MATERIAL AVAILABLE IN THE LABORATORY

LABORATORY EQUIPMENT

- 1-2 microwaves to dissolve agarose solution
- 2 thermocyclers for PCR
- 1 fridge freezer (working)
- 1 ice machine (working)
- eppendorf centrifuges
- 1 transilluminator (under the hood)
- 4 power supplies

COMMUNAL MATERIAL ON THE BENCH

- Electrophoresis grade agarose
- Balance
- Spatulas
- Paper sheets for agarose weighting
- Parafilm
- Scissors
- Gloves (S-M-L)
- Deionized water (1 tank each 2-3 groups)
- Tape
- Blue or black Magic marker (medium tip)
- 96 well plates + lids to store the reactions to be sent to the GATC facility for sequencing

COMMUNAL MATERIAL UNDER THE HOOD

- 2-20µl micropipette
- tips for 2-20µl micropipette
- tank for liquid waste
- transilluminator

PER GROUP ON THE BENCH

- 1 icebox
- 250ml flask
- 100 ml cylinder
- 500ml cylinder
- 20ml pipette tips
- pipette
- a falkon type tube with 50 ml of TAE 50X
- a set of micropipettes (p1000, p200, p20, p2 o p10)

- a box of filtered tips for p1000; p200; p20; p10 micropipettes
- 2 strips of tubes for PCR (12 tubes per strip)
- rack for eppendorf type tubes
- 4-5 sterile “DNA low-bind” eppendorf type tubes (2ml)
- rack for PCR tubes
- blue or black magic marker (fine tip)
- a falkon type tube with 50 ml of ultrapure nuclease free water
- a complete set for agarose gel electrophoresis (box, gel holder and combs)

PER GROUP IN THE FREEZER

- 1 aliquot of 5X GoTaq buffer (colourless)
- 1 aliquot of dNTPs mix (10mM each)
- 1 aliquot of GoTaq DNA Polymerase (5U/μl)
- a full 96 well plate of primer for (plate A)
- a full 96 well plate of primer rev (plate B)
- a full 96 well plate of primer for and rev (plate C)
- 1 aliquot of Template DNA (genomic DNA from *N.gaditana*)
- 1 aliquot of loading dye 2.5X (green)
- 1 aliquot of ladder (blue)

EXPERIMENTAL PROCEDURES

1ST DAY

Each student will perform 5 PCR reactions, using the four primer pairs the he/she has designed during the bioinformatics laboratory. In the "supplementary information" section you will find the coordinates of each of the 3 plates (A, B and C) containing the primer pairs designed by the students. Primers were received in lyophilized form and resuspended to a final concentration of 10μM. We suggest you to look for your primers and highlight their coordinates in the scheme, in order to find them quickly when they will be needed.

Overview: the group will prepare a master mix containing all the reagents needed for the PCR reaction (including the polymerase). The master mix will be used to perform the following reactions:

- **the positive control** where the master mix is added to a solution containing the template DNA (genomic DNA from *N.gaditana*) and a couple of primers that has been already tested by your teachers
- **the negative control** where the master mix will be added to the same mix of primers provided by the teachers. This reaction will be performed in the absence of the template.
- **the actual PCR reactions:** in each tube the students will put a couple of their primer pairs and then will add an aliquot of DNA template and master mix

NOTE: prepare the mix by adding everything but the Template DNA. Transfer an aliquot the mix in the negative control tube. Then add the template DNA to the remaining mix and proceed with transferring aliquots of the mix in all the remaining tubes.

Step by step instructions:

1. Fill your icebox with ice
2. Bring your filled ice box with you and go to the freezer. In the freezer drawers you will find 5 racks containing aliquots of reagents. Collect the following:
   - 5X GoTaq buffer (colourless)
   - 1 aliquot of dNTPs mix (10mM each)
   - GoTaq DNA Polymerase (5U/μl)
3. While the reagents are thawing calculate the volume of each of the reagent for your **master mix**. The following table reports the amount of each component as recommended by the polymerase supplier. Add the volume of genomic DNA and Primers needed for the reaction and compute the amount of water to bring each PCR reaction to the right final volume. After you obtain the correct PCR recipe multiply it by the number of reactions that you group has to perform. The total number of PCR will be the number of students in the group multiplied by 5, plus 2 (the two controls), plus 1 (the bed volume).

(5 x …..) + 2 + 1 = ….. <- number of PCRs

We suggest you to multiply all the components of the master mix by the number of PCRs, while the template DNA has to be multiplied by the total number of PCRs – 1 (because it wont be added to one of the reactions. Which one?)

Component	**Final concentration**	**Final Volume in 1 PCR (μl)**	**Final Volume in the MIX (μl)**
Ultrapure water	-		
5X colourless buffer	1X	10	
dNTPs mix, 10mM each	0.2mM each	1	
GoTaq DNA polymerase (5u/μl)	1.25 U	0.25	
Template DNA 50ng/μl	1ng/μl		(x n PCR - 1)
(Upstream Primer) 10 μM	1μM		---
(Downstream Primer) 10μM	1μM		---
Total Volume	-	50	

Table 1 Fill the cells of the column "Final Volume in 1 PCR" with the right volumes of water, Template and Primers. Fill the cells of the "Final Volume in the MIX (μl)" with: the volumes of water, buffer, dNTPs and Polymerase multiplied by the number of reactions; the volume of Template multiplied by the number of reactions -1; the final volume of the mix.

How much mix you're going to transfer in each of the reactions tubes?

- negative control …………..
- positive control …………..
- PCR reactions …………..

4. Once you're done with the calculations write carefully on each of the tubes of the strip the reaction that you're going to load. FROM NOW AND ON BE CAREFUL NOT TO TOUCH THE TUBES WITH YOU BARE HANDS, YOU MUST ALWAYS WEAR GLOVES WHILE WORKING WITH PCR TUBES AND REAGENTS, IN ORDER TO PROTECT THE DNA FROM DEGRADATION. Be careful to write on the dedicated part of the tube and not on the lid (if you write on the lid your label will probably be deleted while in contact with the hot thermocycler lid). The PCR strips have position numbers impressed on each tube (from 1 to 12) write down on you notes the number impressed on the tube associated to the PCR that you will load inside that tube. Once you will put the strip inside the thermocycler take also note of the position of your strip in the thermocycler. All these information will be of capital importance if your label will be deleted during the reaction (as it happens very often!).
5. Once your tubes will be ready the teacher will come to your bench carrying the plates with your primers (A, B, C). Each of you will find his/her primers and will load the right

amount of primers necessary for one reaction in the right tube (the one marked with the associated name)

6. Once all the tubes contain the primers, put the tube strips on ice (refill the ice when necessary)
7. Before you start with the assembly of the reaction we suggest you to set the thermocycler with the right program ready to start

Step	Temperature °C	Time	Number of Cycles
Initial denaturation	95	2 min	1
denaturation	95	30 sec	35
Annealing	60	30 sec	35
Extension	72	5 min	35
Final Extension	72	10 min	**1**
Soak	4	Indefinite	**1**

8. Make sure that all the reagents are perfectly thawed (do not pipet from half frozen solutions!)
9. Now you're ready to assemble the master mix in a 2ml tube. Proceed with adding the reagents in the following order, tick after you've added:

Ultrapure water	
5X colourless buffer	
dNTPs mix	
GoTaq	

10. Mix the solution using the p1000 sat at a volume of ~300µl
11. Briefly spin the solution using the eppendorf centrifuge to collect all the liquid to the bottom of the tube
12. Transfer the right volume of master mix into the negative control tube
13. Add to the negative control a volume of water equivalent to that of the DNA Template (since you did not include it in this reaction. Pipet throughly
14. Add the DNA template to the remaining mix
15. Mix the solution using the p1000 sat at a volume of ~300µl
16. Briefly spin the solution using the eppendorf centrifuge to collect all the liquid to the bottom of the tube
17. Transfer the right volume of master mix into the positive control and PCR tubes. MAKE SURE TO CHANGE TIP AT EVERY TRANSFER IN ORDER TO AVOID CONTAMINATION

BETWEEN THE PCRs. Pipet thoroughly after you add the mix to the primers. Try to avoid the formation of bobbles.

18. Collect all the PCR volume to the bottom of the tubes and bring them to the thermocycler (keep on ice in the meantime). Load the samples and start the program.
19. Once your PCRs are running we start preparing the gel for the next day. Use the tape to seal the gel holder.
20. We want to produce a 1.1 % agarose gel in TAE buffer. Compute the amount of agarose needed to prepare a 70ml gel. Go to the balance and bring the flask with you. Weight the agarose on the paper shit and pour it in you flask. Seal the flask with parafilm. Use tape and magic marker to label the flask (indicate group number and content of the flask)
21. The gel will be run using TAE 1X solution. Prepare 500ml of TAE from the concentrate stock (TAE 50X). Dilute in deionized water. Seal carefully the cylinder using parafilm and mix by inverting the solution a few time. To label the cylinder paste a piece of tape and write on it the content of the cylinder and the group number
22. Transfer 70ml of TAE 1X solution to the 100ml cylinder. Label the cylinder again using tape and magic marker. Seal both the cylinders using parafilm.

2ND DAY

Overview: we will check the results of our PCR reactions using agarose gel electrophoresis. We will choose which PCRs will be sent for sequencing and we will prepare the samples for shipment to the sequencing service.

Step by step instructions:

1. Fill your icebox with ice
2. Wear gloves and make sure that you will be wearing gloves during all the exercitation
3. Collect your PCRs from the thermocycler
4. Collect one aliquot of 2.5X loading dye (green) from the freezer and one aliquot of ladder (blue)
5. While your samples are on ice and your loading dye is thawing, pour the 70ml of 1X TAE buffer into the flask containing the agarose. Dissolve the agarose by heating the solution in the microwave. Once it is completely dissolved (watch carefully through) bring the hot solution under the hood.
6. Collect your gel holder (already sealed) and bring it also under the hood together with the combs. Place the combs on the holder.
7. Compute the amount of DNA colouring solution that has to be added to the gel: the stock solution available is 10.000X. Add the colouring solution to your gel solution using the pipet and the tips available under the hood. Carefully mix the solution
8. Pour a small amount of the gel solution onto the gel holder. Make sure that the apparatus is not leaking
9. Once you've checked that the holder is sealed properly pour the rest of your gel solution onto the holder and leave the gel under the hood to solidify.

10. Bring the flask with you and wash it thoroughly using soap and water. Rinse using deionized water.
11. Once you're done throw away your gloves into the chemical waste and wear a new pair of gloves
12. Back to your bench, prepare as many eppendorf tubes as you PCR reaction and label each of them with the name of one of the PCR reactions
13. Compute the amount of 2.5X loading dye necessary to load 6 µl of your PCR reaction into the gel. Pipet the correct amount of loading dye (remember it is the green one) into each of the eppendorf tubes (you can use the same tip but be careful not to pipet a wrong volume in case you carried bobbles inside he type)
14. Add 6µl of each PCR reaction to the corresponding tube containing the loading dye
15. Briefly spin the tubes in the eppendorf centrifuge to collect the liquid at the bottom of the tube
16. Once your gel will be completely solidified (the gel will turn cloudy) collect it from the hood. Remove the tape and place it into the box.
17. Cover the gel with 1X TAE buffer.
18. Only after the gel will be soaked in buffer, delicately remove the combs
19. Change your gloves again (through away the used gloves into the chemical waste)
20. Load you samples into the gel and write down carefully the position of each sample in the gel
21. Load 5µl of the ladder in two or more lanes of the gel (at least 1 per level of the gel)
22. Make sure that the gel is completely immersed in TAE buffer, close the box and connect the cables to the power supply
23. Switch on the electricity and switch on the power supply
24. Set the supply to a constant voltage of 100V while leaving the Amperes free to float according to the resistance of the gel
25. When the yellow marker will have reached the end of the run switch of the power supply, unplug the cables and open the box lid
26. Collect the gel and bring it to the transilluminator under the hood to see the results
27. We will chose together the PCRs that gave a successful result and that seem suitable for sequencing
28. Once PCRs have been chosen we will write the loading order of the PCRs into the 96 wells plates for sequencing. Each of the student will carefully load his PCR samples into the plate's well and will take note of the position
29. On a parallel plate the sequencing primer will be loaded in the same position as the relative PCR reaction
30. The two plates will be sealed and sent off for sequencing