BIOTECHNOLOGY EXERCISES, 2025-26

WET LAB TECHNIQUES

Brief overview:

1. **amplification of two fragments**
2. **extraction of PCR products from agarose gel**
3. **ligation of two fragments in pGEM plasmid using Gibson Assembly cloning method**
4. **amplification of plasmid region with inserted DNA and sequencing in order to confirm if the construct was correctly assembled**

1. Exercise: PCR - amplification of two regions (microsatellite markers) from two olive varieties

Plant material and experiment design

List of samples: črnica and štorta

List of loci: EMO3 and DCA15

Preparation of the reaction mixture
Calculate the concentration of dNTPs, MgCl2 and primers in the final reaction volume.

Temperature profile for PCR

A touchdown protocol will be used.

Initial denaturation: 5 min at 94 ºC
5 cycles:
45 sec at 94 ºC,
30 sec at initial annealing temperature 57 ºC (annealing temperature drops for 1 ºC at each following step),
1 min and 30 sec at 72 ºC,
35 cycles:
30 sec at 94 ºC,
30 sec at 52 ºC,
1 min and 30 sec at 72 ºC,
and final incubation at 72 ºC for 10 minutes.

Comment: Microsatellite markers are usually used for genotyping. However, in order to explore other techniques, like genetic engineering, sequencing, etc. primers, designed with SnapGene were used for amplification of DCA15 and EMO3 loci to add overlapping flanking regions. PCR products will be inserted to pGEM plasmid with Gibson Assembly cloning technique. Inserted fragments will be amplified with SP6 and T7 primers (annealing sites are on the pGEM plasmid) and finally, the product will be sequenced to verify sequence of the

inserted fragments. (Same technique could be used for example to merge a plant promoter with a CDS of a bacterial gene).

2. Exercise - Cleaning PCR products obtained with DCA15 and EMO3 primers with QIAquick PCR Purification Kit

After PCR reaction PCR products will be loaded on an agarose gel and excised from it.

Write a recipe for an agarose gel. …

Describe main steps of the protocol, used for extraction of DNA from agarose gel. …

Insert image of the gel.

Questions:

a) What is the role of loading dye? …
b) What is the role of gel dye (SYBR Safe DNA gel stain)? …

3. Exercise - Assembly of PCR products and pGEM plasmid with Gibson Assembly Kit

Describe main steps of the protocol, used for insertion of fragments into a plasmid.

GeneArt™ Gibson Assembly® HiFi cloning reaction

1. Thaw GeneArt™ Gibson Assembly® HiFi Master Mix on ice.
2. Vortex GeneArt™ Gibson Assembly® HiFi Master Mix immediately before use.
3. In a microcentrifuge tube on ice, set up the GeneArt™ Gibson Assembly® cloning reaction as described in the table below:

	1-3 Inserts Assembly	4-5 Inserts Assembly	Positive Control [1]
Recommended DNA molar ratio	vector:insert = 1:1	vector:insert = 1:1	–
Amount of each fragment	0.08 pmol vector 0.08 pmol each insert X µL	0.08 pmol vector 0.08 pmol each insert X µL	10 µL
GeneArt™ Gibson Assembly® HF Master Mix	10 µL	10 µL	10 µL
Deionized water volume	(10 – X) µL	(10 – X) µL	–
Total volume	20 µL	20 µL	20 µL
Incubation time at 50°C	15 minutes	60 minutes	15 minutes

[1] The positive control reagents contain all necessary fragments.

(Optional) For the Positive Control, combine 10 µL of the Positive Control and 10 µL of GeneArt™ Gibson Assembly® HiFi Master Mix in a tube on ice. Mix the reaction by vortexing.

4. Mix the reactions by vortexing, spin down and incubate at 50°C for the recommended time. For Positive Control, use 15 minutes incubation time.
5. After incubation, place the reaction mix on ice and immediately proceed to the transformation step.

Note: Reactions can also be stored at –20°C for later use.

Check page 13 for the equation how to calculate pmoles.

PCR with SP6 and T7 primers

1) What is the aim of this PCR? …

Checking the PCR products on an agarose gel

After PCR reaction, and before cleaning of PCR products, we will check the PCR products on an agarose gel.

Write a recipe for an agarose gel and provide a scheme with samples order (mark the lanes with a ladder and samples). …

Insert image of the gel.

4. SEQUENCING WITH OXFORD NANONPORE TECHNOLOGIES

Link to the protocol which we followed for sequencing of DCA15 and EMO3 amplicons:
https://e.famnit.upr.si/mod/resource/view.php?id=121996