BIOTECHNOLOGY EXERCISES, 2025-26

DRY LAB TECHNIQUES

1. Exercise: GIBSON-ASSEMBLY CLONING

Plasmid: pGEM-T Easy Vector
*The pGEM(R)-T Easy Vector has been linearized with EcoRV at Base 60 of this sequence (indicated by an asterisk *) and a T added to both 3' -ends.*

GenBank accession numbers for DCA15 and EMO3 loci: FJ492763.1 and AJ416321.1

Primers for amplification of DCA15…

DCA-15-for	GATCTTGTCTGTATATCCACAC
DCA-15-rev	TATACCTTTTCCATCTTGACGC

…and EMO3

EMO-03-for	GGTGTAGCCCAAGCCCTTAT
EMO-03-rev	GCATGACCGTGGTGTAAGT

SnapGene workflow

1. Import pGEM-T Easy Vector sequence from “SnapGene Online Sequences…”
2. Linearize the vector (Actions/Linearize…) and add T to the 3’ ends (two AA should be on each 5’ end with blunt ends)
3. Open fasta files with DCA15 and EMO3 sequences and add/annotate (Primers/Add primer…) the primers
4. Simulate PCR to get final sequences for both fragments (Actions/PCR…)
5. Design Gibson Assembly cloning experiment (Actions/Gibson Assembly) and save file Assembled.dna
6. Annotate the sequences of DCA15 and EMO3 with function (Features/Add Feature…)
7. Select “Map” tab, take a screenshot and include it in the notebook. Go to “Primers” tab and near the Save button click the arrow and select Export Primer Data…. Paste designed primers in the notebook.
8. Add primers on the site of T7 and SP6 promoters and simulate the PCR.

Questions:

1. What will the length of the PCR product be using T7 and SP6 primers?
2. Which part of the assembled vector will be amplified with SP6 and T7 primers?
3. Export the amplified region as a fasta file and paste the sequence to your notebook.

2. Exercise: Insertion of gene for cry protein in a plasmid with restriction ligation cloning technique

2.1 Insertion of gene for cry protein into a pGA643 plasmid.

Link to the plasmid:
https://www.snapgene.com/resources/plasmid-files/?set=plant_vectors&plasmid=pGA643

Nucleotide sequence of cry protein is available in GenBank under the accession number EA295176.1.

SnapGene workflow:

1) Import fasta sequence of cry protein in SnapGene.
2) Select the first few nucleotides (until melting temperature 60 °C is reached) and click “Add primer”. Click the tab “Insertions” and select **HindIII** sequence to be attached to the 5’ end to the forward primer and since restriction will be more efficient if recognition site is not at the beginning of the DNA, add additional restriction site of any other restriction enzyme. Repeat the process for the reverse primer, except that the restriction site at the other site of DNA should be **BglII** (Bg”L”2).
3) Simulate PCR to get DNA with elongated primers.
4) Import pGA643 plasmid.
5) Click “Actions”, then “Restriction and Ligation cloning” and “Insert fragment”. Select vector in the first tab, amplified DNA in the second tab and press “Clone”.
6) Select window with pGA643 and inserted DNA. Select inserted sequence from ATG to the TAG codon. Click “Features”, “Add feature”, rename the inserted sequence and select CDS for the “type”.

Questions:

1. How can pGA643 plasmid be inserted into a plant cell? Explain.
2. Which elements are in front of the start codon and downstream of the stop codon? Provide a screenshot (Map tab).
3. Paste sequence of the two primers and for each primer explain which restriction enzyme sites were added to the 5’ end.
4. Provide a screenshot of the History tab.
5. Repeat the process with another gene (mRNA up to 5 kb) and describe the steps.

2.2 Insertion of gene for cry protein in a plasmid with the sequence of GFP protein, called 35S-eGFP-nosT, and used for transient expression

Link to the 35S-eGFP-nosT plasmid
https://www.addgene.org/80127/

Why transient expression could be used for?

To complete this task follow the steps described in 2.1 section, except two important things:

- sequences for the restriction enzymes should be replaced. Which restriction enzymes can be used to open the plasmid between CaMV 35S promoter and eGFP gene? You have to select restriction enzymes which don't have recognition sites in the cry gene.
- when you will design reverse primer you should skip the last three nucleotides (represent stop codon) in order to keep ORF opened.

3. Exercise: Construction of CRISPR/Cas9 plasmid

The aim of this exercise is to create plasmid with CRISPR/Cas9 components to knock out gene for beta-gliadin and consequently to develop gluten-free cultivar.

SnapGene workflow
1) Design gRNA (sequence complementary to targeted gene attached to the scaffold)
Import pUC119-gRNA - plasmid, designed by https://pubmed.ncbi.nlm.nih.gov/23929339/
Check Figure S5 to see how to produce plasmid with the gRNA of interest
https://static-content.springer.com/esm/art%3A10.1038%2Fnbt.2654/MediaObjects/41587_2013_BFnbt2654_MOESM17_ESM.pdf

Addgene: https://www.addgene.org/52255/

Beta-gliadin - https://www.ncbi.nlm.nih.gov/nuccore/AB982279.1?report=fasta
https://www.frontiersin.org/articles/10.3389/fnut.2020.00051/full

Final fasta sequence - within the CDS region (HERE)

Fasta sequence can be paste to one of the tools for designing gRNA (target sequences):
https://zlab.bio/guide-design-resources
https://chopchop.cbu.uib.no/

PrimerF1 and primerR2 must be elongated with HindIII (primerR2) and SbfI (primerF1) recognition sequences in order to be suitable for insertion into a pRGEB31 plasmid.

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PrimerF1 is in a format (from 5' to 3') of "NNN + Restriction site + AGAAATCTCAAAATTCCG" and PrimerR2 is in a format (from 5' to 3') of "NNN + Restriction site + TAATGCCAACTTTGTACA", where NNN represents necessary 5' sequence for a given restriction site to ensure efficient restriction digestion of PCR products.

The PrimerR1 is in a format (from 5' to 3') of "reverse complement of the 5'-most N20 of a gRNA target site + AATCACTACTTCGTCTCT". PrimerF2 is in a format (from 5' to 3') of "the 5'-most N20 of a gRNA target site + GTTTTAGAGCTAGAAATAGC".

Determined by chop chop: AATTGTTGTTGTTGTACCAA

reverse complement: TTGGTACAACAACAACAATT
#
In order to generate fused DNA use the method "Overlap Extension PCR…" in the "Actions" menu.

2) Final amplified DNA fragment using two step PCR can be inserted into a pRGEB31

Questions:

1) Which pRGEB31 plasmid components are removed with HindIII and SbfI restriction enzymes?

2) How long is the sequence of complete gRNA (target region and gRNA scaffold)?
3) Provide History tab screenshots for both plasmids, all primers, gRNA sequence, etc.

Notes:
ChopChop identified sequence ACAACAACTGATTCCATGCA[GGG] (the last three nt represent PAM site - they are not included in the first 20 nt of target sequence of gRNA) as the most appropriate

Reverse complement of the target gRNA: TGCATGGAATCAGTTGTTGT

Final plasmid (modified pRGEB31) could now be used with *A. tumefaciens* to edit a gene in *T. aestivum* to develop gluten-free cultivar.