The protocol to follow in the virtual lab

There might be several differences between this protocol and the virtual lab, so please follow the instructions of the virtual lab.

1. **Diluting oligonucleotides**

- At the table will be tubes with oligonucleotides. Each oligonucleotide will be labelled with name, sequence and information on which amount of water should be added to obtain a 100 µM solution.
    - gRNA_OsbZIP69_F (GGCACCGCCGGCGTCGTTTCAGGA) - add 890 µl to get 100 µM
    - gRNA_OsbZIP69_R (AAACTCCTGAAACGACGCCGGCGG) - add 910 µl to get 100 µM
    - pRGE31_F (GCCCATTACGCAATTGGACG) - add 940 ul to get 100 µM
- To dilute the oligonucleotides, you have to use a new (unopened) box of tips and add fresh DEPC H_2O.

a. Make aliquots of at last 2 tubes with roughly 1 ml of DEPC H_2O. (Even you are not going to use all of the aliquots, it is always good to prepare them once you are opening the main stock of the water to avoid repetitive opening of the stock.)
    i. Prepare clean autoclaved 1.5 Eppendorf tubes into a stand.
    ii. Open a new bottle of DEPC H_2O.
    iii. Pipette with clean tips DEPC H_2O into Eppendorf tube.
    iv. Close the bottle.
    v. Close the tubes.
    vi. Label the tubes.
        - It is good to label all your tubes with name and date - DEPC 210707
        - The format of the date should be year-month-day, because if you are going to sort your samples in PC according to their name, the samples will be sorted also by the date.

b. Add water from aliquoted DEPC water into tubes with oligonucleotides to dissolve them.
c. Vortex the tubes approximately 3 times for 5 sec.
d. Short-time centrifuge tubes on the table centrifuge = short-spin (hold for 10 secs).
e. Let oligonucleotides completely dissolve at room temperature (RT) for about 20 min.
f. Use the oligonucleotides directly or store them at -20 °C.

2. **Annealing of oligonucleotides - oligo-dimer formation**

a. Label a new 0.2 PCR tubes for the pair of oligonucleotides.
    - example - dimer_gRNA_OsbZIP69_210707
b. Mix at room temperature (RT) 50 µl of the reaction:
    i. x µl of each stock oligonucleotide (gRNA_OsbZIP69_F/R)
        - to get 20 µM final concentration
    ii. y µl of DEPC H_2O
    iii. z µl of NEBufer 3 (stored in freezer at -20 °C)
        - This buffer is stored in the freezer. It must be defrosted and vortexed before use. It is 10-times concentrated, and the final reaction should be 1-time concentrated.
c. Vortex the solution.
d. Short-spin.
e. Insert into the PCR machine and set up the program for:

i. Cycle 1 -> 90 °C 3 min
ii. Cycle 2 -> 4 °C for infinity

f. Use directly for the next step (and keep in cooler stand - can be found in the freezer) or store at minus 20 °C.

3. Phosphorylation of the oligo

- The DNA ends of oligo-dimers must be phosphorylated before ligation.

a. Label a new 0.2 PCR tube.
b. In the labelled 0.2 PCR tube in ice-cold stand mix:
   i. 7.5 µl od DEPC H_2O
   ii. 1 µl of oligo-dimer (to final 2 µM)
   iii. 1 µl of 10x T4 DNA ligase buffer with ATP (stored in the freezer, vortex it and make a short-spin)
   iv. 0.5 µl of T4 Polynucleotide Kinase (PNK - stored in the freezer, must be kept in a cooler stand container all the time except for pipetting)
c. Vortex.
d. Short-spin.
e. Set PCR machine for a program: 37 °C for 30 min; 65 °C for 20 min; 4 °C infinity.
f. Run the program with the prepared tube.
g. Short-spin.
h. Store in -20 °C or use directly; keep in the cooler stand from now on.

4. Check the oligo-dimers on ElFO.

- Firstly, prepare gel for the electrophoresis.

a. In an Eppendorf tube mix:
   i. 4 µl of water (any kind of clean water)
   ii. 1 µl of phosphorylated oligo-dimers
   iii. 1 µl of 6x loading DYE (stored in freezer -> defrost, can be used directly)
b. Pipette the solution into the well in the gel.
c. Pipette 6 µl of diluted 100 bp DNA ladder next to the sample (can be found in the freezer).
d. Run the ElFO for 20 - 40 min at 80 V.
e. Take a picture of the gel in the G:box under UV light.

5. Restriction of the entry vector *pRGE31*

- The entry vector must be restricted with *Bsa*I. This restriction enzyme makes overhangs that can not reassociate, thus it is not necessary to dephosphorylate the vector before ligation.

a. Mix at RT 50 µl of the reaction:
   i. x µl of DEPC H_2O
   ii. y µl of 10x NEBuffer 2.1
   iii. z µl of pRGE31 vector (stored in the freezer -> defrost -> vortex -> short-spin)
      - add 3000 ng; the concentration is 77 ng/µl
   iv. 15 U of *Bsa*I (10 U/ul; stored in the freezer)
b. Vortex.
c. Short-spin.
d. In the PCR machine, cultivate 1.5 hours at 37 °C; 4 °C infinity.
   - Note: the inactivation of the *Bsa*I restriction enzyme is not necessary since you are going to load the reaction in electrophoresis gel and separate restricted vectors from unrestricted.

6. **ElFO of the vector and gel extraction**
   - It seems that the TBE buffer is gone and you have to make a fresh stock solution.

a. Run the restricted vector on the gel. (Hint: Use the same procedure as with the oligo-dimers. Just change 100 bp DNA ladder for 1 kbp DNA ladder.)
b. Prepare an Eppendorf tube and label it. This tube will store the digested vector.
c. After the run, do not use G:box with UV light; it could damage the vector. Use blue light transilluminator instead.
d. Cut out the indicated fragmented vector using a scalpel.
e. Put the fragment into the labelled tube.
f. Isolate the vector DNA from the gel using the gel extraction kit.
   - There are several companies providing gel extraction kits. As the procedure is different according to the company and it is repetitive work when you are pipetting liquid from tube to tube, you are not going to do it in this virtual lab, because it would be too boring and you would not learn anything new. Let us assume that you magically did it.

g. Measure the concentration of the digested vector on the nanodrop.
   i. Open the lid of the nanodrop.
   ii. Pipette to the nanodrop 1 µl of DEPC H_2O.
   iii. Calibrate.
   iv. Pipette to the nanodrop 1 µl of the vector.
   v. Write the concentration on the tube with the vector.

7. **Ligation of the oligo-dimer into the entry vector *pRGE31***
   a. Mix on the cooler stand 10 µl reaction:
      i. 150 ng of the digested vector
      ii. 2 µl of the oligo-dimers (phosphorylated)
      iii. 1x T4 DNA ligase buffer (10x concentrated; stored in the freezer)
      iv. 0.5 µl T4 ligase
      v. DEPC H_2O up to 10 µl
   b. 4 °C overnight (at least 8 hours) in the fridge.

8. **Heat-shock transformation into *E. coli***
   a. Turn on the laminar flow box (hood).
   b. Add to competent cells the ligation mixture
      i. 50 µl of heat-shock competent cells (stored in 50 µl aliquots in the deep-freeze)
      ii. 1-2 µl of the ligation mixture
   c. Keep 30 min in the fridge in the cooler stand.
   d. Incubate 30 sec at 42 °C in the water bath.
   e. Immediately add 250 µl of ice-cold SOC media (can be found in the fridge).
   f. Put in cooler stand for 1 min.
   g. Cultivate 1 hour at 37 °C with 220 rpm shaking.
   h. Sow on LB media with antibiotics in laminar flow box:
   i. Cultivate overnight at 37 °C.

9. **Picking up colonies from the plate.**
   a. Turn on the fume-hood.
   b. Prepare PCR tubes.
   c. Transfer small amount of bacteria into the PCR tubes.
   d. Test at last 8 colonies.

e. Close the bacteria plate.

10. Making PCR reaction.

a. According to the amount of the colonies to be tested, prepare the master mix for PCR.
b. Into new Eppendorf tubes, dilute pRGE31_F and gRNA_OsbZIP69_R primers from stock (100 µM) to 10 µM. Vortex. Short-spin. Store at the ice-cold stand; for a long term at -20 °C.
c. Mix in the cooler stand PCR premix
    - example for 50 µl reaction - recalculate for your master-mix -> 1 reaction 20 µl volume, make a 10% reserve volume for pipetting error.
    i. w µl of DEPC H_2O
    ii. x µl of 10x Taq polymerase Buffer (stock is in the freezer)
    iii. y µl of 10 mM dNTP (stock is in the freezer; final concentration 200 µM)
    iv. z µl of pRGE31_F (to final 0.2 µM)
    v. a µl of gRNA_OsbZIP69_R (to final 0.2 µM)
    vi. b µl of Taq polymerase (stock concentration 5,000 units/ml, final concentration in the reaction should be 25 units/ml; stored in the freezer)
d. Pipette 20 µl of the master-mix into tubes with collected bacteria from colonies. Avoid cross-contamination.
e. Vortex.
f. Short-spin.

11. Set up the PCR reaction.

a. Initial denaturation 98 °C 5 min.
b. 23 cycles of:
    i. denaturation - 98°C 30 sec,
    ii. annealing – x °C 30 sec (calculate the temperature according to the sequence of the primers – the general formula is -> Tm = 4*(G+C) + 2*(A+T))
    iii. elongation - 68 °C for y (decide the time according to the length of the expected product – 125 bp).
c. Final extension 68 °C 3 min.
d. Hold 4-10°C.

12. Run the PCR reaction on ElFO

- Run the PCR products on electrophoresis gel and decide which *E. coli* colonies are positive.
- Follow the instruction as described above in part 4.
- Decide which colonies are positive.
- Pick up 3 positive colonies.

13. Cultivation of *E. coli*

- You picked up 3 colonies that contain plasmid from which you were able to amplify the expected PCR product. Those plasmids codes Cas9 exonuclease and sgRNA, which will target the desired locus. Before you could transform this vector into Agrobacterium and transform the plant you would like to edit, you should get the plasmid sequence. It is expected that the backbone of the plasmid is fine, but the position of ligation is worth sequencing.
- The process would be as follows:
    - You would independently inoculate the bacteria from positive colonies into 3 ml of LB media with diluted antibiotics (For the positive selection of

bacteria containing the plasmid. Without antibiotics, some of the bacteria could drop the plasmid as they would not need it for their life.)

- You would let them cultivate overnight (cca 16 hours) at 37 °C with shaking around 220 rpm.
- The next day you would isolate the plasmid using one of the commercial MiniPrep kits.
- For example:
  - https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/plasmid-dna/qiaprep-spin-miniprep-kit/
  - https://international.neb.com/tools-and-resources/video-library/monarch-plasmid-miniprep-kit-protocol
- Finally, you would send the plasmid for sequencing.

14. Analyse the Sanger-seq data.

- We will do this work together tomorrow on a synchronous lecture. We will show you basic *in silico* work with sequences as plasmids and primers; how to analyse the real sequencing data and how to design gRNA targets using the Geneious software. 14 days trial version could be downloaded here: https://www.geneious.com/free-trial/
- Please, install the program on your computer in advance and try if it works.