS*

Now, you have to do precise and accurate measurements of your constructs to communicate the results of your work with the iGEM community. This step is even more important as it is a criterium for the silver medal! To help you, here is some common experiments that you can do to measure your constructs expression in *Chlamydomonas reinhardtii*.

If you work with microorganisms, you need to have a method to count them at any point in your project. This is important if you do some physiological test with your organism like a growth-test, toxicity-test or if you want to incorporate a safety part in your project like an auxotrophic strain. It is also important to know your exact cell number if you have to adjust your culture to a specific cell density for further screening or analytic experiments. There are several methods to determine the cell number of a culture. Particularly for Chlamy you have three options. The first one isn't recommendable but for the sake of completeness you will find it here. It's the way of counting with a microscope and a counting chamber or also known as hemocytometer. This method is very time consuming if you are new to it. You need a lot of practice to avoid mistakes, for example counting twice or missing out cells. Therefore, your results might be insignificant. Another disadvantage is that it is needed to fix your samples on the hemocytometer. The second method is to determine the cell number by the measurement of the optical density (OD). It is a good opportunity and a fast way of counting. The third counting method is the use of an automatic culture counter which is the one we used during our project phase.

1. Cell concentration

Protocol:

1. Prepare 2 mL tubes for cell counting.
2. Add 32 µL of TAP medium, 4 µL of cell culture and 4 µL 12 (dilution 1/10).
3. Deposit 15 µL between the cell counting slide and the cover glass, 2 measures per culture.
4. Count cells on automatic cell counter or a Hemocytometer (final concentration needed is 6.10^6 cells/mL).
5. Harvest the volume needed to have 1.106 cells.
6. Centrifuge cells at 3000 g for 3 min, discard the supernatant.
7. Resuspend in 1 mL of TAP-N+NH_4 or TAP-N+NO_3 medium (both conditions for each culture).
8. Incubate for 4h to 26h under constant light (100 µmol photon.m^{-2}.s^{-1}) at 25°C.
9. Recount cells.

2. How to Culture-counter

For our experiments we used the Z2 series coulter counter by Beckman. Working with a culture counter is a bit more time consuming than measurement by optical density but therefore, your results will have a higher quality. But if you choose this way you will have to pay attention to a couple of things. The following protocol includes some tips:

1. Prepare your culture and mix it gently before taking a 100 µl sample to avoid cells from settling down at the bottom of your flask. Transfer your sample into a container for analysis.

(Usually the counter device comes with special containers for your analysis.)
(Take your samples under sterile conditions if necessary.)

2. Fill up the sample to a final volume of 10ml with the counter solution. Close the sample with the lid and gently invert three times.
3. Prepare the counter device by flushing the system with the pure counter solution in a fresh container. Check if the glass electrode is free of dust and impurities.

(If you want to remove the last drop of solution on the glass electrode always use a precision wipe as paper towels may clog the glass electrode.)

4. Place the sample into the device and wait until all air bubbles have disappeared.
5. Measure the cell number. The results will be shown in cells per ml.

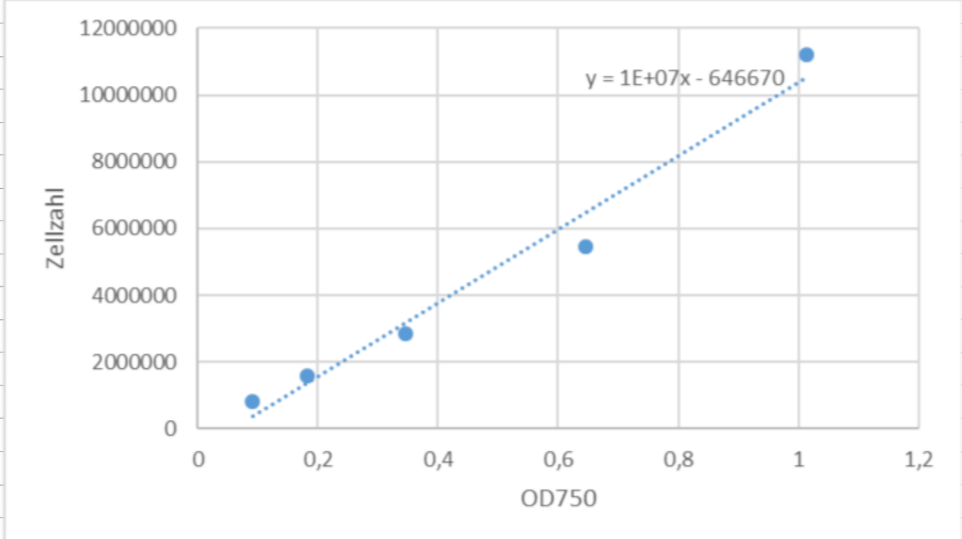
(There are different options to adjust the dilution factor before measurement.)

6. After measurement take out your sample and clean the glass electrode by letting counter solution run over the glass electrode.
7. Flush the system after every measurement to ensure that the device stays clean.
8. Important: Think while measuring! Always have a look on your results. If you see any abnormal values or if you have strong fluctuations in your results this could be a hint that something is wrong with the device.
9. After measuring flush the system again with pure counter solution in a fresh container. Leave the container in the device.

(Never leave a sample in the device when you have finished your measurements.)

10. Then check everything again, clean the probe, flush the apparatus for a second time, check the counter solution with the filter and exclude blockages in the apparatus

If you have no culture-counter available and you measuring your culture via OD, here is a standart curve for it. But attention: this standart curve was done for the cc4533 strain of *C. reinhardtii*.



3. Electrophoresis

a. Cell preparation protocol

Materials needed:

- 1.5 mL Eppendorf tubes
- 50 mL tubes
- Ice bucket
- Hepes_KOH 1M at pH7.5 (kept at 4°C)
- Protease inhibitors 50X: dilute 1 tablet of Roche protease inhibitor cocktail mix EDTA free (ref: 05056489001) in 1 mL of MilliQ water (kept at -20°C)
- DTT 1M (kept at -20°C)
- Na_2CO_3 1M (kept at room temperature)

Detailed protocol:

1. Grow cultures to 2.10^6 cells/mL

Your cells must reach exponential growth phase

2. Harvest cells by centrifugation of 30-40 mL of each culture in 50 mL tubes 5 min at 5500 rpm at 4°C
3. Discard the supernatant
4. Resuspend cells in 1 mL of Hepes (20 mM) + protease inhibitors (Roche EDTA free cocktail mix 50X stock) at 4 °C

To prepare 10 mL of Hepes 20 mM + protease inhibitors, add:

- 200 µL of Hepes 1 M at pH 7.5
- 200 µL of protease inhibitors (Roche EDTA free cocktail mix 50X)
- QSP 10 mL of water

Hepes KOH is a buffer and it is supplemented with protease inhibitors to prevent the degradation of your proteins

5. Vortex
6. Transfer to 3 x 1.5 mL Eppendorf tubes
7. Centrifuge 1 min at maximal speed in bench centrifuge
8. Discard supernatant
9. Resuspend in 100-150 µL of DTT-carbonate 0.2 M + protease inhibitors at 4 °C

To prepare 1 mL of DTT-carbonate 0.2 M + protease inhibitors, add:

- 200 µL DTT 1 M
- 100 µL Na_2CO_3 1 M
- 20 µL protease inhibitors 50X
- QSP MQ water

DTT is used to reduce disulfide bonds and Na_2CO_3 provides an alkaline pH that decreases non-covalent protein-protein interactions. This buffer is used to prevent aggregation between proteins

10. Prepare 3 aliquots of 100 mL each and freeze in liquid nitrogen before congelation at - 80°C

This step allows the conservation of your samples for several days.

b. Sample preparation protocol before gel loading

1. Remove your samples from - 80°C congelation
2. To each sample, add 60 µL of SDS 5% + sucrose 20%

SDS is used to alter your proteins and sucrose is used to weigh samples down for a better migration in the gel.

3. Vortex
4. Make holes in eppendorf tops and heat at 100°C in boiling water
5. Quickly vortex
6. Heat again at 100°C in boiling water during 50 sec
7. Cool on ice
8. Centrifuge 15 min at 13200 rpm at 4°C.
9. Transfer 100 µL supernatants in new 1,5 mL eppendorfs

These samples will be deposited in the gel.

10. For chlorophyll concentration determination, dilute 5 µL of the supernatant with 1000 µL H_2O milliQ and measure the optical density at 680 nm (optical path: 1cm).

An OD_{680} of 0.11 corresponds to 1 $\mu g.\mu L^{-1}$ of chlorophyll in the sample. 15 µg of chlorophyll are typically loaded on a large lane. The volume of sample to load on a large lane is, thus, $v(\mu L) = 15 \times 0.11/OD_{680} = 1.65/OD_{680}$ and on a small lane $0.825/DO_{680}$

Caution: It is important to deposit the samples with the same chlorophyll concentration to deposit the same quantity of proteins in the gel!

4. Measurement of your construct using the MoClo kit

Now, here is an overview of parts that you can use to measure your constructs expression in *Chlamydomonas reinhardtii*. These parts were developed for the MoClo kit (ACS Synth. Biol. 2018, 7, 2074-2086) through recoding to match the codon bias of *Chlamydomonas reinhardtii*, and cloned at different positions within the standard.

a. Fluorescence

This is a list of reporter genes that you can assemble in your constructs to measure protein expression by fluorescence.

Parts	Number of level 0	Positions	Excitation peak (nm)	Fluorescence peak (nm)
Clover (i2)-Strep	3	B2, B3-B5 and B5	505 (green)	515 (green)
mCerulean (i2)-Strep	3	B2, B3-B5 and B5	433 (cyan)	475 (blue)
mCherry (i1)	4	B2, B3-B4 B3-B5 and B5	587 (red)	610 (pink)
mRuby (i2)-Strep	3	B2, B3-B5 and B5	558 (yellow)	605 (red)
mVenus (i2)+StrepII	3	B2, B3-B5 and B5	515 (green)	527 (yellow)
mVenus (i2)+ StrepII, with restriction sites	4	B2, B3-B4 B3-B5 and B5	515 (green)	527 (yellow)
mVenus + StrepII, with restriction sites	2	B2 and B5	515 (green)	527 (yellow)

Note:

- (i1): with the intron 1 of RBCS2
- (i2): with the intron 2 of RBCS2
- Strep: StrepII tag in C-terminal, included within the part

Measurement of fluorescence by microscopy (Crozet et al. 2018):

Take images using a TRITC filter with a BX53F microscope (Olympus).

b. Luminescence

This is a list of reporter genes that you can assemble in your constructs to measure protein expression by luminescence.

Part	Number of level 0	Positions
NanoLuc	6	B2, B3-B4, B3-B5, B4-B5 and B5
gLuc (i2)-Strep	3	B2, B3-B5, and B5
GUS (CAT1i)	1	B3-B4
HiBiT	2	B2 and B5

Note:
The HiBiT tags have been developed by the iGEM Sorbonne Université 2019 team for N-terminal (BBa_K2909000) and C-terminal (BBa_K2909001) fusions, in B2 and B5 positions, respectively, according to the Open Plant standard (Patron et al, 2015, New Phytol.).

Detailed protocol for HiBiT and NanoLuc measurement in *C. reinhardtii*:

HiBiT screening:

1. Count cells in order to harvest 10^6 cells.
2. Centrifuge and discard supernatant.
3. Resuspend in 1 mL of TAP.
4. Transfer 25 µL of liquid culture on opaque 96-well plate.
5. For HiBiT screen, add 25 µL (1:1) Nano-Glo® HiBiT Lytic Buffer and 1 µL LgBiT Protein in each well.
6. Incubate 10 min.
7. Add 25 µL Nano-Glo® HiBiT Lytic Buffer and 1 µL Nano-Glo® HiBiT Lytic Substrate in each well.
8. Measure the luminescence with a luminometer (such as CLARIOstar®).

Nanoluciferase screening (control):

1. Transfer 25 µL of liquid culture on opaque 96-well plate.
2. Add 25 µL (1:1) Nano-Glo® Lytic Buffer and 1 µL Nano-Glo® HiBiT Lytic Substrate in each well.
3. Read luminescence with a luminometer (such as CLARIOstar®).

c. Immuno-blotting

This is a list of immuno and purification tags that you can assemble in your constructs to measure protein expression by immuno-blotting.

Part	Number of level 0	Positions
3xMyc	2	B2 and B5
3xHA (with internal Lys)	2	B2 and B5
3xFlag (with internal Lys)	2	B2 and B5
3xHA	2	B2 and B5
6xHis	2	B2 and B5
HA	2	B2 and B5
StrepII	2	B2 and B5
StrepII-8xHIS tag	1	B5

Detailed protocol for immunoblot analysis in C. reinhardtii (Crozet et al. 2018) :

1. Prepare proteins (see paragraph 2. Electrophoresis)
2. Transfer proteins to nitrocellulose membranes (Bio-Rad, 162-0115 or Amersham Protran).
3. After blocking with 5% low-fat milk in TBS for 1 h at RT, incubate membranes with primary antibody in 1% low-fat milk in TBS for 16 h at 4 °C.
4. After four washes in TBS-0.1% tween-20 (TBS-T), incubate the membranes with appropriate secondary antibody coupled to HRP in 1% low-fat milk in PBS for 1 h at room temperature.
5. Wash four times in TBS-T.
6. Reveal chemiluminescence using ECL (Amersham).

CHLAMY SCREENING AND IMMUNOFLUORESCENCE

Screening is an important part of each project to search for positive transformants. Especially with *Chlamydomonas* it can happen that the desired gene is integrated into the genetically inactive heterochromatin. Therefore, this step is essential to perform further tests such as analytical methods.

1. Screening for cytosolic expressed proteins

The following protocol can be used to analyze whether transformants express the desired proteins. It is describing the cell lysis and the subsequent chlorophyll determination.

At first the transformed *Chlamys* have to be inoculated in 10 ml TAP medium. After about 2 days they can be adjusted to 2.10^5 cells per ml in 20 ml TAP. After another 4 days the transformants can be screened for proteins. Therefore, you have to harvest 1.5 ml culture by centrifugation at 4000 rpm for 30 sec. Discard the supernatant and keep the pellet which contains the cells. Add 60 µl DTT-Carbonate buffer and 55 µl SDS-Sucrose buffer onto the pellet. Vortex briefly for at least 30 sec to lyse the cells. The samples have then to be denatured by heating them for 1 min at 95 °C. Finally, the cell fragments have to be removed by centrifugation for 2 min at full speed. Now the amount of proteins of the cell lysate can be identified by doing the chlorophyll determination.

For chlorophyll determination 190 µl Millipore water, 10 µl of your sample and 800 µl acetone have to be added in to a 1.5 ml reaction tube. Vortex your samples properly. To remove leftover residues of cell fragments the samples have to be centrifuged at full speed for 5 min (otherwise cell fragments could falsify the following measurements). Afterwards the absorption has to be measured at 645 nm, 663 nm and 750 nm. The amount of µg chlorophyll per µl can be calculated by using the following formula: $[(A_{645}*17.76)+(A_{663}*7.34)]/10.2$ µg chlorophyll can now be loaded onto your gel but be sure that you also load at least one negative and one positive control

2. Screening for secreted proteins

a. TCA-precipitation

Because *Chlamy* is also well suited for secretion and since this has certain advantages over protein extraction by cell lysis, it is important to know how to screen for protein secretion. For this we followed two different protocols, on the one hand precipitation by using TCA and on the other hand lyophilization of the samples.

The following protocol explains the screen for proteins by TCA precipitation. Like in the protocol above, the transformants at first have to be inoculated in 10 ml TAP and after 2 days adjusted to 2.10^5 cells per ml in 20-50 ml TAP. After another 4 days 10 ml of each culture can be harvested by centrifugation at 3800 rpm for 2 min. Save the supernatant and repeat the last step to remove residual cells from the medium (You can freeze the cell pellet for further investigations in case there is no secreted protein detectable). Transfer the supernatant to a 50 ml reaction tube and add 10 ml 20% (w/v) TCA solution for precipitation (Take care TCA is a strong acid. Wear googles, gloves and lab coat). Incubate the samples for 30 min on ice and centrifuge them afterwards for 15 min at 20.000 g at 4°C. Carefully remove the supernatant and resuspend the pellet in 400 µl 1x PBS (Take care the pellet which now contains the precipitated proteins can be very small). Now the resuspension can be transferred into 2 ml reaction tubes. For further purification a subsequent acetone precipitation will be performed. Therefore 1.6 ml ice cold acetone has to be added to the resuspension. Centrifuge the samples for 5 min at full speed at 4 °C. Afterwards the supernatant can be discarded. Try the pellet under the hood and resuspend it in 2xSDS loading buffer. Denature the samples by incubating one minute at 95°C. Finally, the samples can be loaded onto the gel and again do not forget to load at least one negative and positive control.

b. Lyophilization and acetone precipitation

Another method to screen transformants which are secreting proteins is the lyophilization of the samples from the supernatant. It is easier as the TCA-precipitation but requires enough time for the lypophilizer to freeze dry your samples.

The preparation of the cultures is the same as mentioned in the previous protocol on TCA-precipitation. Instead of 10 ml, 2 ml of culture are harvested and centrifuged twice. Transfer 2 ml of supernatant into a 15 ml reaction tube. To prevent loss of sample whilst lyophilization put on 4x4 cm of nylon tights and fix with a rubber band. The loss of sample happens when your sample is not completely frozen to solid and the consequence might be a boiling retardation which causes your sample to shoot out of the reaction tube. Put your samples in the -80°C freezer for one to two hours remove the caps and place the tubes in the lyophilizer overnight. We recommend you to let the lyophilizer cool down before you place your samples in it as well as working fast to prevent your samples from melting.

Take your samples out of the lyophilizer and resuspend the dried protein in 100 µl Millipore water. (Depending on the solubility of your protein you might need to resuspend in a special buffer). Spin down the samples for a few seconds to ensure that the whole sample is getting processed and no leftover protein is on the upper part of the reaction tube. Transfer the 100 µl into a 2 ml reaction tube and add the 6-fold amount (in this case 600 µl) of ice-cold acetone to it. Incubate samples at -80°C for 20 minutes and afterwards centrifuge at high speed (25.000 g) at 4 °C for 30 minutes. Remove the supernatant and dry the pellet under the hood for approximately 15 minutes. (Do not wait too long -the drier the protein sediment the harder it is for you to resuspend it.) You can also add an additional washing step after the acetone precipitation by adding 500 µl 95% EtOH onto your pellet and centrifugation at 25.000 g 4 °C for 10 minutes. After the additional washing step, you can proceed with the desiccation of the pellet as described.

Resuspend your protein pellets in 2x SDS loading buffer. (Volume depends on the desired concentration for your analysis. For example: We resuspend in 25 µl loading buffer loading 10 µl onto the gel to have some sample leftover for further analysis.) If the sediment is hard to resuspend in your loading buffer you will have to sonificate your samples. Denature the samples by incubating one minute at 95°C. Samples are now ready for SDS-PAGE.

3. Visualization of protein expression by immunofluorescence

Testing your transformants for the secretion of proteins might lead to negative results. Problems might occur during the secretion process in the secretory pathway. For troubleshooting we recommend you to check the location of your target protein by visualization via immunofluorescence.

The following protocol can be used to fix your cells on microscope slides, using antibody staining and DNA staining.

Inoculate your cultures in 10 ml TAP medium and let them grow for four to five days. (Depending on your growth rate you can vary in growth days.) Take 108 µl of 37% formaldehyde solution and add 892 µl cells. For other volumes: the final formaldehyde concentration of your sample should be 4%. Calculate by the formula:

$4/37 * x = y$

With x = volume of the final sample and y = volume of 37% formaldehyde you need to add. You can use formaldehyde stocks with other concentrations if the final concentration is 4%.

Incubate the samples one hour at 4°C while inverting your sample. In the meantime, prepare the microscope slides by washing three times in a 50 ml reaction tube filled with 100 % ethanol. Dry the slides and spread 10 µl 0,1% Poly-(L)-Lysin (1:10 dilution) on the microscope slide. Let dry for 5 minutes at room temperature.

After the incubation time add 40 µl of the cell-formaldehyde mixture onto the microscope slide. Let stick for 15 minutes and discard the supernatant carefully by pipetting at the edge of the drop without touching the glass surface. (Whenever it is necessary to wash or add solutions to your samples make sure you are not scratching/touching the sample with your pipette tip!) Incubate in a 50 ml reaction tube filled with methanol for 6 minutes at -20°C. Wash the microscope slide five times by pipetting 40 µl 1x PBS onto your samples and removing the buffer carefully. (It is recommended to write down the washing steps which have already been done.) For permeabilization incubate your samples on the slide with 2% TritonX-100 in PBS at room temperature. The next washing step is done as described but with the buffer 1x PBS-Mg (5mM $MgCl_2$).

Pipette 40 µl of PBS-BSA 1% onto your samples for blocking. Remove carefully and add 40 µl of your primary antibody solution to every sample, incubate at 4°C overnight. (Dilution factor depends on the used antibodies; they are diluted in PBS-BSA.) To ensure that the staining is specific prepare a control which is a sample with the antibody solution not containing any *Chlamydomonas* cells. After incubation overnight remove the antibody solution and wash your samples five times as described above using PBS 1% BSA.

From now on it is indispensable avoiding light whilst working since the secondary antibody is conjugated to a fluorophore which is highly sensitive when exposed to light! Add 40 µl secondary antibody solution (Dilution depends on your antibody.) Incubate at 1,5 h in the dark. Remove antibody solution carefully and wash your samples as described five times with 1x PBS. Dry the microscope slides and add 3,2 µl DAPI with mounting solution. Place the cover glass on your samples and let dry. Seal the corners of the cover glass with a clear nail polish.

Samples are now ready to be analyzed by fluorescence microscop

HOW TO CULTURE CHLAMY IN A BIOREACTOR

Being able to do a bioreactor experiment is in most cases the icing on a project. It is often not necessary, since you can do the most experiments on a shaker. Still it has a couple advantages. Having *Chlamy* in a bioreactor ensures a steady cell density and a constant light source. Furthermore, you can collect far more data, because the bioreactor is constantly measuring as many parameters as you want. The most important thing, if you consider experimenting in a bioreactor is, that you first discuss it with a supervisor or someone that has already worked with your laboratory's bioreactor. The following steps will also help you to start and execute your experiment successful.

1. Things you need to choose before you start a bioreactor experiment

First, you need to choose what results you want to gain out of your experiment. Alongside you will have to decide what sensors you want or need, since you cannot exchange them after you filled the bioreactor. Also make sure that you have a strain that has a cell wall, otherwise it would not survive inside the bioreactor!

2. Things you need to do before you start the bioreactor

To be able to inoculate the bioreactor you need a preculture. In our case the cultures were inoculated a week prior in 50ml HMP-medium. Four days later we diluted 25ml of the preculture in 100ml HMP-medium. The last time we diluted the preculture one day before inoculating the bioreactor 1:4 in HMP-medium. You also have to make sure that you prepared enough medium.

3. Things you should do while the experiment

Of cause this point depends on your experiment but measuring OD and counting the number of cells per ml at least once a day is in most cases very important, since you often link your results to it. If you are working with proteins and protein production, it is also helpful to take a sample at the same time to measure its quantity.

SUPPLEMENTARY PROTOCOLS

Ligation:

To ligate a level 1 construct, use BsaI and to ligate a level 2 construct, use BbsI. The Volume of the ligation is 20 µl and will be filled up with ddH_2O. To calculate the quantity of the constructs, use the concentration [ng/µl] and length of the construct in [kDa]. Follow the link: http://www.molbiol.ru/ger/scripts/01_07.html and calculate the amount of the construct in [ng] that is needed to get 40 fmol. Then, dived the result [ng] threw the concentration [ng/µl] to get the amount in [µl]. For high-fidelity enzymes, use cut-smart-buffer.

Ligation of a MoClo construct	
Ingredient	Amount [µl]
parts	40 fmol
Buffer	2
ATP	2
T4-ligase	0,5
Enzyme	0.5
ddH_2O	20-X

Place the ligation into the Thermocycler for

a) 1 min. -> 95°C
b) 30 sec. -> 95°C
c) 30 sec. -> 55°C
d) 30 sec. -> 72°C
e) 5 min. -> 72°C
f) Infinit -> 4°C

Cycle b),c),d) repeat for 30 times.

Transformation in *E.Coli*:

Use 100 µl of competent *E.Coli* Top 10 cells and handle carefully. Do not touch the Eppy at the botten and keep them on ice. Do not pipet them up and down. Put 10 µl of the ligation onto the competent cell and keep them for

a) 30 min. -> on ice
b) 90 sec. -> at 42°C
c) 5 min. -> on ice

Add 600 µl of LB-Media onto the cells and keep them for 45 min. at 37 °C.

Plating out:

Depending on the efficiency of the ligation use 200 µl to 400 µl of the LB-ligation mixture. Spread them carefully onto a LB-Agar plate (1.5%) under the clean-bench. Stroke out the mixture with a

bend tip of a pipette. Keep the Plate lid down at 37°C over night for 12-15 h. Do not keep the plate too long at 37°C to avoid satellite cultures.

Picking:

To pick colonies, prepare LB-media with the according antibiotics. For a level 1 construct, use ampicillin and for a level 2 construct, use kanamycin. For every colony you pick + one as a buffer, use 5 ml LB and 5 µl stock of the antibiotics. Add them sterile into a falcon and invert the falcon. Pour 5 ml of the LB-antibiotics mixture into a reaction tube (sterile) and pick a single colony with the tip of a yellow pipette.

Keep the reaction tubes shaking at 37°C over night (12-15 h).

Antibiotic stocks:

antibiotics	Stock concentration [mg/ml]	dissolved	Concentration of the media [µg/ml]
spectinomycin	100	ddH_2O	100
ampicillin	100	50% EtOH 50% ddH_2O	100
kanamycin	50	ddH_2O	50
hygromyin	50	SDS	50

Miniprep:

Perform the Miniprep according to the instruction.

Control digestion:

First, perform the control digestion with a computer program such as serial cloner. Chose up to two enzymes that create bands not smaller than 1000 bp. Chose the enzymes to get two bands that differ in size by 1000 bp. Use 500 ng of the construct and calculate the amount needed by dividing the 500 [ng] threw the concentration [ng/µl] of the construct.

Control digestion	
Ingredient	Amount [µl]
construct	500 ng
Buffer	2
Enzyme	0.5
ddH_2O	20-X

Keep the digestion for 1 h at 37°C.

Loading a gel:

Prepare a mastermix (MM) with ¾ Loading dye, ¼ Gel Red (1:1000 stock)

Mix 5 µl of the control digestion and 1.5 µl of the MM.

Use a 1% agarose-gel and place the gel in a gel chamber. Fill the chamber with 1XTAE buffer. Push out any air bubble of the chambers and run the gel with 100 mV and 150 mA for 45min.-55min.

Retransformation:

Perform a retransformation according to a transformation but only use 1 µl of your plasmid and only plate out 50 µl of the picked construct.

Transformation into *Chlamydomonas reinhardtii*:

Before the transformation:

Prepare some 2 ml eppis with glasbeat. Therfore, put a tip of glasbeats in the eppi and wrap the eppis airtight in tinfoil. Autoklave the eppis and dry them for 2 days at 50°C. Do not close the lids of the eppis until they are dry. Then close them and remove the tinfoil. One day before the transformation dilute the algae to a concentration of 2*10^6 cells/ml and use 100 ml for each transformation as well as the control. Two hours before the transformation, run a linearization of the construct. Chose an enzyme that cuts only once and that cuts in the backbone. Run a control gel of the linearization as described above. 30 min. before the transformation sterilize a 1000 µl an 20µl pipette by pipetting up and down 70% EtOH without a tip.

The actual transformation:

Work steril. Measure the concentration of the algae and use 1*10^8 cells per transformation. Centrifuge them down in a 50 ml falcon for 5 min. at 4000 g. Discard the supernatant and resuspend the pellet in 330 µl per 1*10^8 cells. Therefore, first use the remaining supernatant that was not successfully discarded. Full up the lid of the bottle of TAP-Media with the Media and put your pipette to 330 µl. Suck up the resuspended pellet and fill the rest of the tip with the TAP-Media from the lid, then put everything back in the falcon and resuspend again. For each transformation fill 330µl of the chlamys with 1*10^8 cells into a 2 ml glasbeat-eppi and add 10 µl of the linearized construct. For the control use TAP-Media. Do not touch the eppy on the insight of the lid. Vortex the cells for 15 sec. (UVM4) or 25 sec. (clip) by holding two eppies against each other at an angle. Plate out the cells carefully onto a TAP-spec-plate and try not to pick up the glasbeats. Stroke out the mixture with a bend tip of a pipette. Only tough every area one no not kill the algae. Let the plate dry and then close it with a strip of parafilm. Keep the plates dark over night and then place them lid down for 8-10 day under light.

Picking of chlamy-cultures:

When you see single colonies on your plate you can start picking them. Therefore, use a TAP-agar plate and label it with 1 to 30. Pick sterile under the clean bench. Be careful to only pick single colonies. Use sterile toothpicks or the yellow pipette tips to pick the colonies. Pick a colony in one

swipe and turn your picker to not destroy your cells. Place the colony on TAP-Plate. When finished, close the plate with parafilm and place it under light for 3-4 days. Transfer the colonies on a fresh plate every three weeks.

Cell harvest and cell lysis for chlorophyll determination

Solutions

DTT-Carbonate buffer	
Ingredient	Amount
1 M DTT	100 µl
1 M Na-Carbonate	100 µl
Millipore water	800 µl
Final	1 ml

SDS-Sucrose buffer	
Ingredient	Amount
10% SDS	25 ml
Sucrose	15 g
Millipore water	Add to 50 ml
Final	50 ml

Cell harvest and cell lysis

1. Prepare 1.5 ml reaction tubes according to numbers of transformants
2. Harvest 1.5 ml at 4000 rpm for 2 min (if necesary harvest 3 ml)
3. Discard supernatant, centrifuge again at 4000 rpm for 30 sec and discard supernatant
4. Add DTT-Carbonate buffer (60 µl), vortex properly, add SDS-Sucrose buffer (55 µl) (add both buffers in the same ratio up to a bright green colour)
5. Vortex each sample for 30 sec
6. Denature samples for 1 min, let cool down, centrifuge at full speed for 2 min

Chlorophyll determination

1. Add 190 µl millipore water into fresh 1.5 ml reaction tubes
2. Add 10 µl oft the supernatant, vortex properly and add 800 µl acetone, vortex again
3. Centrifuge at full speed for 5 min
4. Meassure the absorption at 645 nm and 663 nm (use glass cuvettes)
5. Calculate chlorophyll concentrations in excel by using the following formula: $[(A_{645}*17.76)+(A_{663}*7.34)]/10$
6. Load 2 µg Chlorophyll onto the SDS-gel

Screen for protein secretion and TCA precipitation

Solutions

2xSDS loading buffer		
Ingredients	Volume	Final concentration
1 M Tris ph 6.8	600 µl	60 mM
Glycerol	5 ml	50%
20% SDS	1 ml	2%
Bromphenolblue	a really small tip of a spatula	-
1 M DTT	1 ml	100 mM
H_2O	2.4 ml	-
Final	10 ml	-

Screening

1. Inoculate cultures in 10 ml TAP
2. Dilute pre cultures to 2*10^5 in 50 ml TAP
3. Let them grow for 4-6 days
4. Harvest 10 ml in non-sterile 15 ml falcons at 3800 rpm for 2 min
5. Transfer supernatnant to fresh 15 ml falcons and freeze cell pellet, repeat centrifugation to remove residual cells from medium
6. Transfer supernatant to fresh 50 ml falcon
7. Add 10 ml 20% TCA solution to the falcons with supernatant
   (20% w/v in H_2O, CAUTION: TCA is a strong acid, wear gloves, googles and handle with care!)
8. Incubate for 30 min on ice
9. Transfer supernatant and TCA to 40 ml Avanti tubes, centrifuge 15 min at 20.000 g, 4°C
10. Carefully remove the supernatant
11. Add 400 µl 1x PBS to the the respective Avanti tubes. Thoroughly resuspend the pellet and wash the bottom of the tube
12. Transfer the resuspension to the respective 2 ml tubes
13. Add 1.6 ml cold acetone to each 2 ml tube and spin for 5 min at full speed, 4°C
14. Remove supernatant and dry pellet under the hood (takes about 30 min)
15. Resuspend protein pellet in 2xSDS loading buffer
16. Load samples on SDS gel and do western blot

Screening

Solutions & Materials

2x SDS loading buffer		
Ingredient	Volumes	Final concentration
1 M Tris pH 6.8	600 µl	60 mM
Glycerol	5 ml	50%
20% SDS	1 ml	2%
Bromphenolblue	small tip of spatula	-
1 M DTT	1 ml	100 mM
H_2O	2,4 ml	-
Final	10 ml	-

Other materials: acetone, 95% ethanol, Milli-Q water, rubber bands, transparent nylon thighs

Freeze-drying / lyophilizer

1. Transfer 2 ml of your culture into a 2 ml reaction tube.
2. Centrifuge at 5.000 g for 5 minutes. Transfer supernatant into a fresh tube.
3. Repeat step 2, transfer the supernatant into a 15 ml centrifuge tube.
4. Cut transparent tights (nylon tights) into 4 x 4 cm squares.
5. To prevent loss of sample whilst lyophilization put on the squares and fix with a rubber band. Close the centrifuge tube and put your samples in the -80°C freezer at least 2 hours.
6. Remove the caps from the tubes, place the tubes in the lyophilizer overnight.

Protein precipitation (desalting)

1. Take your samples out of the lyophilizer. Resuspend dried protein in 100 µl Milli-Q water or the compatible buffer for your analysis. Spin down for a few seconds to get the whole sample into the bottom of your tubes.
2. Transfer sample into a 2 ml reaction tube.
3. Add 6 times the amount of acetone to your sample (600 µl).
4. Incubate 20 minutes at -80°C.
5. Centrifuge samples at high speed (25.000 g) at 4°C for 30 minutes.
6. Remove the supernatant. Be careful, don't remove the sediment.
7. *Optional washing step*: Add 500 µl 95% Ethanol and centrifuge 10 minutes, 25.000 g at 4°C.
8. Place your samples with opened caps under the hood to dry the pellet.

Loading preparation

1. Resuspend protein pellets in 2x SDS loading buffer. (Volume depends on the desired concentration)
2. Denature the samples by incubating 1 min at 95°C.
3. Run SDS-PAGE (see Protocol *SDS-PAGE*)

SDS-PAGE

Solutions & Materials

Note: Stand, clamps, combs etc. were purchased by BIO-RAD (Mini-PROTEAN®)

Mini-Gels (four)		
Ingredient	Stacking gel (≈3%)	Separating gel (≈10%)
dd H_2O	6,1 ml	8 ml
0,5 M Tris-HCL pH 6.8	2,5 ml	-
1,5 M Tris-HCL pH 8.8	-	5 ml
30% Acrylamide	1,1 ml	6,8 ml
20% SDS	50 µl	100 µl
0,5 M EDTA pH 8	50 µl	-
APS (100 mg/ml)	200 µl	100 µl
TEMED	20 µl	12 µl
Final	≈ 10 ml	≈ 20 ml

5x SDS Stock		1x Running buffer	
Ingredient	Amount	Ingredient	Volume
Tris	75,1 g	5x SDS Stock	200 ml
Glycin	360,35 g	20% SDS	5 ml
dd H_2O	5000 ml	0,5 M EDTA	2 ml
		dd H_2O	793 ml
Final	5 l	**Final**	1 l

2x SDS loading buffer		
Ingredient	Volumes	Final concentration
1 M Tris pH 6.8	600 µl	60 mM
Glycerol	5 ml	50%
20% SDS	1 ml	2%
Bromphenolblue	small tip of spatula	-
1 M DTT	1 ml	100 mM
H_2O	2,4 ml	-
Final	10 ml	-

Other materials: isopropanol, 70% Ethanol, dd H_2O

Working with gloves is necessary – Unpolymerized acrylamide is toxic

Pouring gels:

1. Wash glass plates with water and 70% Ethanol.
2. Assemble glass plates into the pouring device.
3. Fill with dd H_2O to check if the construction is leaking.
4. Dry the space between the glass plates with Whatman-paper.

5. Prepare the solutions for the mini gels without adding APS & TEMED (these ingredients start the polymerization).
6. Add APS & TEMED to the **separating gel** solution (the lower gel).
7. Pour the separating gel solution in between the glass plates. Let 2,5 – 3 cm free space on the upper part of the glass plates.
8. Overlay with isopropanol using a pasteur pipette.
   Let polymerize for 45 – 60 minutes.
9. Decant the isopropanol, rinse the space between the plates with dd H_2O, dry with Whatman-paper (avoid touching the gel as it may rip apart)
10. Add APS & TEMED to the **stacking gel** solution (the upper gel)
11. Pour solution onto the separating gel, slide in the combs (avoid bubbles).
    Let polymerize for 30 – 45 min.
12. Remove comb. If necessary, mark the pockets on the outer glass.

Storage: Fill the pockets with 1x Running buffer. Wrap up the gels in wet paper tissues (dd H_2O) and store them in the refrigerator (4°C).

<u>Loading & running</u>

For electrophoresis the Mini-PROTEAN Tetra Vertical Electrophoresis Cell (BIO-RAD) was used.

1. Use the 2x SDS loading buffer for your sample (as described in the protocol you used to prepare your proteins).
2. Assemble glass plates with gels into the running device and place it into the running chamber. If you have an unequal number of gels, you need to use a buffer dam.
3. Fill the chamber with the 1x running buffer. (The pockets should also be filled with the running buffer)
4. Use 3 µl marker (PageRuler™ Prestained Protein Ladder by Thermofisher Scientific #26616) for the first pocket.
5. Load your samples into the remaining pockets.
6. Close the device. Make sure the cables are plugged in right.
7. Conduct electrophoresis at 150 V for 55 minutes or until the samples have reached the bottom of the gel.
8. Turn off the device. Disassemble your gel. Open the gel by using a spatula as a lever in between the glass plates.
9. Use the gel for further experiments.

TAP-Medium

TAP- NH4 Medium		
Tris	2,42g	12,1g
4x Beijerinck- NH4	25ml	125ml
1M Phosphat-buffer pH7	1ml	5ml
„special K“ Trace Elements:		
EDA-Na2	1ml	5ml
(NH4)6Mo7O24	1ml	5ml
Na2SeO3	1ml	5ml
Zn EDTA	1ml	5ml
Mn EDTA	1ml	5ml
Fe EDTA	1ml	5ml
Cu EDTA	1ml	5ml
acetic acid	1ml	5ml
Aqua dest	ad 1l	ad 5l
Final	1l	5l

Adjust to pH7 with acetic acid

TAP- Plates

TAP- Plates	
TAP- NH4 Medium	1l
Agarose	1,5%
Final	1l

HMP-Medium

Ingredient	Amount
Hepes 5mM [20mM]	5,96g [23,83g]
4xBeijerinck-NH_4	125ml
1M KPO_4 pH7	5ml
EDTA-Na_2	5ml
$(NH_4)_6Mo_7O_{24}$	5ml
Na_2SeO_3	5ml
Zn EDTA	5ml
Mn EDTA	5ml
Fe EDTA	5ml
Cu EDTA	5ml
Aqua dest	5l
Final	5l

Ad KOH until you reach pH 7,02

4xBeijerinck-NH_4

Ingredient	Amount	Amount
NH_4Cl	16g	80g
$CaCl_2$ $2H_2O$	2g	10g
$MgSO_4$ $7H_2O$	4g	20g
Aqua dest	Ad 1l	Ad 5l
Final	1l	5l

1M Phosphat-buffer pH 7 (1M KPO_4)

Ingredient	Amount
1M K_2HPO_4 (alkaline)	500ml
1M KH_2PO_4 (acidic)	Ad 200ml until pH 7 is reached

Immunofluorescence

Solutions & Materials

Ingredients	10x PBS	1x PBS
NaCl	80 g	100 ml 10x PBS
KCl	2 g	
Na_2HPO_4	14,6 g	
KH_2PO_4	2 g	
dd H_2O	1 l	900 ml
Final	1 l	1 l

Calculate the amount of special buffers/solutions:
1x PBS-Mg (5mM $MgCl_2$)
2% Triton X-100 in 1x PBS
1% BSA in 1x PBS
Antibody solutions in PBS-BSA

Other materials:
nail polish (clear)
microscope slides
cover glasses

Fixation of cells

1. Add 108 µl 37% formaldehyde to 892 µl of cells. (For other volumes add the right amount to reach the final concentration of 4% formaldehyde in your culture)
2. Incubate one hour at 4°C while inverting your sample.

Preparation microscope slides

1. Wash three times in 100% Ethanol. Let dry.
2. Spread 10 µl 0,1% Poly-(L)-lysin (1:10 dilution) on the microscope slide. Let dry for 5 minutes at room temperature.

Fixation and Permeabilization

1. Add 40 µl of the cell-formaldehyde mixture on the microscope slide.
2. Let stick for 15 minutes.
3. Discard the supernatant carefully.
4. Incubate in methanol for 6 minutes at -20°C.
5. Washing step: Add 40 µl 1x PBS and remove carefully. Repeat 5 times.
6. Permeabilization: Incubate 10 minutes in 2% Triton x-100 in PBS at room temperature.
7. Washing step: Add 40 µl 1x PBS-Mg and remove carefully. Repeat 5 times.

Staining

1. Incubate with PBS-BSA 1% (blocking).
2. Add 40 µl primary antibody solution to every sample (primary antibody in PBS-BSA) and incubate at 4°C overnight.
   Add 40 µl primary antibody solution on a spot without cells to create a control.
3. Washing step: Add 40 µl PBS 1% BSA and remove carefully. Repeat 5 times.

Secondary antibody

Avoid light! The secondary antibody is conjugated to a fluorophore.

1. Add 40 µl secondary antibody solution (1:200 diluted in PBS-BSA) to every sample.
2. Incubate 1,5 h in the dark.
3. Remove secondary antibody solution carefully.
4. Washing step: Add 40 µl 1x PBS and remove carefully. Repeat 5 times.
5. Dry the microscope slides.
6. Add 3,2 µl DAPI + mounting solution per sample
7. Place cover glass on your samples.
8. Seal the corners with nail polish.