Universitat d'Alacant
Universidad de Alicante
Departament de Fisiologia, Genètica i Microbiologia
Departamento de Fisiología, Genética y Microbiología

Divisió de Genètica
División de Genética

# Molecular Techniques
## Third-year Biology

## Genetics Lab practices
## PCR-DIRECTED MUTAGENESIS, MOLECULAR CLONING AND RESTRICTION ANALYSIS

### PLANNING

- **Sessions 1 & 2 (2x3 hours):**
  **PCR-directed mutagenesis. Purification and digestion of the PCR product.**
- **Sessions 3 & 4 (2x3 hours):**
  **Purification, quantitation and DNA insert cloning.**
- **Sessions 5 & 6 (2x3 hours):**
  **Transformation and screening of recombinant colonies. Plasmid DNA isolation.**
- **Session 7 (2 hours):**
  **Restriction analysis of plasmid DNA.**

### PCR-DIRECTED MUTAGENESIS

**Site-directed mutagenesis** can be used to introduce subtle (point) and previously designed changes in a DNA molecule. This methodology is a very powerful tool to study gene function. Several methods have been described in order to introduce point mutations. In these lab practices, we will use a strategy based on **PCR** (*polymerase chain reaction*): **PCR-directed mutagenesis**.

The objective is to create, using PCR-directed mutagenesis, target sites for restriction endonucleases at both ends (5' and 3') of a selected gene, which will allow us to clone it into a vector for its further manipulation and study. To accomplish this, we will carry out a PCR reaction, purify the resulting PCR product, clone it in an appropriate vector and check the cloning results.

PCR is a method to amplify *in vitro* a specific DNA sequence, using two primers (oligonucleotides) whose sequence is complementary to the 3' end of both strands of the target DNA. The multiple repetition of a three-step cycle (denaturation of template DNA, annealing of primers and extension by ***Taq* polymerase**), will result in a massive exponential amplification of the DNA between the two oligonucleotide annealing sites.

In this lab practice we will amplify by PCR the *glnB* gene of the cyanobacterium *Synechococcus elongatus*, coding for a nitrogen regulatory protein named PII. We will use two mutagenic primers, GlnB-1F and GlnB-1R, containing a restriction site for *Eco*RI and *Pst*I enzymes, respectively, none of which is present in the wild-type *glnB* allele. The resulting PCR product will contain both restriction sites flanking the coding region (**ORF**) of the gene.

**Primers**:

*Eco*RI
GlnB-1F: 5'-GGC TTA AGG AGA ATT CCC TTG AAG AAG-3'

*Pst*I
GlnB-1R: 5'-AAC TGC AGT CGA GCG TGA CTT AGA TTG CGT CG-3'

***glnB* PCR product:**

5'GGCTTAAGGAGAA**A**TTCCCTTGAAGAAG.....CGACGCAATCTAAGTCACGCTCGA**CT**GCAGTT 3'

Expected PCR product size: 376 bp.

At the end of the PCR reaction, almost every strand produced will have the above primer sequences incorporated at both extremes of the molecule.

## SESSION 1

### 1. PCR amplification of the *glnB* gene

Mix in a 0,2 ml Eppendorf microtube the following reagents:

| | |
|---|---|
| *BioTaq* polymerase buffer (10×) | 5 µl |
| 50 mM $MgCl_2$ | 2 µl |
| dNTPs (5 mM) | 2 µl |
| Primer 1F (4 µM); forward | 5 µl |
| Primer 1R (4 µM); reverse | 5 µl |
| BioTaq pol (1 U/µl) | 1 µl |
| *S. elongatus* genomic DNA (20 ng/µl) | 2 µl |
| mQ $H_2O$ | 28 µl |
| Final volume | 50 µl |

Place the tubes in the PCR machine, programmed to perform the following cycles:

- Initial denaturation 95ºC 4 min
- Amplification (30 "3-step" cycles):
  - Denaturation 95ºC 1 min
  - Annealing 50ºC 1 min
  - Extension 72ºC 1 min
- Final extension 72ºC 4 min
- Hold at 4ºC

## SESSION 2

### 2. Purification of the PCR product

- Add 100 µl (2 vols.) of capture buffer to the PCR mixture. Mix by pipetting (5-6 times) until homogeneous. Load the mixture into a DNA binding column assembled in a collection tube (remember to label your tube!) and centrifuge at 13000 rpm for 1 min.
- Discard the flow-through. Add 700 µl of washing buffer to the binding column, close the cap and centrifuge at 13000 rpm for 1 min.
- Remove the binding column from the collection tube and discard the flow-through. Place the binding column back into the collection tube and centrifuge (uncapped) again for 1 min without any additional wash solution to remove excess ethanol.
- Label a new a fresh 1.5 ml Eppendorf microtube and transfer the binding column to it. Add 50 µl of elution buffer to the centre of the column. Incubate at room temperature for 1 min and centrifuge at 13000 rpm for 1 min.

A ~50 µl volume of eluted (purified) DNA will be obtained.

**3. Digestion of PCR product with *Eco*RI and *Pst*I**

Add to a new 1.5 ml Eppendorf microtube:

| | |
|---|---|
| Purified DNA | 40 µl |
| Restriction buffer (10×) | 6 µl |
| *Eco*RI (7.5 U/µl) | 1 µl |
| *Pst*I (7.5 U/µl) | 1 µl |
| mQ $H_2O$ | 12 µl |
| Final volume | 60 µl |

Incubate at 37ºC overnight. Store at –20ºC until next lab session.

## SESSION 3

**4. Purification and quantitation of DNA insert:**

**4.1 Purification**

- Add 120 µl (2 vols.) of capture buffer to the PCR mixture. Mix by pipetting (5-6 times) until homogeneous. Load the mixture into a DNA binding column assembled in a collection tube (remember to label your tube!) and centrifuge at 13000 rpm for 1 min.
- Discard the flow-through. Add 700 µl of washing buffer to the binding column, close the cap and centrifuge at 13000 rpm for 1 min.
- Remove the binding column from the collection tube and discard the flow-through. Place the binding column back into the collection tube and centrifuge (uncapped) again for 1 min without any additional wash solution to remove excess ethanol.
- Label a new a fresh 1.5 ml Eppendorf microtube and transfer the binding column to it. Add 50 µl of elution buffer to the centre of the column. Incubate at room temperature for 1 min and centrifuge at 13000 rpm for 1 min.

A ~50 µl volume of eluted (purified) DNA will be obtained.

**4.2 Gel quantitation of insert**

We will use agarose gel electrophoresis to quantify the amount of DNA present in our sample:

- Sample preparation: Mix 5 µl of digested purified DNA with 5 µl of mQ $H_2O$ and 2 µl of 6× loading buffer.
- Load each sample in a gel lane. Load DNA molecular weight standards in the two lanes flanking simple Wells.
- Run the electrophoresis in 0.5× TBE buffer at 120 V for at least 1 h.
- Photograph gel under a UV light transilluminator.

## SESSION 4

**MOLECULAR CLONING**

The objective of the second part is to clone the amplified gene in the **expression vector** pUC18. This plasmid contains a multiple cloning site (**MCS** or *polylinker*) with unique restriction sites for *Eco*RI and *Pst*I. We can introduce our **DNA insert** (*glnB gene*) in the MCS of pUC18, since both molecules will exhibit cohesive compatible ends that can be covalently sealed by **DNA ligase**. The pUC18 MCS is located within the *lacZ* **reporter gene**, which will provide a direct way to identify transformants carrying the recombinant plasmid.

**5. Ligation**

Mix in a 1.5 ml Eppendorf microtube:

| | |
|---|---|
| Vector digested with *Eco*RI and *Pst*I (__ ng/µl) | __ µl |
| DNA insert (__ ng/ µl) | __ µl |
| Ligation buffer (10×) | 2 µl |
| T4 DNA ligase (60 U/µl) | 2 µl |
| mQ $H_2O$ | __ µl |
| Final volume | 20µl |

The volume of insert and $H_2O$ will be different for each subgroup, depending on the concentration obtained for the insert DNA sample.

Mix carefully, give a short pulse centrifugation and incubate at 4ºC overnight.

## SESSION 5

### 6. Transformation

We will use **competent cells** from E. coli strain DH5α, obtained upon treatment with TSS solution (containing PEG, DMSO and $Mg^{2+}$).

- Add 150 µl of competent cells to the ligation mixture from previous session.
- Incubate on ice for 20-30 min.
- Heat shock by placing the tube into a 42ºC water bath for 90 s.
- Put the tubes back on ice for 2 min.
- Add 1 ml of sterile LB liquid medium.
- Incubate at 37ºC for 45 min-1 h.
- Add 100 µl of the transformation mixture to a LB plate (supplemented with 100 µg/ml ampicillin and 40 µg/ml X-gal) and spread using a glass rod (spreader).
- Centrifuge the remaining transformation mixture at 13000 rpm for 1 min. Discard supernatant, resuspend the cell pellet in 100 µl of liquid LB medium, and spread it on another plate.
- Incubate at 37ºC overnight.

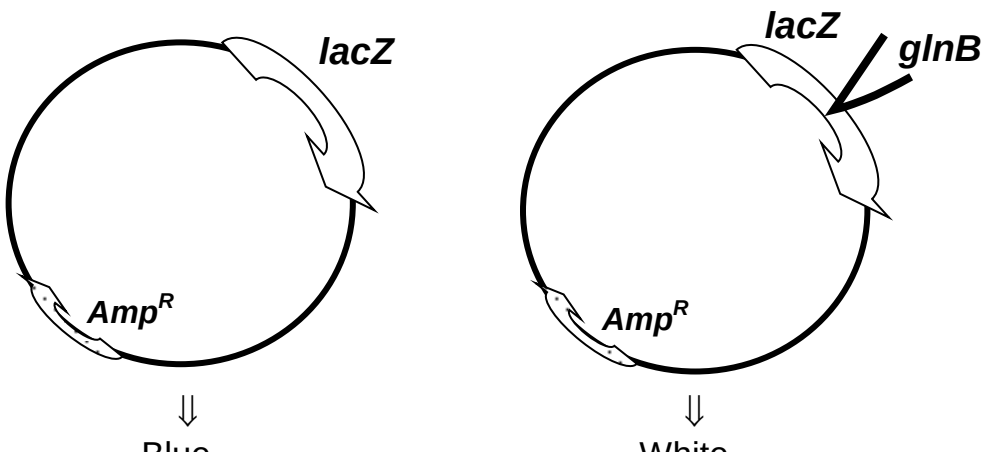
## SESSION 6

**MUTATIONAL ANALYSIS**

### 7. Screening of recombinant colonies

The *lacZ* gene encodes β-galactosidase, an enzyme able to use as a substrate the colourless molecule X-Galto yield an insoluble blue-colored product. This gene is used in pUC18 plasmid to discriminate between colonies containing an unmodified vector and colonies containing a plasmid with DNA insert in its MCS.

Colonies containing the **recombinant DNA molecule**, i. e. the vector-insert ligation product, will not produce β-galactosidase since the insert interrupts *lacZ* coding region (**insertional inactivation**). In those colonies, no cleavage of X-Gal will take place and, therefore, they will remain white in X-Gal-containing plates. Colonies containing self-ligated or uncleaved vector will produce fully active β-galactosidase, thus exhibiting blue colour.



Prior to the lab session, liquid cultures from both white and blue colonies will be inoculated by the teacher in LB medium (with ampicillin) and incubated at 37ºC overnight with shaking. These cultures will be the starting material for plasmid DNA isolation.

### 8. Plasmid DNA isolation using the alkaline lysis method

- Transfer 1.5 ml of each culture, coming from either blue or white colonies, to a new Eppendorf microtube.
- Centrifuge at 13,000 rpm for 1 min.
- Discard supernatant carefully.
- Transfer again 1.5 ml of culture, centrifuge and discard supernatant. Remove the latter supernatant completely by pipetting.
- Add 0.2 ml of GTE buffer (solution I), resuspend cell pellet by vortexing and incubate at room temperature 5 min.
- Add 0.4 ml of a 0.2 M NaOH/1% SDS solution (solution II). Mix gently by inverting the tube.
- Incubate on ice 5 min.
- Add 0.3 ml of 3 M potassium acetate pH 5.4 (solution III). Mix gently by inverting the tube.
- Incubate on ice 5 min.
- Centrifuge at 13000 rpm for 5 min.
- Transfer 0.75 ml of supernatant to a new Eppendorf microtube.
- Add 0.8 ml of 96% ethanol. Mix gently and incubate at room temperature for 1 min.
- Centrifuge at 13000 rpm for 10 min.
- Carefully, discard the supernatant and add 1.5 ml of 70% ethanol to the pellet.
- Centrifuge at 13000 rpm for 5 min.
- Remove the supernatant completely by pipetting (very carefully!). Dry the pellet keeping the tube open in a 37ºC incubator for 15 min.
- Add 50 µl of TE. Keep in a water bath at 37ºC for 15 min. Vortex the final prep gently to dissolve.

### 9. Restriction of plasmid DNA with *Eco*RI and *Pst*I

Add to a new Eppendorf microtube:

| | |
|---|---|
| Plasmid DNA | 2 µl |
| Restriction buffer (10×) | 2 µl |
| *Eco*RI (7.5 U/µl) | 1 µl |
| *Pst*I (7.5 U/µl) | 1 µl |
| RNAse (10 µg/ml) | 2 µl |
| mQ $H_2O$ | 12 µl |
| Final volume | 20 µl |

Incubate at 37ºC in a water bath for at least 1 h. Store at -20ºC.

## SESSION 7

### 10.- Separation and visualization of DNA fragments using agarose gels

- Thaw the DNA digestion mix and add 4 µl of 6× loading buffer.
- Load 24 µl (the full volume) from each sample into a gel lane.
- Run electrophoresis in 0.5× TBE buffer at 120 V for at least 1 h.
- Photograph gel under a UV light transilluminator.

# CULTURE MEDIA, REAGENTS AND BUFFERS

**5×TBE Electrophoresis buffer (for 1 L):** Trizma base 60.55 g, EDTA·$Na_2$ 3.73 g, boric acid ($H_3BO_3$) 28.75 g.

**6×Loading buffer:** 15% Ficoll 400, 0.25% bromophenol blue and 0.25% xylene cyanol.

**1,2% agarose gel**
- Weight 1.2 g of agarose powder
- Add 100 ml 0.5× TBE buffer
- Microwave the mixture until the agarose melts completely.
- Cool in a water bath at 50ºC.
- Add 4 µl of 10 mg/ml EtBr (final concentration in gel: 0.4 µg/ml).
- Set a gel running tray into a casting tray. Set the appropriate comb on the casting tray. Pour the agarose solution into the casting tray.
- Allow the gel to set/solidify (30 min).
- Remove the comb carefully to avoid well damage. Transfer the running tray along with the gel to the electrophoresis buffer chamber and fill with 0.5× TBE buffer until the gel is completely submerged.

**LB liquid medium (1 L)**
Dissolve in 1 L mQ $H_2O$
- 10 g tryptone
- 5 g yeast extract
- 5 g NaCl

Autoclave at 120ºC for 20 min.

**LB solid medium (1 L)**
Add 15 g of agar to LB liquid medium before autoclaving.

**X-Gal 40 mg/ml**
Dissolve 160 mg of X-Gal in 4 ml DMF (dimethylformamide). Wrap the tube containing the solution in aluminium foil to prevent damage by light and store at -20°C.

**Ampicillin 100 mg/ml**
Dissolve 500 mg of ampicillin in 5 ml mQ $H_2O$. Filter sterilise through a 0.45 µm filter and store at –20ºC.

**LB + 100 µg/ml ampicillin + 40 µg/ml X-Gal (1 L; enough for 40 plates)**
Cool LB-agar to 50ºC. Add 1 ml of 100 mg/ml ampicillin and 1 ml of 40 mg/ml X-Gal. Pour 25 ml of medium on each plate.

**Plasmid "miniprep" solutions**

**Solution I**
- 50 mM Glucose
- 25 mM Tris pH 8
- 10 mM EDTA pH 8

For 100 ml, mix:
5 ml 1 M Glucose
2.5 ml 1 M Tris pH 8
2.5 ml 0.4 M EDTA pH 8
90 ml mQ $H_2O$

**Solution II**
- 0.2 M NaOH
- 1% SDS

For 10 ml, mix:
0.2 ml 10 M NaOH
1 ml 10% SDS
8.8 ml mQ $H_2O$

**Solution III**
- 3 M Potassium acetate
- 2 M Acetic acid

For 100 ml, mix:
60 ml 5 M Potassium acetate
11.5 ml Glacial acetic acid
28.5 ml mQ $H_2O$

**10× TE**
- 0.1 M Tris-HCl pH 7.5
- 10 mM EDTA

For 100 ml, mix:
10 ml 1 M Tris-HCl pH 7.5
2.5 ml 0.4 M EDTA pH 8
87.5 ml mQ $H_2O$