Cloning protocols

Making media petri dishes

1. Melt LB media in the microwave:
   - Loosen lid
   - Place in microwave. Check and homogenize by moving bottle from side to side every 2 minutes. It takes around 8 minutes to melt
   - Wait to proceed to the next step until the media has cooled down enough to touch the bottle comfortably

2. Take antibiotic stock out from the freezer and melt in your hands.
   Ex : kan 50 stock, kanamycine at a concentration of 50mg
   The antibiotic stock is 1000 times more concentrated than necessary for it to function. This stock gives a media concentration of 50 µg/mL
   - With 200mL of LBA, we add 200µL of the antibiotic stock (1/1000)

3. Preparing the plates
   - Put the name of the antibiotic on the side of the petri dish (example : Kan50)
   - Over bunsen burner: using a pipette, measure the necessary amount of antibiotic and add to the bottle of LBA. Mix by moving the bottle from side to side on the bench
   - Still over bunsen burner, pour approximately 25 mL of media in the plates starting from the bottom of the stack. When you are finished, move the stack around on the bench to get rid of bubbles

4. Storage
   - Leave the plates on the bench for two days
   - After two days, put them in the plastic bag they came in and store them in the fridge, media facing up

If you only want two plates: pour LBA in a 50mL falcon tube, add the antibiotic, then mix by turning the tube from side to side. Pour 25mL in each petri dish.

How long can the petri dishes containing media be kept in the fridge?

This depends on the antibiotic used. Ampicillin is less stable than some other antibiotics, and it is better to only keep it for a couple of weeks, at the maximum one month, in the refrigerator.

Plated media should always be kept in the refrigerator.

PCR amplifying fragments

1. **Diluting the primers**

   Take out the 100 µM primer stock solution, kept in the freezer. We want to make 100 µL of primer solution at 10 µM for PCR.

   - Add 90 µL of H_2O, then 10 µL of the primer stock solution.

   We do this with all the primers that are necessary.

2. **Diluting the DNA template**

   We need to dilute our original DNA solution (containing plasmid). If not, we will have a large quantity of untransformed plasmids at the end of the Gibson.

   To dilute the DNA to the right concentration:

   - Measure the initial concentration using the nanodrop
   - We are aiming for a concentration of 1ng/µL. To reach it we use the following formula: (Measured Concentration - 1) of water + 1 µL of DNA solution

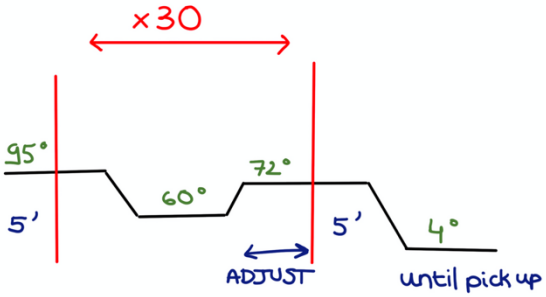
3. **Making the PCR mix**

   In a PCR tube, add the following components:

   - 25 µL master mix (contains polymerase)
   - 20 µL H_2O
   - 1 µL DNA (see section *DNA template*)
   - 2 µL primer 1
   - 2 µL primer 2

   Vortex this mixture, then centrifuge in baby centrifuge for 1 second, so that the liquid collects at the bottom of the tube.

- 95°C : denatures DNA
- 60°C : primers adhere to DNA. This temperature can change according to the Tm of the primers.
- 72°C : polymerase is at its most efficient. We adjust the time at this temperature according to the length of DNA we are looking to replicate. For example, if our polymerase has a speed of 30 sec/kB and our DNA fragment is 1kB, we leave this temperature for 30 seconds. If you need to round the time, round up.



4. **Set up PCR machine**

   - When loading the machine, load the tubes in the middle and not on the sides.
   - Make sure to close the tubes well (otherwise the contents will dry during the PCR).
   - Adapt the time at 72°C according to the enzyme efficiency.
   - If you need a new template: Create a new template —> choose time at 72°C, etc. —> done —> save as —> start
   - Wait until the machine is preheated to add the samples.
   - When the samples are added, close the lid and screw tightly.
   - Check that the PCR has worked by doing an electrophoresis and checking that the fragments have the desired sizes.

Electrophoresis

This allows us to see the size of the fragments and check that they are as expected if the colonies had integrated the desired plasmids.

If the fragments are the right size, we can then proceed to PCR cleanup and sending for sequencing, then creating a bigger 5mL culture.

Preparing the Gel

1. If using the stock, dilute the buffer TAE 50 times.

2. Mix 0.5 g of agarose and 50 mL of properly diluted TAE buffer in an erlenmeyer. Melt in the microwave, checking every minute until crystal clear.

3. Add 2 µL of Sybr dye (do NOT use loading dye).

4. Pour the gel in the mould. Make sure to use the well mould necessary to create the right number of wells (amount of PCR products + 1 for the ladder). After pouring the gel, make sure there are no bubbles in or between the wells by lightly rotating the well mould.

5. When the mould is set, put it in the electrophoresis tank and pour TAE buffer until the gel is covered. You can keep the buffer in the tank and use it to run multiple gels.

Preparing the PCR product for electrophoresis:

1. In a clean PCR tube, mix
    - 5 µL of PCR product
    - 1 µL of loading dye (kept in the refrigerator)

2. Load ladder in the first well
    - The stock ladder is kept in the refrigerator. A properly diluted tube is also ready to use and placed in the refrigerator as well.

3. Load these 6 µL in the wells, then run electrophoresis at 100V for around 40 minutes (or until the dye is reaching the edge of the gel)

4. When the gel has run, view in the transilluminator using white light in order to center it and zoom on the gel properly. Then shut the transilluminator door and turn on UV light to view DNA.

PCR cleanup

This allows us to purify our PCR product in order to only keep the desired sequence.

1. Ratio 1 to 2 of the PCR product and NTI binding buffer, mixed in the tube used for the PCR. The binding buffer allows the DNA to bind to the membrane in the column.
    - 45 µL PCR product (left after electrophoresis)
    - 90 µL NTI buffer

   Add to the column, then centrifuge for 30 seconds at 11'000g so that the DNA goes through the membrane. Discard flow through from collection tube.

2. Add 700 µL NT3 wash buffer to the column. Centrifuge for 30 seconds at 11'000g. Discard flow through.

3. Add 700 µL NT3 wash buffer to the column. Centrifuge for 30 seconds at 11'000g. Discard flow through.

4. Dry centrifuge for 1 minute at 11'000g. Discard flow through. Transfer column from collection tube to a clean eppendorf.

5. Elude DNA: Add 30 µL NE elution buffer to the column. Wait 5 minutes. Centrifuge for 1 minute at 11'000g, making sure the eppendorf lid is facing the right way.

Gibson assembly

We start by measuring the concentration of our cleaned fragments using the nanodrop. The nanodrop gives us the concentration in ng / µL.
For an optimal Gibson, you need the vectors to be half as concentrated as the inserts.

$$pmol = \frac{(Weight\ in\ ng) \cdot 1000}{base\ pair \cdot 650Da}$$

1. We always want 100 ng of the vector. Knowing the concentration (calculated with the nanodrop), calculate the desired volume of the vector solution.

2. Using the above formula, calculate the amount of pmol (picomol) we have in the volume of vector we will use.

3. Multiply this value by two, to get the amount in pmol for the insert.

4. Calculate the weight in ng of the insert using the above formula.

5. Knowing the amount of ng and the concentration of the insert, calculate the volume of the insert solution needed.

6. Make the mix in a PCR tube :
    - 10 µL of Gibson Master Mix (kept in the freezer)
    - x µL of DNA (vector and insert volumes calculated above)
    - 10 - x µL of H_2O
    - for a total volume of 20 µL.

7. Put the tube in the PCR machine, on the Gibson program (it stays at 50°C for 15 to 60 minutes).

Negative Control: make a Gibson, but only with one of the two fragments (replace the volume from one of the fragments with H_2O).

We can then use an electroporation to transform the plasmid in the bacteria.

Plating

1. Label the plate: date, name, bacterial strain (eg: E Coli), plasmid used.

The whole process is done above the bunzen burner to avoid contamination.

2. Remove 900 µL from the 1'000 µL tube using the pipette, making sure not to disturb the residue at the bottom of the tube. Pour the 900 µL in biological waste recipient.

3. Use 100 µL pipette to mix the 100 µL leftover in the tube, homogenizing the residue of cells at the bottom with the liquid. Do this gently to avoid the cells getting stuck in the pipette.

4. Use the 100 µL pipette to transfer the contents of the tube to the petri dish containing antibiotic. Only open the lid of the petri dish when you are about to add the cells.

5. Spread the cells on the petri using the L-spreader. Do so until the liquid is mostly absorbed. Whilst doing this, keep the lid of the petri dish hovering over it to avoid contamination from your mouth.

6. Away from the bunsen burner, cut a piece of parafilm with scissors and use it to seal the petri dish.

7. Plate the dish overnight (at least 12 hours) at 37°C, agar facing up.

Colony PCR

We are doing a PCR using cells as the starting product instead of using purified DNA. The goal is to check that the cells have been transformed with the desired plasmid by checking that a fragment has the right size and producing enough fragments to be able to send them to sequencing.

1. **LB liquid + amp**: Aliquote LB liquid and add ampicillin stock to make 15mL (we need 100 µL of this solution for each colony we want to multiply and test with PCR).
    - In sterile conditions (around bunsen burner): measure 15 mL of the LB liquid using a big pipette. Keep cap from the LB recipient elevated on pipette box close to the flame to avoid contamination.
    - Measure 15 µL of ampicilline stock (around bunsen burner), then add to the 15mL of LB liquid. Vortex.

2. **PCR master mix**: Make master mix for all the samples to test with PCR:
   Volume MasterMix = m(n+1)x50 = ... µL, with m de number of petri dishes and n the number of colonies tested per petri dish.
    - For 3 petri dishes, and 3 PCR per petri dish (we calculate master mix for 4 PCR/dish to have extra) we need enough master mix volume for 12 PCR with 50 µL per PCR, so a total of 600 µL.
    - Knowing the total volume, we can then calculate the amount of each element needed:
        - 240 µL H_2O
        - 24 µL of each primer (we use the primer from each side of the insertion/deletion site)
        - 300 µL Master mix
    - Keep master mix on ice until ready to use.

3. **Colony aliquote**: inoculate 100 µL LB liquid + amp with the colony in a PCR tube (we will let them grow for a day after using some for the PCR).
    - In sterile conditions (around bunsen burner) measure 100 µL of the LB liquid + amp and put in a PCR tube
    - In sterile conditions (around bunsen burner) take sterile loop and touch the desired colony with the tip.
    - Put colony in the 100µL tube and spin the loop around in the tube to make sure it detaches.
    - Vortex

4. **PCR**:
    - In PCR tubes, add 1µL of the colony aliquote and 49µL of the PCR master mix. Keep on ice until ready to load in machine.
    - Settings: 95°C for 10 minutes (in a colony PCR, we want to kill the cells, so we double the time at this temperature). Adjust extension time at 72°C for the longest fragment you want to replicate (then click *done*, *save as*). Tighten the top of the PCR machine when it is shut to avoid the lids of the PCR tubes opening.

Do a PCR cleanup, then use an electrophoresis to check that the fragments are the right size and send to sequencing if needed.

PCR product: measure concentration (nanodrop), send to sequencing

Measure concentration with nanodrop

After doing the PCR cleanup, we can measure the concentration of the product collected using the **nanodrop**. This allows us to check that our product is in the right concentration range to send to sequencing.
Blank the nanodrop with 1µL of elution buffer, then run with your DNA and check that it is properly concentrated for sequencing (see concentration chart). If it is too concentrated, dilute using elution buffer.

Send to sequencing

Purified DNA is sent to sequencing to make sure there are no unwanted mutations, and we have the right plasmid.
We can sequence about 1kb: if you have larger sequences, you have to add primers. It is better to have a primer for 800bp, allowing some overlap in the sequences.

In this case, we have 2kb so we have to use 2 primers.

Per colony :

- Tube 1 : 12 µL PCR product + 3 µL primer 1
- Tube 2 : 12 µL PCR product + 3 µL primer 2

For each tube, add a bar code sticker and write down what the 8-number code corresponds to in your notes. Then fill out online form in Microsynth using iGEM account and put the tubes in Microsynth drop box at the DMF (pickup at 15h15).

The next day, download the files on your computer.

- **ab1 files** : open with snap gene viewer. This shows a chromatogram for the sequencing. This allows us to check the quality of the sequencing. Defined peaks show a very clean sequencing. If the concentration sent to sequencing is too low or too high, the peaks are more difficult to read. If the peaks overlap, we aren't as confident in the sequencing.

- **fasta files** : nucleotide sequence.

Glycerol stock

These are used to preserve cells. We should make a glycerol stock every time we receive new cells or plasmids (transform it in cells, then make a glycerol stock).

Make two glycerol stocks for each cell culture to have a backup in case of contamination.

We want a 1 mL solution containing 25% glycerol. In the lab, we have a bottle of 80% glycerol.

1. Calculate the volume of glycerol stock and cell solution needed:
   Calculation necessary volume of glycerol:
   C1 x V1 = C2 x V2
   V1 = 0.25 x 1 / 0.8 = 0.3125 mL = 312 µL
   Calculation for the volume of cell solution:
   1- 0.312 = 0.668 mL = 688 µL

2. Around bunsen burner: add glycerol and culture in cryotube. Vortex for a long while (glycerol is denser than water, so you need to vortex for a long time for the two to mix).

3. Label the tube properly, then store it on ice until you put it in the -80°C freezer. When you do so, be very careful to shut the freezer properly.

To use the glycerol stock, DO NOT DE-FREEZE. Just scrape off some of the surface and put it in a liquid growth media.

Competent cells

You want five times more volume of air than of solution in the flask so that the cells get enough oxygen. For this reason, we use 1 liter baffled Erlenmeyer flasks to make a solution of 200mL of competent cells. The ridges in the flask create bubbles and air flow, so better growth conditions.

1. In a baffled Erlenmeyer flask, over bunsen burner, we add:
    - 200 mL of media (2XYT media for our strain)
    - 1 : 100 dilution of cells, so 2 mL of E. Coli. At this concentration, E. Coli takes about 3 hours to reach mid to end of exponential growth. At this growth stage, the cells are metabolically active, and their membranes are fragile since they are dividing, making them more competent.

2. Put the flask in the shaking incubator at 37°C and 180 rpm for approximately 3 hours.

3. After around 3 hours, using a spectrophotometer, measure the OD (optical density) of the cell solution at 600nm to see if they are at the right replication stage (correlates with the cell concentration).
    - Fill cuvettes around bunsen burner
    - Blank the machine using 1 mL of the growth media used to incubate the cells
    - Measure OD of 1 mL of the culture

    If the absorbance is between 0.4 and 0.6, proceed to the next step. If not, put the cell cultures back in the shaking incubator and wait until the OD is in this range.

4. When the OD is in the right range, put the cells on ice so that they stop growing and proceed to do the washes. The washes are used to remove the salts so that the cells can be electroporated.

    Over bunsen burner:
    - Split the 200mL cell culture in four 50mL falcon tubes. Centrifuge them for 5 minutes at 4'000 rpm and at 4°C.
    - Throw out supernatant. Resuspend cells in 50mL of a solution of H_2O and 10% glycerol kept in the refrigerator. The glycerol is used to freeze the cells. If you do not want to freeze the cells, only use water for these washed.
    - Centrifuge these four tubes for 5 minutes at 4'000 rpm and at 4°C. Throw out the supernatant. Resuspend cells in 25mL of 10% glycerol, then combine the contents of the tubes to have two 50mL tubes.
    - Centrifuge these two tubes for 5 minutes at 4'000 rpm and at 4°C. Throw out the supernatant. Resuspend cells in 25mL of 10% glycerol, then combine the contents of the tubes to have one 50mL tube.
    - Centrifuge this tube for 5 minutes at 4'000 rpm and at 4°C. Throw out the supernatant. Resuspend cells in 1mL of 10% glycerol.
    - Put 70-100 µL of this solution in small eppendorf tubes. These tubes can be kept in the freezer at -80°C until needed.
    - To make sure our cells are in fact competent, we can transform them using a purified plasmid.

Miniprep

This process is used to extract plasmids from a solution of cells. The cells are lysed, then a long centrifugation separates the plasmids from the rest of the cell content.

During these steps, do NOT use the vortex! It can cause shredding of genomic DNA that can then be confused with plasmid.

1. To extract plasmids from cells, we first need to concentrate the cells. Centrifuge 1-5 mL of culture for 30 seconds at 11'000g.

2. Add 250 µL of resuspension buffer A1. Resuspend cells by pipetting up and down gently. Keep A1 in the refrigerator.

3. Add 250 µL of lysate buffer A2. Mix by inverting the tube. Incubate at room temperature for 5 minutes or until lysate appears.

4. Add 300 µL of neutralisation buffer A3. Mix by inverting the tube until the sample is colorless.

5. Centrifuge 5 minutes at 11'000 g. Make sure the supernatant is clear.

6. Load 700 µL of supernatant in the column, placed in the collection tube. Do not add white residue. Centrifuge 1 minute at 11'000 g and discard flow through.

7. Add 500 µL AW buffer to the column. Centrifuge 1 minute at 11'000 g and discard flow through.

8. Add 600 µL A4 buffer to the column. Centrifuge 1 minute at 11'000 g and discard flow through.

9. Dry spin: centrifuge 2 minutes at 11'000 g and discard flow through.

10. Replace the collection tube with an eppendorf tube. Add 50 µL elution buffer AE. Incubate 1 minute, then centrifuge 1 minute at 11'000 g.

11. Measure the concentration of the liquid collected with the nanodrop to make sure it contains DNA.

We can keep the plasmids from the miniprep in the fridge, or in the freezer at -20°C if you want to preserve them for longer.

Transforming competent cells with plasmids – electroporation

Control: we proceed with the same treatment of the competent cells, but do not add the plasmids. We want to make sure that the cells are not resistant to the antibiotic without plasmid transformation. We electroporate the cells without adding DNA, then plate them on different antibiotics to make sure that they are not resistant.

Always keep the competent cells on ice (including in the cuvette).

Around bunsen burner :

1. Add 1-2 µL of plasmids (depending on their concentration, for example add 1 µL if they are at 300ng/µL and 2 µL if they are at 20ng/µL) to 70 µL of competent cells.

2. Put this solution in electroporation cuvette and wipe down the sides using a tissue. Make sure the liquid is evenly distributed in the cuvette. Then place the cuvette in the machine. Pulse. Normally, the time constant on the machine should be between 4 and 6. If the liquid isn't properly distributed in the cuvette, the time constant could be much lower.

3. Take the cuvette out and add 1 mL of growth media. Pipette up and down. Then put the solution in a new falcon tube.

4. Incubate for an hour.

5. Centrifuge at 8'000 rpm for 3-4 minutes. Remove around 850 µL of supernatant. Resuspend the cells.

6. Around bunsen burner, pipette 100 µL of this solution on a petri dish containing media with the proper antibiotic (only cells that have integrated our plasmid can grow). Use an L- spreader to absorb the liquid in the media.

7. Incubate the cells at 37°C, media facing up, overnight.

The petri dishes can then be kept in the fridge for 2-3 weeks (for E. Coli).

Restriction Digestion plasmid DNA

The protocol is an example using the restriction enzymes XhoI and HindIII. You must adapt the protocol to the restriction enzymes you are using for your (double) digestion.

In this webpage, enter enzymes I and II. Then click in the button “show detailed protocol”.
http://nebcloner.neb.com/#!/redigest

1. Set up reaction as follows in PCR tubes:

Make sure the total volume in the tubes is 50µL.

COMPONENT	50 µl REACTION
DNA	1 µg
10X NEBuffer r2.1	5 µl (1X)
XhoI	1.0 µl (20 units)†
HindIII	1.0 µl (20 units)†
Nuclease-free Water	to 50 µl

We often have 50ng/µL of DNA (plasmids). To measure the necessary volume of DNA to get a mass of 1µg, we used the following calculation:

$$Volume = \frac{masse}{concentration} = \frac{1\mu g}{50ng/\mu L} = 20\mu L$$

† For convenience, 1.0 µl is specified; adjust as needed. In general, we recommend 5–10 units of enzyme per µg DNA, and 10–20 units for genomic DNA in a 1-hour digest. Enzyme volume should not exceed 10% of the total reaction volume to prevent star activity due to excess glycerol.

2. Incubate at 37°C for 1 hour in the PCR machine. After the hour-long incubation, the enzymes need to be inactivated. To do so, set up the PCR machine for it to follow the 37°C incubation with an enzyme inactivation period at a certain temperature. The temperature and duration necessary for inactivation depend on the enzyme and are summarized in the following table:

https://www.neb.com/tools-and-resources/usage-guidelines/heat-inactivation

GOLDEN GATE ASSEMBLY (for sgRNAs):

Oligos annealing:

- Resuspend with H2O the oligos to get a 100 µM concentration;
- In three PCR tubes, mix together 5 µL of each oligo with 90 µL of 30 mM HEPES at pH 7.8 (to get to the working concentration of 10 µM)

- Heat the mixture in a thermal cycler at 95°C for 5 minutes, and set a linear decrease in temperature of 0.1°C/sec until reaching 4°C

Golden Gate Assembly reaction:

Prepare the following reaction mixture:

Component	***Volume [µL]***
pJH1 (backbone)	1 µg DNA
sgRNA (insert)	0.3
T4 Ligase Buffer (NEB)	2
T4 Ligase (NEB)	1
BsaI-HFv2	1
Nuclease-free water	complete to 20 µL

Table 1: Golden Gate reaction mixture

This cloning method has been carried out using a thermal cycler which followed the following settings:

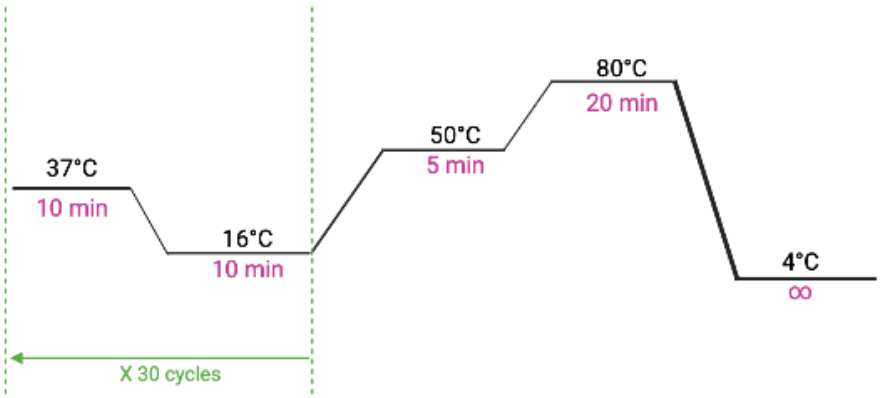


Fig. 1: Representation of the thermal cycler program set, allowing both the backbone digestion and the insertion of the three sgRNAs into pJH1

The resulting plasmid can then be transformed in *E. coli* NEB5α

Competent cells & electroporation – P. syringae

Note: All manipulations must be done on sterile conditions (ex. Around a Bunsen burner)

Competent cells *p. syringae* :

1. 50 mL of King's B overnight culture of bacteria
2. Throw away the supernatant and wash twice with 50 mL of sucrose 300 mM at 4°C for 5min at 4000 rpm.
3. Resuspend in 800 µL of sucrose 300 mM

Electroporation:

4. Add 1-2µL of DNA (depending on its concentration) to 70 µL of competent cells
5. Put the mix in an electroporation cuvette and wipe down the sides using a tissue. Make sure that it is evenly distributed in the cuvette. Then put it the electroporation and electroporate at 1.2-1.8 kV.
6. Add 1 mL of media in the cuvette and mix using the up-and-down method before putting the whole solution in a new falcon tube.
7. Incubate for 2-3 hours at 30°C, 180 rpm.
8. Centrifuge at 8000 for 3-4min and remove 850 µL of supernatant, then, resuspend the cells.
9. Finally, take 100 µL of the final solution and plate it on selected medium and spread it all over the plate using a L-spreader.
10. Incubate for 24h to 48h at 30°C with the lid facing down.

The plate can be kept in the fridge at 4°C for 10 days maximum.