Cloning

PCR reaction

KAPA HiFi and Phusion DNA polymerase were used for DNA amplification. Colony PCR was performed with DreamTaq DNA polymerase.

- The master mix for reactions using Phusion DNA polymerase contained:

  DNA (10-25 ng)
  Forward and reverse primer (0.4 pmol/µL)
  1x Phusion HF or GC buffer
  0.2 µM dNTPs
  Phusion DNA polymerase (0.02 U/µL)
  MQ up to final volume of 25 µL

- The master mix for reactions with KAPA HiFi contained:

  DNA (10-25ng)
  Forward and reverse primer (0.4 pmol/uL)
  KAPA HiFi HotStart ReadyMix (2X)
  MQ up to final volume of 25 µL

- The master mix for reactions with DreamTaq DNA polymerase contained:

  Forward and reverse primer (0.4 pmol/µL)
  DreamTaq (green or colorless) PCR Master Mix (2x)
  MQ up to final volume of 25 µL.
  Then the bacterial colony or 1 µL of over-night culture grown in mini prep was added.

All temperature and time profiles were optimized according to manufacturer's protocol for optimal DNA polymerase activity, the melting temperature of primers, and the length of the desired PCR products. Reactions were performed in the Applied Biosystems 96 well thermal cycler.

PCR product purification. Completed PCR reactions were loaded on the agarose gel, desired fragment was excised and purified by kit according to the manufacturer's protocol.

DNA concentration. Samples of isolated DNA fragments was analyzed using NanoDrop.

DNA fragment isolation from agarose gel

Agarose electrophoresis

- A mixture of different sized DNA fragments was separated on an agarose gel (from 0.8 to 1.5% agarose in 1x TAE buffer and 0.1 µg/mL ethidium bromide) at a constant voltage of 100 V.

- To visualize and determine the size of DNA fragment with intercalated ethidium bromide UV light (254 nm) was used.

Fragment isolation from agarose gel

- The band with desired DNA fragment was excised from the gel, using a scalpel.
- DNA was isolated from gel slice with GeneJET PCR Purification Kit (Thermo Scientific) according to the manufacturer's protocol.
- Amount and purity of isolated DNA was determined using NanoDrop.

Restriction digest

To digest the desired DNA, restriction reactions were prepared as followed:

- for analysis of cloned DNA (control restriction)

  2 µL of the appropriate 10x restriction buffer
  0.5 µL of each restriction enzyme
  MQ up to final volume of 20µL

- for isolation of specific DNA fragment

  10 µL of the appropriate 10x restriction buffer
  1.5 µL of each restriction enzyme
  MQ up to final volume of 100µL

Samples were incubated at 37°C for 1 h (control restriction) or 2 h (for isolation of specific DNA fragment).

Analysis of fragmented DNA was done by gel electrophoresis. Desired DNA fragments were excised and purified using suitable DNA purification kit.

Ligation

T4 DNA ligase ligates the 5' phosphate and the 3'-hydroxyl groups of DNA.

- Vector and insert concentrations were estimated and joined in a molar ratio 1:3 (50 ng vector DNA).
- A ligation mixture was prepared:

  1x Rapid ligase buffer (5x)
  0.75 µL T4 DNA ligase
  MQ up to final volume of 15 µL

- Reactions were incubated at room temperature for 25 minutes.
- After incubation the ligation mixture was used for the transformation of bacterial cells.

Gibson assembly

Gibson assembly master mix was prepared according to the protocol published in Gibson et al. (*Gibson, D. G., Young, L., Chuang, R.-Y., Venter, J. C., Hutchison, C. A., & Smith, H. O. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. Nature Methods, 6(5), 343–345. doi:10.1038/nmeth.1318*).

- 40 ng of each PCR product was added to the Gibson assembly master mix and incubated 1 h at 50°C.
- After incubation, the entire master mix volume was transformed into competent bacterial cells.

Transformation of bacteria

- 60 µL or 200 µL of competent cells were thawed on ice for 30 minutes.
- 10 – 200 ng of DNA was added to competent bacterial cells.
- A mixture of cells and DNA was incubated on ice for 30 minutes.
- The mixture was heat-shocked for 2 minutes at 42°C and then cooled on ice for 2 minutes.
- 500 – 1000 µL of LB medium was added and incubated for one hour at 37°C with agitation.
- The selection for plasmid and therefore antibiotic resistant bacteria was conducted by plating them on LB-agar plates containig appropriate antibiotic.

Culturing bacteria

For plasmid DNA propagation two bacterial strains were used: **DH5alpha** [fhuA2Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17] and **TOP10** [mcrA, Δ(mrr-hsdRMS-mcrBC), Phi80lacZ(del)M15, ΔlacX74, deoR, recA1, araD139, Δ(ara-leu)7697, galU, galK, rpsL(SmR), endA1,nupG].

Growth media for bacteria:

Luria Broth (LB): 10 g/L trypthone, 5 g/L yeast extract, 10 g/L NaCl, media is supplemented with suitable antibiotic depending on the selection marker present on the transformed plasmid.

LB agar plates: LB with 1.5% agar supplemented with suitable antibiotic depending on the selection marker present on the transformed plasmid.

Plasmid DNA isolation

- A single colony was picked from LB-agar plate and inoculated into mini prep containing 10 mL of LB-medium and appropriate antibiotic for selection.
- Bacteria were grown overnight at 37°C with agitation.
- Plasmid DNA was isolated from 10 mL of over-night culture with GeneJET Plasmid Miniprep Kit (Thermo Scientific) according to the manufacturer's protocol.
- Concentration and purity of the isolated plasmid DNA was determined by NanoDrop.
- Amounts around 5 µg of plasmid DNA were obtained.
- To determine correctness of an insert plasmids with appropriate sequencing primers were sent to for sequencing (GATC Biotech).