Date:

Investigators:

Title:

Theoretical Cloning: (with Geneious)

1st Step: Vector-cut: (Tasten-Kombi)

1) Cloning
2) Digest into fragments
3) Enzyme set: RFC 25 (iGEM) (don't mark "Exclude enzymes cutting between bases")
4) Options: mark the restriction enzymes → click ok
5) Choose the necessary fragment and annotate (Annotation: e.g. pMA_LongLinker+FokA_cut_Age+Spe, p=plasmid, MA= name of vector, LongLinker+FokA=constructs, cut= what you've done with the vector, Age+Spe= restriction enzymes)
6) cut your insert (step 1-5)
7) mark vector and insert
8) cloning
9) ligate sequences (annotate: e.g. pMA_LongLinker_FokA_HisII, HisII=Insert)

NOTE: For cloning with iGEM restriction sites we have our own enzymes in the freezer (-20°C). you always need buffer 4, which is stored in the same freezer. If you are working with EcoRI, NotI,XbaI, AgeI, SpeI, and PstI, DO NOT use the lab stock enzymes!!!

Practical Cloning:

- Plasmid's name
- Buffer used: (stored at iGEM's -20°C; box iGEM2010 stocks)
- BSA: (stored at iGEM's -20°C; box iGEM2010 stocks)
- DNA-Concentration:
- Measure DNA-concentration with Nanodrop
- Restriction-enzyms used → see: http://www.neb.com/nebecomm/DoubleDigestCalculator.asp

Enzyme1 (Nr. Lab:)
Enzyme2 (Nr. Lab:)

To obtain distinct bands, Buffer and DNA amount need to be adjusted

Remember: add more insert (1.5 - 2.5µg) than vector (1 - 1.5µg)

components	Vector/µL	Insert / µL
DNA		
BSA (10x iGEM **stock!**)		
Buffer … (10x **iGEM stock**)		
Enzyme1 (Nr. Lab:)		
Enzyme2 (Nr. Lab:)		
H_2O		
Endvolume (e.g 15, 20, 25, 30µl)		

Total enzyme volume shouldn't be more than 10% of endvolume

Comments:

- Incubation at 37°C, 1.5-2h (Thermoblock with lid)
  - Remember: for a preparative digestion incubate for *at least* **1,5h**
- While waiting for digestion, prepare an agarose gel (1%)

Agarose-Gel:

- 100 - 1000bp can be separated using 1% Agarose-Gel (0,5g Agarose + 50ml TAE + 3 µL)
- thick and small combs (preparative and analytic gels)
- microwave is next to the "communist-freezer"
- **be aware of delay in boiling ("Siedeverzug")**

Ingredient	Volume/weight
Agarose	g
Gelred (ethidiumbromid-ersatz)	µL

- wait until the agarose is at ~ 40°C
- add 3µl GELRED => mix it
- spill the gel

Loading Dye

- 6x (or 5x) loading dye (stored in small tube-rack in coldroom iGEM shelf) need to be 1x
- Calculate volume

Sample/µL	Loading dye µL

Expected size of Fragments (Geneious)

- Size of the fragments:

Sample	Expected size

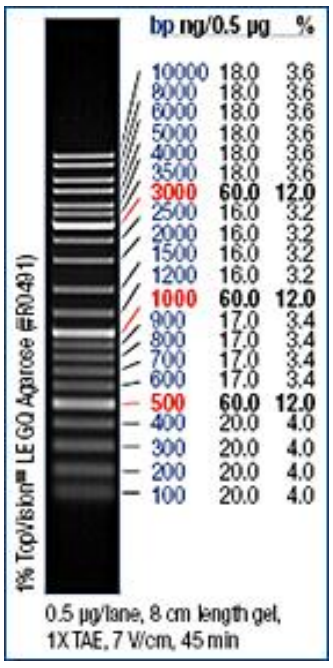
Running the Gel

- 10µL marker (stored in small tube-rack in coldroom iGEM shelf) Generuler DNA-Ladder-Mix Fermentas
- 115V (at the beginning ~ 90V until samples are in the gel); Running about 30-45 min

Loading plan:

Marker µL									

Generuler DNA-Ladder-Mix Fermentas



Cutting the Gel

- You need: scalpel, EtOH, Tissue, gloves, glasses, Eppis (labeled)
- Using the ‘‘outer‘‘ camera
- Take pictures with the left machine which is independent on the computer
- Work **fast** under UV-light, protect yourself => sunburn! (**70% intensity**)

- **<u>Don't overexpose the DNA to UV-light and don't take too much agarose</u>**

Gelextraction

- Measure the weight of Gel-fragments (use empty tube for calculating the weight of the fragments as a blank)
- Transfer gel fragments to 2ml Eppendorf tubes

<u>Gel measurement</u>:

sample	**weight**

Following Standard Protocol (Bench Protocol: QIAquick Gel Extraction Microcentrifuge and Vacuum Protocol)

- Located above our Bench on the left side
- Measure DNA-concentration (NANODrop): 1.5 µL
  → H_2O
  → EB ("Blank")
  → sample ("Measure")
  <u>Important:</u> Before measurement: enter name of sample into the program! Clean NANODrop between each step!
  Print results.

Ligation

- Ratio insert/vector: 3 molecules insert/1 molecule vector <u>→ use LabTools program on left computer to calculate exact volume of insert and vector</u> which should be together 9 µL
  - c( ):
  - c( ):

  volume insert:

  volume vector:

<u>Quickligation:</u>

- 10 µL QuickLigase buffer (2x),
- 9 µL (vector + insert)mix
- 1 µL Quickligase

- Incubate for 10 minutes @RT.

In the meantime prepare cells for transformation!!

Transformation

- Thaw cells for transformation (-80°C freezer) on ice. Important: Cells should be always on ice.
- Per ligation: add 2 µL DNA sample to cells, mix via soft flicking (Remember: amount should always be xx pg!!).
- Incubate for 20-30 minutes on ice.
- In the meantime prepare the agar plates (see protocol for preparative agarose gel)
- Heat shock: Put eppis on 42°C-thermoblock or use waterbath for better results for 40 seconds (**exact durance is important**!)
- Put eppis on ice for 4 minutes.
- Add 700 µL LB medium (**w/o antibiotics**), incubate for 1 hour (for establishing antibiotics-resistance), shaking in thermomixer (37°C) 700-900 rpm.
- Centrifuge for 3 minutes (6000 rpm), decant supernatant (leaves automatically 100 µL, which is necessary for resuspending).
- Resuspend gently (!) with pipette.
- Plate on agar plates (flame trigalsky spatula, cool it on agar plate before plating).
- Incubate at 37°C over night in a small box to prevent drying of the plates.

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Picking clones

- Prepare 10 ml LB-Medium or DYT-Medium in Erlenmeyer-flasks for each clone
- Add 10 µL of antibiotics (ratio always 1:1000).
- Label the flasks with
    - Your Name (Namenskürzel)
    - Date
    - Strain
    - Plasmid
    - Clone number
- Pick clones from agar plate and inoculate media (one clone – one flask!!!) – **try to NOT to touch the flask with your pipette**
- Incubation over night at 37°C (do not prolong incubation for more than 16 hours)
    - Note: If you are not working immediately with the cells, place them on ice or at 4°C (cold room)

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Plasmid Mini-Prep

- Before starting with mini-prep: prepare glycerol stock of over-night culture in 2ml Eppis (1/3 glycerol, 2/3 cells (**cut approx. 1cm from the tip before pipetting glycerol**) and freeze them at **-80°C Vortex before!**
    - Discard negative clones after test digestion (stored in -80°C)
- Add 2 ml of overnight culture in 2 ml Eppendorf-tubes
- Centrifugation 8000 rpm, 3 minutes
- Discard supernatant
- Resuspend in 250 µL P1 buffer (stored in coldroom), vortex until all clumps are dissolved
- Add 250 µL P2 buffer, DO NOT VORTEX, invert tube 6-10 times
    - Do not wait with next step longer than 5 minutes (Buffer P2 contains NaOH)
- Add 350 µL N3 (Neutralization buffer), invert tube 6-10 times, centrifuge at 13000 rpm, 10 minutes
- Apply **supernatant** on spin column, centrifuge at 13000 rpm, 1 minute; discard flow-through
- Add 500 µL PB buffer, centrifuge at 13000 rpm, 1 minute; discard flow-through

- Add 750 µL PE buffer (**check if ETHANOL is in solution**), centrifuge at 13000 rpm, 1 minute; discard flow-through **& remove remaining buffer in waste tube by beating tubes on Küchenrolle**
- Centrifuge at 13000 rpm, 1 minute to remove residual wash buffer
- **place column in fresh 1.5ml Eppendorf tube (labeled)** Elute DNA with 60 µL EB buffer, wait 1 minute, centrifuge at 13000 rpm, 1 minute
- Measure DNA concentration with Nanodrop

Nandodrop concentrations

Sample	**Concentration /ng*µL-1**

Test digestion

- For test digestion volume of enzyme can be reduced down to 0.5 µL

Components	**Volume/µL**	**Mastermix**	**Sample:**	**Sample:**
DNA	Variable (800-1000 ng)	-		
BSA (100x)				
Buffer no. (10x)				
Enzyme 1 (no. Lab:)				
Enzyme 2 (no. Lab:)				
H2O	Variable	-		
Total volume	**x**	-		

Incubation time: Incubation temperature:

- While waiting for test digestion, prepare an analytical agarose gel (percentage depends on fragment size) and run it for 30-45 minutes at 115 Volt (check brome phenol blue migration after 30 minutes – if it ran not far enough, run for additional ~ 10 minutes.)
- Total amount of DNA to recognize a distinct band should be more than 50 ng (remember insert size)

<u>Loading plan</u>

Marker: (8- 10µL)	(~20 µL)	(~20 µL)	(~20 µL)	(~20 µL)	(~20 µL)	(~20 µL)

- Take a picture of the gel and analyze results.

Sequencing (www.GATC-biotech.com)

- Samples for sequencing are collected above the enzyme-freezer (responsible for sequencing: Tobias or Christina)
    1. PLasmid-concentration: 30 – 100 ng/µL
    2. 1,5 ml eppendorf tubes
    3. Total volume: 30 µL
    4. DNA dissolved in H20
- Calculate volume needed for sequencing:

$$c_1 * v_1 = c_2 * v_2$$

Calculation:

Volume Plasmid:

Volume H_20:

- Add H20 up to 30 µL
- Label eppi just with name and number of sample
    1. Namenskürzel_number (e.g. BK_1) (on the lid!)
        - Name:
- Fill in the list above enzyme freezer
    1. Date
    2. Name of eppi
    3. Primer

- Finding appropriate primers:
    1. Search in Geneious:
        - "Primers"
        - "Test with saved primers"
        - "Search for saved primers"
        - Just select: "Target region"
    2. For custom primers send 30µl total volume (3µl of 100µM in 27µl H2O)

Results: Search on homepage: www.GATC-biotech.com

- Login
- "Sequences, Watchboxes"

- Watch Box → search for your sequencing
- Choose file ….ab1 → paste into geneious
- Analysis of sequencing data