

Agarose Electrophoresis

Preparing the gel

- Weight the desired amount of agarose. The concentration range normally used is 0.7% - 2% g/L. At the lab we standardised using 100 mL of 1% agarose gel, as to simplify the amounts;
- Add the weighted agarose to the desired volume 1X Sodium Borate buffer;
- Heat 1-3 min in the microwave, or until the mixture is homogeneous and no clumps are visible. Careful with overheating the mixture, as it may splash outside the container;
- Carefully pour the liquid solution onto the tray with the comb, and let it solidify; (should take 20-30min)
- Remove the comb and place the tray in the cell, and add 1X SB buffer until both electrodes are covered;

Adding samples

- Aliquot the sample to be tested by adding 4 μ L of the template DNA, 1 μ L 10X Loading Buffer and 1 μ L 5X Gel Red dye, and mix by pipetting;
- Ladder aliquot should follow the same steps, with DNA Ladder being used as template, while the negative control should be only Loading Buffer and Gel Red;
- Pipette the corresponding samples into their lanes (including Ladder and negative control) and mix with pipette;

Running samples

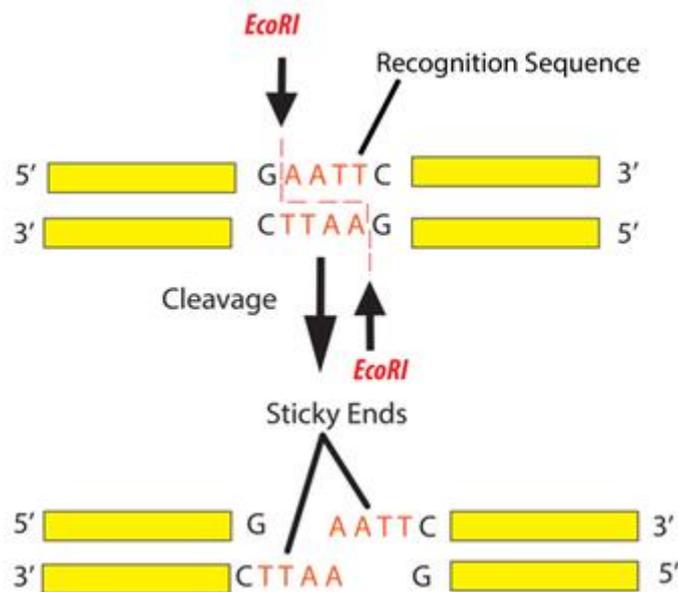
- Run electrophoresis at 80 - 150V;
- Stop the run when the dye reaches 70%-80% of the gel; (~ 1h - 1h30min)

Notes

1X TAE and 1X TBE buffers used to be previously used, but better results and faster running times were perceived with SB buffer, and so the protocol was adapted.

DIGESTION

- 1- Select the enzymes to digest the material.
- 2- Select the appropriate reaction buffer to the set of selected enzymes. Sometimes in the double digests, the enzyme digestion buffer is not optimal for another enzyme; it is possible to verify the efficiency of the enzyme in selected buffer and increasing the amount of enzyme added, as well as increasing digestion time.



- 3- In a tube of 0,2 mL mix:
Reaction example

Volume	Concentration	Final quantity	Item
0.5 µL	20000 U**/mL	10 U	XhoI
0.5 µL	20000 U**/mL	10 U	BamHI
2.0 µL	10 X	1 X	Cutsmart
7 µL	500 ng/ µL	3,5 µg	DNA a ser cortado
sqt*10-50 µL	0	0	H ₂ O

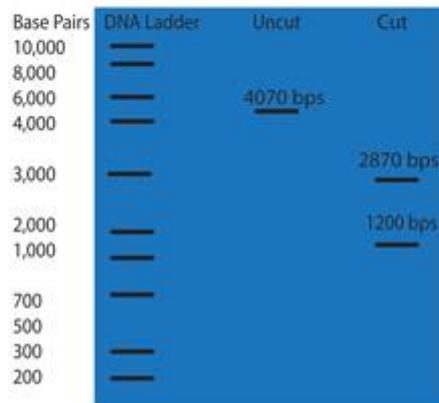
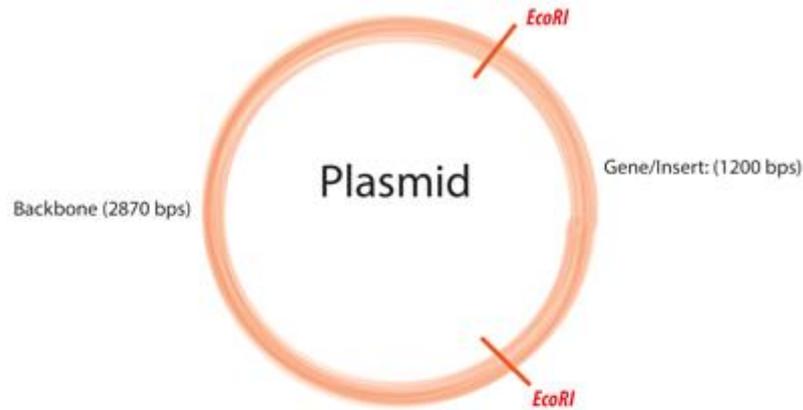
***sqt:** sufficient quantity to

****U:** It is defined as the amount of enzyme required to cut 1 µg of DNA λ (HindIII digest) fragments in 1h at 37 ° C in a total volume of 50 µl

Note: A quantity of DNA than will be cut depend of the application. To analytics cuts, ~ 500 ng of DNA and for the molecular cloning usually are need 1-3 µg of DNA. The volume of the reaction varies between 10-50 µL, and depending the application is determined the DNA volume to be cut.

- 4- Mix gently by pipetting
- 5- Incubate at the appropriate temperature (typically 37 ° C) for 1h (could be increased to ensure complete cut, with the exception that some enzymes can lose specificity and cut randomly "Star Activity"). Always follow the manufacturer's instructions.
- 6- To view the result of digestion, prepares an electrophoresis gel.

Example



Source: Add gene

Note 1: The restriction enzymes MUST be placed on ice IMMEDIATELY after removal from the -20°C freezer, because the heat can denature the enzyme and lose the activity.

Note 2: The amount of restriction enzyme to be used for a digestion depends on the amount of DNA to be cut. By definition: A U enzyme cuts 1 μg of DNA in 50 μL of reaction for 1h. Using this ratio, you can determine the minimum amount of enzyme for the reaction, but remember that this value is for optimal reaction conditions, with pure DNA. It is recommended to use more enzyme. Moreover, it is difficult to pipette less than 0.2-0.5 μL and is therefore common to use something in this range with a minimum.

Note 3: One option for the choice of enzymes is to use various enzymes that form cohesive ends.

Note 4: For reactions with blunt or enzyme-cutting a vector, it is necessary to use a phosphatase to prevent recircularization of the vector if you are cloning an insert. CIP (Calf alkaline phosphatase) or SAP (shrimp alkaline phosphatase) are commonly used. Follow the manufacturer's instructions.

Note 5: If your enzyme did not cut, check if it is not sensible to methylation. Plasmids produced in Dam or Dcm strains than are positive methylase, will be tough to cut.

Note 6: Sometimes enzymes cut sequences than are similar, but not identical, to their recognition sites. This is by "Star Activity" and can occur for different reasons, like high concentrations of glycerol.

Note 7: To digest a large amount of plasmids with the same enzymes (e.g. in analytic digests), is possible to create a "master mix" with all reaction components except the DNA. Aliquot DNA into individual tubes and add the appropriate amount of Master Mix to each tube. This will save time and ensure consistence between the reactions.

DNA Quantification

In order to accurately measure the concentration of a substance based on its absorbance, you need to know the wavelength of light that your substance maximally absorbs. In the case of nucleic acid (DNA and RNA), the maximal absorbance is at 260nm. Protein maximally absorbs at 280nm and the ratio of nucleic acid to protein (260/280) is generally used as an indicator of the purity of DNA samples.

These days, many labs have a NanoDrop, which is a very small spectrophotometer that can accurately read DNA concentration and purity in as little as 1µl. Regardless of whether you have a NanoDrop, follow the manufacturer's instructions for the spectrophotometer specific to your lab.

Spectrophotometer measurement

1. Before measuring any samples, be sure to 'blank' the spectrophotometer using the solution the DNA is resuspended in, but with no DNA added. 'Blanking' measures the background inherent to the machine and your solvent.
2. Place 1-2µL of mini-prepped DNA onto the pedestal.
3. Close the lid and click measure, be sure to record the concentration and purity.

Note: Purity is measured under the 260/280 column (A good purity ranges from 1.80-2.00).

4. Repeat for each sample.

Note: Keep in mind that despite the accuracy of the NanoDrop, if two consecutive samples have significantly different concentrations, it is possible that the difference between them has affected the accuracy of the NanoDrop. It is a good idea to re-zero any spectrophotometer between samples if they are expected to vary significantly in concentration.

Electrotransformation Protocol

adapted from "Enhancing DNA electrotransformation efficiency in Escherichia coli DH10B electrocompetent cells" (Ning Wu et al, 2010) by Tiago Lubiana

(yields 8 ready-to-transform tubes)

Keep everything as cold and as sterile as possible.

Previous preparation

- Prepare glycerol stocks of your favourite *E. coli* strain (in our case, DH5a and JM110) **or** plate your stocks, pick colonies and grow overnight in day 0 in 10 mL of LB;
- Autoclave 1 1L Erlenmeyer flask and prepare 400 mL sterile LB media;
- Autoclave 10% glycerol in milliQ water;

Day 1

- Put 8 50 mL Falcon tubes in the -4°C;
- Inoculate (either glycerol stock or 2mL of overnight grown cells) in sterile flasks with 400 mL of LB at 37°C and 250rpm until the culture reaches an OD₆₀₀ close to 0.15 (until 0.6 it is in the exponential phase, but the efficiency is better for the beginning);
Obs: make sure that you have a proper, calibrated OD600 curve;
- Split the culture in 8 Falcon tubes of 50ml. Centrifuge at 4°C (pre-cold the centrifuge and the tubes) at 3200 rpm for 20 minutes and remove the LB;
- Remove the supernatant and perform three washes (reinforcing, **3 washes!**) with 15mL of ice-cold 10% glycerol in each flask, followed by centrifugation at 3200 rpm for 20 minutes (each wash);
- Resuspend the cells in 10% ice-cold glycerol, 100 µl for each 50ml Falcon. Each tube will have $\sim 0.6 \times 10^{10}$ electrocompetent cells;
- Electroporate with 10 to 40 pg of DNA (OBS: More plasmidial DNA lowered efficiency in the paper, but this step might require optimization for ligation products, for example) with 0.2-cm-gap cuvette (BIO-RAD, Hercules, CA) at 12.5 KV/cm (field strength), 200 Ω (resistor), and 25 µF (capacitor) or your electroporation protocol of choice.;

Obs: Electroporation at room temperature (incubate samples at RT 15 minutes before electroporation) seems to improve efficiency ([Tu et al. 2016](#)). Actually, according to this article, everything at RT seems to improve efficiency. The protocol was later tested entirely at room temperature and seemed to work more efficiently than at 4°C. Despite efficiency not being directly calculated, the notable

improvement led to the team deciding to adapt the protocol to room temperature. The only downside of such adjustment was the incapability of using frozen (-70°C) cells, so we had to prepare electrocompetent cells previously on the same day.

GC Rich PCR

Roche, Cat. No. 12 140 306 001

Reaction designed for DNA templates with high GC content.

- Mix 1

Component	Volume	Final Concentration
DEPC Water	To total 35 μ L	-
10 mM dATP	1 μ L	200 μ M
10 mM dTTP	1 μ L	200 μ M
10 mM dGTP	1 μ L	200 μ M
10 mM dCTP	1 μ L	200 μ M
Downstream primer	5 μ L	0.2 μ M
Upstream primer	5 μ L	0.2 μ M
Template DNA	Variable	10 - 500ng (gDNA) 1 - 100ng (cDNA)
GC-Rich Resolution solution	Variable	0 to 2 M
Final Volume	35 μ L	-

- Mix 2

Component	Volume	Final Concentration
DEPC Water	4 μ L	-
5 \times GC-RICH PCR Reaction buffer with DMSO	10 μ L	1X
GC-RICH PCR System enzyme mix	1 μ L	2U / 50 μ L
Final Volume	15 μ L	-

- PCR

Combine contents from both mixes and set on the thermocycler, as follows:

Initial denaturation --- 3 min --- 95°C

10 cycles of:

Denaturation --- 30 sec --- 95°C

Annealing --- 30 sec --- 45°C to 65°C

Elongation --- 45 sec/kb --- 68°C to 72°C

20 to 25 cycles of:

Denaturation --- 30 sec --- 95°C

Annealing --- 30 sec --- 45°C to 65°C

Elongation --- 45 sec/kb --- 68°C to 72°C

+5 sec for each cycle in addition

Final Elongation --- 7 min --- 68°C to 72°C

QIAquick[®] Gel Extraction Kit

The QIAquick Gel Extraction Kit (cat. nos. 28704 and 28706) can be stored at room temperature (15–25°C) for up to 12 month.

Further information

- *QIAquick Spin Handbook*: www.qiagen.com/HB-1196
- Safety Data Sheets: www.qiagen.com/safety
- Technical assistance: support.qiagen.com

Notes before starting

- This protocol is for the purification of up to 10 µg DNA (70 bp to 10 kb).
 - The yellow color of Buffer QG indicates a pH ≤7.5. DNA adsorption to the membrane is only efficient at pH ≤7.5.
 - Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
 - Isopropanol (100%) and a heating block or water bath at 50°C are required.
 - All centrifugation steps are carried out at 17,900 $\times g$ (13,000 rpm) in a conventional table-top microcentrifuge.
1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.
 2. Weigh the gel slice in a colorless tube. Add 3 volumes Buffer QG to 1 volume gel (100 mg gel ~ 100 µl). The maximum amount of gel per spin column is 400 mg. For >2% agarose gels, add 6 volumes Buffer QG.
 3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). Vortex the tube every 2–3 min to help dissolve gel. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose). If the color of the mixture is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The mixture turns yellow.

4. Add 1 gel volume isopropanol to the sample and mix.
5. Place a QIAquick spin column in a provided 2 ml collection tube or into a vacuum manifold. To bind DNA, apply the sample to the QIAquick column and centrifuge for 1 min or apply vacuum to the manifold until all the samples have passed through the column. Discard flow-through and place the QIAquick column back into the same tube. For sample volumes of >800 µl, load and spin/apply vacuum again.
6. If DNA will subsequently be used for sequencing, in vitro transcription, or microinjection, add 500 µl Buffer QG to the QIAquick column and centrifuge for 1 min or apply vacuum. Discard flow-through and place the QIAquick column back into the same tube.
7. To wash, add 750 µl Buffer PE to QIAquick column and centrifuge for 1 min or apply vacuum. Discard flow-through and place the QIAquick column back into the same tube.
Note: If the DNA will be used for salt-sensitive applications (e.g., sequencing, blunt-ended ligation), let the column stand 2–5 min after addition of Buffer PE.
Centrifuge the QIAquick column in the provided 2 ml collection tube for 1 min to remove residual wash buffer.
8. Place QIAquick column into a clean 1.5 ml microcentrifuge tube.
9. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 µl Buffer EB to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min. After the addition of Buffer EB to the QIAquick membrane, increasing the incubation time to up to 4 min can increase the yield of purified DNA.
10. If purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.



Scan QR code for handbook.

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Heat-shock Transformation

1. Take competent cells out of freezer and let them defrost at room temperature (~20-30min for -70°C);
2. Take LB Petri dishes (with antibiotic) out of freezer and let them defrost at room temperature;
3. Mix 1-5 μL of desired DNA and 20-50 μL of competent cells solution into a sterile tube;
4. Cool the sample in ice for 20-30 min;
5. Heat-shock the sample by placing the tube (not completely) in a 42°C hot-bath for 30-60s; (45s seemed to be the most effective for us)
6. Cool the sample in ice for 2min;
7. Add 250-500 μL of LB medium and incubate sample in the shaker at 37°C for 45min;
8. Plaques the cells on the Petri dishes and incubate at 37°C.

Ligation

- Prepare solution as follows:
 - 10X T4 DNA Ligase Buffer --- 2 μ L
 - DNA Vector (4kb) --- 50ng (0.020 pmol)
 - DNA Insert (1kb) --- 37.5ng (0.060 pmol)
 - DEPC Water --- Total to 20 μ L
- Add 1 μ L of T4 DNA Ligase;
- For cohesive ends, incubate overnight at 16°C or at room temperature for 10 min;
- For blunt ends, incubate overnight at 16°C or at room temperature for 2h;
- Inactivate with heat at 65°C for 10min;
- Cool the solution with ice and transform 1-5 μ L of the solution with 50 μ L of competent cells;

QIAprep[®] Spin Miniprep Kit

The QIAprep Spin Miniprep Kit (cat. nos. 27104 and 27106) can be stored at room temperature (15–25°C) for up to 12 months.

For more information, please refer to the most recent version of the *QIAprep Miniprep Handbook*, which can be found at: www.qiagen.com/handbooks.

For technical assistance, please call toll-free 00800-22-44-6000, or find regional phone numbers at: www.qiagen.com/contact.

Notes before starting

- Optional: Add LyseBlue reagent to Buffer P1 at a ratio of 1 to 1000.
 - Add the provided RNase A solution to Buffer P1, mix and store at 2–8°C.
 - Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
 - All centrifugation steps are carried out at 13,000 rpm (~17,900 $\times g$) in a conventional table-top microcentrifuge.
1. Pellet 1–5 ml bacterial overnight culture by centrifugation at >8000 rpm (6800 $\times g$) for 3 min at room temperature (15–25°C).
 2. Resuspend pelleted bacterial cells in 250 μ l Buffer P1 and transfer to a microcentrifuge tube.
 3. Add 250 μ l Buffer P2 and mix thoroughly by inverting the tube 4–6 times until the solution becomes clear. Do not allow the lysis reaction to proceed for more than 5 min. If using LyseBlue reagent, the solution will turn blue.

4. Add 350 μ l Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times. If using LyseBlue reagent, the solution will turn colorless.
5. Centrifuge for 10 min at 13,000 rpm (\sim 17,900 \times g) in a table-top microcentrifuge.
6. Apply 800 μ l supernatant from step 5 to the QIAprep 2.0 spin column by pipetting. For centrifuge processing, follow the instructions marked with a triangle (\blacktriangle). For vacuum manifold processing, follow the instructions marked with a circle (\bullet). \blacktriangle Centrifuge for 30–60 s and discard the flow-through, or \bullet apply vacuum to the manifold to draw the solution through the QIAprep 2.0 spin column and switch off the vacuum source.
7. Recommended: Wash the QIAprep 2.0 spin column by adding 0.5 ml Buffer PB. \blacktriangle Centrifuge for 30–60 s and discard the flow-through, or \bullet apply vacuum to the manifold to draw the solution through the QIAprep 2.0 spin column and switch off the vacuum source.
Note: This step is only required when using endA+ strains or other bacteria strains with high nuclease activity or carbohydrate content.
8. Wash the QIAprep 2.0 spin column by adding 0.75 ml Buffer PE. \blacktriangle Centrifuge for 30–60 s and discard the flow-through, or \bullet apply vacuum to the manifold to draw the solution through the QIAprep 2.0 spin column and switch off the vacuum source. Transfer the QIAprep 2.0 spin column to the collection tube.
9. Centrifuge for 1 min to remove residual wash buffer.
10. Place the QIAprep 2.0 column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 μ l Buffer EB (10 mM TrisCl, pH 8.5) or water to the center of the QIAprep 2.0 spin column, let stand for 1 min, and centrifuge for 1 min.
11. If the extracted DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

Open Wetware Buffers

DEPC-treated Free-RNA nuclease Water

1. Get Milli-Q (reverse osmosis purified) water;
2. Add 1 ml DEPC (Diethylpyrocarbonate) per 1000 ml of MilliQ or double distilled water (i.e. to a final concentration 0.1%) and mix thoroughly;
3. Let the DEPC-mixed water incubate for 12 hours at 37°C;
4. Autoclave DEPC-mixed water for 15 minutes,

DEPC inactivates RNases by covalent modification. The autoclaving step inactivates the DNases and removes all traces of DEPC.

Sodium Borate Buffer

- Curators

Anyone should feel free to add themselves as a curator for this consensus protocol. You do not need to be a curator in order to contribute. The OpenWetWare community is currently discussing the idea of protocol curators. Please contribute.

- Abstract

SB (Sodium Borate or Sodium Boric Acid) buffer is a agarose gel electrophoresis buffer for DNA gels. It has low conductivity and allows for less heat buildup and thus higher voltage and faster runs.

- Reagents

Sodium Borate decahydrate (Borax)
Boric Acid
dH₂O

- Procedure

A simple version of this buffer can be easily made as a 20X (100 mM) concentrate.

38.17 g Sodium Borate decahydrate
33 g Boric Acid
Bring to 1L with dH₂O

Dilute to 1X and use to make gel and running buffer.

Restriction Enzyme Buffers

From Invitrogen's "Restriction Enzyme Buffer Set" (Cat. no. 15241-002). Store at -20°C.
Buffer Compositions

- 10X Buffer H
500 mM Tris-HCl, pH 7.5
100 mM MgCl₂
10 mM Dithiothreitol (DTT)
1000 mM NaCl

- 10X Buffer K
200 mM Tris-HCl, pH 8.5
100 mM MgCl₂
10 mM Dithiothreitol (DTT)
1000 mM KCl

- 10X Buffer L
100 mM Tris-HCl, pH 7.5
100 mM MgCl₂
10 mM Dithiothreitol (DTT)

- 10X Buffer M
100 mM Tris-HCl, pH 7.5
100 mM MgCl₂
10 mM Dithiothreitol (DTT)
500 mM NaCl

- 10X Buffer T (BSA-free)
330 mM Tris acetate, pH 7.9
100 mM Magnesium acetate
5 mM Dithiothreitol (DTT)
660 mM Potassium acetate

Qiagen Buffers

- BufferAE (elution buffer for genomic DNA preps)

10 mM Tris-HCl
0.5 mM EDTA
pH 9.0

- Buffer P1

50 mM Tris-HCl pH 8.0
10 mM EDTA
100 µg/ml RNaseA

- Buffer P2

200 mM NaOH
1% SDS

- Buffer N3

4.2 M Gu-HCl
0.9 M potassium acetate
pH 4.8

- Buffer PB

5 M Gu-HCl
30% isopropanol

- Buffer PE

10 mM Tris-HCl pH 7.5
80% ethanol

- Buffer QX1 (for solution and binding of agarose gels)

7 M NaPO₄
10 mM NaAc
pH 5.3

- Buffer QC (wash buffer)

1.0M NaCl
50 mM MOPS pH 7.0
15% isopropanol

- Buffer QF (elution buffer)

1.25M NaCl
50 mM Tris-HCl pH 8.5
15% isopropanol

PCR for Gene Positive

1. Reaction (1x):

5x GC phusion buffer	5 μ L
dNTP (10mM)	0.5 μ L
MgCl ₂ (25mM)	0.25 μ L
Forward primer (10uM)	1.25 μ L
Reverse primer (10uM)	1.25 μ L
X7	0.5 μ L
DNA	25 ng (calculate the volume needed)
H ₂ O	Calculate to obtain 25 μ L total volume

2. Set up the thermal cycler:

1. 98°C - 3' initial cell breakage and DNA denaturation
2. 98°C - 20" DNA denatures into single strands
3. 60°C - 20" primers anneal to ssDNA template (T_m depends on primers)
4. 72°C - 2' primes are extended from 3'-end by X7 (1 min/2000bp)
5. 72°C - 5' final extension to make all products are full length
6. 12°C - forever

Step 1 = 1 cycle

Steps 2-4 = 30 - 34 cycles

Step 5 = 1 cycle

Preparation of Culture Dishes

Preparation of 500mL of LB media (yields ~25 dishes)

- 5g NaCl
 - 5g Tryptone
 - 2.5g Yeast extract
 - 7.5g Agar
 - Milli-Q water to 500mL
1. Add a magnetic stirrer and autoclave the solution at 121°C for 20-30min;
 2. Stir and let the solution cool to about 55°C. From this point on, all procedures should be as sterile as possible;
 3. Add the appropriate amount of antibiotics (more on that further down) and stir;
 4. Pour ~20mL of medium into each Petri dish;
 5. Let the medium solidify face up for 30-60min, and store normally at 4°C;

Antibiotic concentrations

Recommended concentrations for most commonly used antibiotics:

Ampicillin --- 100 µg/mL
Bleocin --- 5 µg/mL
Carbenicillin --- 100 µg/mL
Chloramphenicol --- 25 µg/mL
Coumermycin --- 25 µg/mL
Gentamycin --- 10 µg/mL
Kanamycin --- 50 µg/mL
Spectinomycin --- 50 µg/mL
Tetracycline --- 10 µg/mL

Preparation of electrocompetent cells

1. Culture target cells into 100 mL LB (no amp). Grow O/N at 37°C with shaking.
2. Next morning, add the O/N 100 mL of culture into 900 mL LB medium and grow at 37°C with shaking until OD600 reaches ~ 0.7 (this takes ~ 2 h);
3. Cool cells in cold room on ice for ~ 20 min;
4. Spin centrifuge rotor for 5 min to pre-cool to 4°C;
5. Pour cells into 4 pre-cooled recipients, 250 mL each; Centrifuge at 5,000 RPM for 15 min at 4°C;
6. Centrifuge at 5,000 RPM for 15 min at 4°C. Discard supernatant and resuspend cells in 250 mL of sterile ice-cold water;
7. Repeat the wash and centrifugation from step 5 twice;
8. Centrifuge at 5,000 RPM for 15 min at 4°C. Discard supernatant and resuspend cells in 20 ml ice-cold 10% glycerol. Transfer cells into a more practical recipient (if needed) and centrifuge on table-top centrifuge, at max speed for 15 min at 4°C. Discard supernatant and resuspend cells in 2 mL sterile 10% glycerol;
9. Aliquot 40 µl of cells into each pre-cooled Eppendorf tubes on ice;
10. Store at -70°C.

Restriction Digestion Protocol

1. Prepare the following mixture:

10x buffer (see table for best enzyme activity)	2 μ L
10x BSA	2 μ L
Enzyme 1	0.5 μ L
Enzyme 2	0.5 μ L
DNA	4 μ L (about 1000ng of DNA)
H ₂ O (nuclease free water)	11 μ L
<hr/>	
Total	20 μ L

2. Place reaction at 37°C for 1 hour (about)

3. Make a 1% agarose gel (0.7g agarose and 70mL TAE buffer)

4. Add to each eppendorf reaction 5 μ L of dye (BB) and mix up and down

5. Add the ladder (4 μ L) to the first well (1kb +)

6. Run the gel at 120 volts

7. Take picture of the gel using UV light.

SLiCE

1. Principle

Bacterial cell extract promoted *in vitro* homologous recombination between short homologies (15bp-52bp) of **linear** DNA fragments

2. Characters

- Seamless Cloning
- Multile-way Cloning
- Batch Cloning
- BAC Subcloning

3. Material

3.1 Preparation of SLiCE extract

- PPY strain
- 2XYT medium
 - Bacto-tryptone 16g
 - Bacto-yeast extract 10g
 - NaCl 5g
 - ddH₂O to 1L
 - Adjust pH to 7.2 with NaOH, autoclave to sterilize
- Streptomycin
- Chloramphenicol
- L-(+)-Arabinose (Sigma, A3256)
- ddH₂O
- CelLytic™ B Cell Lysis Reagent (Sigma, B7435)
- 100% Glycerol
- Protein LoBind Tube 1.5 ml (Eppendorf)
- Protein LoBind Tube 0.5 ml (Eppendorf)

- 50-ml centrifuge tubes
- 250ml Nalgene Lab Quality Wide-Mouth Bottles
- 37°C shaker
- 37°C incubator
- Centrifuge
- Spectrometer

3.2 Maintenance of PPY strain

- PPY strain
- LB medium
- Streptomycin
- Chloramphenicol
- 20% Glycerol
- 37°C shaker

3.3 SLiCE cloning

- PPY SLiCE extract
- 10X SLiCE Buffer (500 mM Tris-HCl (pH7.5 at 25°C), 100 mM MgCl₂, 10mM ATP, 10 mM DTT)
- ddH₂O
- Materials for restriction cutting, PCR, DNA purification and transformation.

4. Protocol

4.1 Preparation of PPY SLiCE extract

1. Streak PPY glycerol stock or fresh culture on a LB agar plate (10ug/ml streptomycin and 12.5ug/ml chloramphenicol) and incubate at 37°C overnight.
2. Inoculate 1 single colony into a 50-ml centrifuge tube with 25-ml 2XYT containing 10ug/ml streptomycin and incubate at 37° and 338rpm overnight.
3. The next day, measure the OD600.

For OD600 measurement:

- (a) Spin down 500-1000ul bacterial culture at top speed for 2 min and aspirate off all of the medium
 - (b) ddH₂O should be used as a black and in resuspension/dilution
 - (c) OD600 readings are calculated by diluting the sample to enable photometric measurement in the linear range between 0.1 and 0.5 OD600.
4. Dilute the o/n culture to 0.03 OD600, i.e. inoculate appropriate volume of the o/n culture into a 250ml Nalgene Lab Quality Wide-Mouth Bottle with 50ml 2XYT medium containing 10ug/ml Streptomycin.
 5. Shake at 37° and 338rpm until the culture reaches an OD600 of 5-5.5.
 6. Add 0.2% L-(+)-arabinose into the culture and continue shaking at 37° and 338rpm for 2h at 37°C, to express λ prophage protein Red. Take out 500ul of the culture to measure the actual OD600.
 7. Transfer 48 ml of bacterial culture into two 50-ml centrifuge tubes (24 ml each)..
 8. Pellet the cells by centrifugation at 5,000g for 20 minutes at 4°C.
 9. Wash the cell pellet from 24ml of original culture with 50 ml ddH₂O for 1 time.
 10. Pellet the cells by centrifugation at 5,000g for 20 minutes at 4°C.
 11. Measure the wet weight.
 12. Resuspend the cell pellet of about 0.23g of wet weight or from 24ml of original culture at OD600 \approx 6 in 300ul CellLytic™ B Cell Lysis Reagent (Sigma, B7435).

10: 2.5
11:20 3.75

13. Transfer the resuspended cells into a low binding 1.5 ml tube (Protein LoBind Tube 1.5 ml, Eppendorf) and incubate at room temperature for 10 minutes to allow lysis to occur.
14. Centrifuge the cell lysate at 20,000g for 2 minutes at room temperature to pellet any insoluble material.
15. Remove the resulting supernatants from the cell debris into a low binding 1.5 ml tube (Protein LoBind Tube 1.5 ml, Eppendorf).
16. Mix the cell extracts with equal volume of 100% glycerol and aliquot into 40-60 ul portions in low binding 0.5 ml tubes (Protein LoBind Tube 0.5 ml, Eppendorf), labeled as "PPY SLiCE extract".
17. Store the PPY SLiCE extract at -20°C for about 2 months or at -80°C for long term storage, which can be thawed on wet ice and refrozen up to 10 times without significant loss of activity.

4.2 Maintenance of PPY strain

1. Inoculate 1 single colony of PPY strain from LB agar plate (10ug/ml streptomycin and 12.5ug/ml chloramphenicol) into 5ml LB medium (10ug/ml streptomycin and 12.5ug/ml chloramphenicol) and incubate at 37° and 225-338rpm overnight.
2. In sterile tube add equal volume of PPY culture and 20% autoclaved glycerol.
3. Mix and store at -80 °C.

4.3 SLiCE Cloning

1. Preparation of linear vector and insert fragments

(1) Linearize the vector used for SLiCE by restriction digestion or PCR amplification

- *The nature of vector and insert ends such as blunt ends or 3' or 5' sequence overhangs don't influence SLiCE efficiency or accuracy.*
- *The use of linearized vectors with complementary 5' or 3' overhangs increase*

the formation of empty vector background colonies. So try to avoid using a single sticky ends restriction enzyme or a pair of restriction enzymes generating complementary 5' or 3' overhangs. If it cannot be avoided, blunt the ends before SLiCE reaction.

(2) Amplify the cloning inserts using PCR with primers containing 5'- end homologies (15-52bp, Counted from 3'-end of the vector if the vector has sticky ends) to the vector or to other inserts for co-assembly.

(3) Treat DNA fragments generated by PCR amplification using plasmid DNA as templates with *DpnI* prior to purification to remove residual plasmid template DNA.

- *1-2 ul DpnI in a standard 30ul PCR reaction, @37°C for 30-60min. DpnI works fine in PCR buffers*

(4) Purify the vector and insert by gel extraction with Qiaquick Gel Extraction Kit (Qiagen) or other reagent kits and elute them in EB (10mM Tris.cl, ph 8.5) or ddH₂O.

- *New prepared agarose gel and unused TAE or TBE for electrophoresis are recommended. Because sometimes old gel and running buffer lead to a low cloning or transformation efficiency*
- *Gel extraction is not necessary and can be replaced by other DNA purification methods such as column based purification methods or phenol/chloroform extraction. But these methods sometimes lead to higher background caused by uncut vector plasmid or unspecific PCR products.*
- *For SLiCE cloning of BAC DNA, the restriction digested BAC DNA is purified by phenol/chloroform extraction.*

2 SLiCE Reaction

(1) Add the following ingredients into a 0.2ml tube in this order (see Table1) and vortex:

Table1 SLiCE reaction ingredients

NO NEED
ORIGIN
IF PLASMID
HAS ≠ SELEC
TION MARKER

Vector	50-200ng
Insert	1:1-1:10 molar ratio of vector: insert
ddH ₂ O	Variable
10xSLiCE buffer	1ul
PPY SLiCE extract	1ul
Total	10ul

(2) Incubate the SLiCE reaction mix as above @37°C for 1 h using a PCR machine or water bath.

3 Standard Transformation

(1) Electroporation:

Transform 1ul SLiCE reaction into 20ul ElectroMAX DH10B™ Cells (Invitrogen) or other electrocompetent cells following the manufacturer's instruction

Or

(2) Chemical transformation:

Transform 1ul SLiCE reaction into 100ul MAX Efficiency® DH10B™ Competent Cells (Invitrogen) or other chemically competent cells following the manufacturer's instruction

- *For large recombinant DNA, Electroporation is required.*
- *For complex Cloning, electroporation is recommend. The transformation efficiency of electroporation is 10-100 times higher than chemical transformation*

USER vector preparation

1. 5 µg (could be 8 µg) of vector Hcr41 in buffer 4 + Bsa (cutsmart)
2. 100 µL reaction - 3 µL PacI
3. 3h digestion (37°C)
4. Add 1.5 µL PacI (overnight at 37°C)
5. 1.5h digestion at 37degree Celsius
6. Add 2 µL of enzyme: Nt BbvCI
7. 2.5h digestion (37°C)
8. Check on gel to ensure complete digestion
9. Cut and gel purify the linearized
10. Use 100ng/µL in USER cloning to guarantee

USER reaction

In the PCR tube:

- 2 µL of each piece
- 1 µL of User vector previously digested
- 1 µL of User enzyme

PCR machine:

- 37°C for 15 minutes
- 25°C for 05 minutes

Subsequently, transform *E coli* competent cells as in a standard protocol.

Chlamy Nuclear Transformation by electroporation

DNA preparation:

1. Digest a given DNA (Plasmid + insert) amount with appropriate enzymes. Digest reaction volume is variable according to your starting material. Incubate 37°C for 3 – 5 hours.
2. Gel/column purify your insert
3. Quantify it

For microalgae electroporation, it's needed at least 500 ng of **digested insert**.
Remember: Maximum volume allowed to transform is 10 µL.

Cell Preparation:

Preparation of *C. reinhardtii* inoculum

4. In hood, our microalgae strains (cc1610 and cc1010) are picked from stock plates and transfer into a 200-ml Tris-Acetate-Phosphate (TAP) culture on a 500 ml flask.
5. Inoculum is put on a shaker at 250 rpm adapted with proper illumination system.

Electroporation of prepared expression plasmid on *C. reinhardtii*

Similar to bacterial transformation, microalgae must reach a certain growth state which allow it to embody and recombine digested insert, which in our case, is actually the whole expression cassette.

Assessing the growing state of algae was standardized by measuring optical density at 750nm.

6. Pick an aliquot from the inoculum and measure on a spectrophotometer. OD should not surpass 0,3 so as to have a reasonable transformation efficiency.
Remember: This is mainly recommended due to the final number of cells per milliliter, which is expected to be **3x10⁶ cells/ml**.
7. According to the final volume of inoculum (in our case, 200ml), distribute it on proper amount of 50-ml falcons for pelleting. Pellet cells 2000 rpms for 10 min.

Based on this volume, it's estimated that 100 ml can yield up to 4 potential transformant cells.

Once pelleted, all further steps must be done on hood.

8. Take into consideration that cell final concentration once centrifuged is 50-fold higher (if start volume of distributed inoculum was 50 ml, final cell concentration is **3x10⁸ cells/ml**). Carefully, remove all TAP supernatant and resuspend cells in 1ml of sterile water.

9. Immediately after, pipette suitable amount of plasmid (**500 ng** at minimum) on the inner wall surface of the electroporation cuvette (0,2 mm). Pipette up and down on DNA, 250 ul of resuspended cells (**3×10^8 cells/ml**). Flick to mix cells and DNA well.

Remember: Do not forget a negative control (no DNA).

10. Incubate on ice for 5 to 10 minutes.
11. Electroporate under the following conditions:

Voltage = 800
Capacitance = 25
Resistance = Infinity
Size (mm) = 0,2 ~ 4 (Varies depending on your cuvettes)

Remember: One critical point that can hamper electroporation itself came with over-carried salts from TAP culture, therefore, supernatant removal must be done carefully as stated before.

12. Leave electroporated cells 15 minutes on recovery phase
13. After this, pour whole cuvette content into a 10-ml TAP culture supplemented with sucrose.
14. Incubate at room temperature on rocker over 24 hours. Constant agitation allows proper and homogeneous growth.

Plating transformed microalgae

15. Pellet cells for 20 minutes at 3500 rpm. Remove supernatant and keep with transformed cells on the bottom.
16. 600 ul of each transformant (from previous 10 ml-TAP sucrose culture, step 13) is fairly distributed into TAP plates containing ampicillin (100 mg/ml) and antifungal solutions, and also zeocin for positive pressure selection of transformant bearing Ble-resistance gene ([BBa_K2136014](#)).
17. (OPTIONAL) To assure positive selection, our lab recommends to employ two different concentrations of zeocin (5 and 10 mg/ml). Under this conditions, it will be needed just 300 ul of each transformant cells for each plate.

Remember: On these steps, cooling is no needed.

Transformação nuclear em *Chlamydomonas reinhardtii* por eletroporação

Preparação de DNA:

1. Digerir uma quantidade determinada de DNA (plasmídeo + inserto) com as enzimas de restrição apropriadas. O volume final de digestão varia de acordo às quantidades iniciais de material genético. Incube a 37°C por 3 a 5 horas.
2. Purificação do seu inserto por coluna ou gel.
3. Quantificação

Para eletroporação de Chlamy é necessário pelo menos 500 ng de inserto digerido.

Lembrar: Máximo volume permitido para transformar é 10 µL.

Preparação de células:

Preparação do inóculo de *C. reinhardtii*

4. Sob fluxo, nossas cepas de microalga (cc1610 e cc1010) são repicadas a partir das placas stock e transferidas para 200 ml de médio TAP (Tris-acetato-fosfato) em um erlenmeyer de 500 ml.
5. O inóculo é colocado no shaker a 250 rpm com iluminação apropriada.

Eletroporação do vetor de expressão em *C. reinhardtii*

Similar à transformação em bactéria, é necessário que a microalga atinja uma fase de crescimento idônea para favorecer a recombinação do inserto digerido, que no nosso caso, é um *cassette* de expressão.

A mensuração da fase de crescimento da alga foi padronizada por quantificação da densidade óptica a 750 nm.

6. Pipete uma alíquota do inóculo e quantifica num espectrofotômetro. OD não deve ultrapassar 0,3, de forma que ainda possa se manter uma eficiência de transformação aceitável.
Lembrar: Isto é altamente recomendado devido à concentração final de células por mililitro. É esperada uma concentração de 3×10^6 células/ml.
7. Segundo o volume final do inóculo (nosso caso é de 200ml), distribuir em falcon de 50 ml. Centrifugar a 2000 rpms por 10 mins para concentrar as células.

A cada 100 ml de inóculo, é estimado um rendimento de 4 conglomerados de células transformantes.

Assim que concentrados por centrifugação, os passos a seguir devem ser feitos sob fluxo.

8. Leve em consideração que a concentração final das células centrifugadas é 50 vezes maior (se o volume distribuído nos falcons foi de 50 mL, a concentração final é de 3×10^6 células/mL). Remover cuidadosamente o sobrenadante de TAP e resuspenda as células em 1 mL de água estéril.
9. A seguir, pipete um volume adequado de plasmídeo digerido (500 ng como mínimo e não maior a 10 μ L) sobre as paredes internas da cubeta de eletroporação (0,2 mm). Pipetar 250 μ L de células resuspendidas (3×10^6 células/ml) encima do DNA. Misture bem

Lembrar: Não esqueça de adicionar um controle negativo (Sem DNA).

10. Incubar em gelo por 5 a 10 minutos.
11. Eletropore sob as seguintes condições:

Voltagem = 800

Capacitância = 25

Resistência = Infinita

Espessura da cubeta = 0.2 ~ 4 (Varia de acordo à cubeta)

Lembrar: Um ponto crítico que pode impedir uma correta eletroporação é controlar a concentração de sal restante nas células resuspendidas. Por tanto, é altamente recomendável remover a maior quantidade de meio TAP.

12. Deixe as células eletroporadas por 15 minutos para recuperação.
13. O conteúdo da cubeta transfira para um falcon com 10 ml de meio TAP suplementado com sucrose.
14. Incube a temperatura ambiente por 24 horas em um *rocker*. Agitação constante permite um crescimento homogêneo.

Plaquear células transformadas

15. Centrifugue as células por 20 minutos a 3500 rpm. Remova todo o sobrenadante.
16. 600 μ L de cada célula transformada é distribuída em placas de TAP contendo ampicilina (100 mg/ml), antifúngico e zeocina. Este último permite a seleção positiva das células que contém o gene de resistência a bleomicina ([BBa K2136014](#)).
17. (OPCIONAL) Para garantir uma seleção positiva, nosso lab recomenda utilizar duas concentrações de zeocina (5 e 10 mg/mL). Sob estas condições, é necessário adicionar 300 μ L de célula em cada placa.

Lembre: Não é necessário mais manter em gelo nestas últimas etapas.