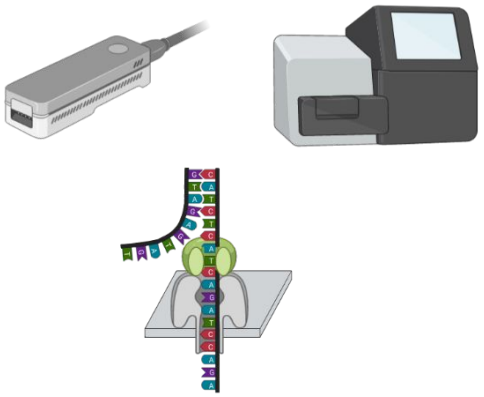
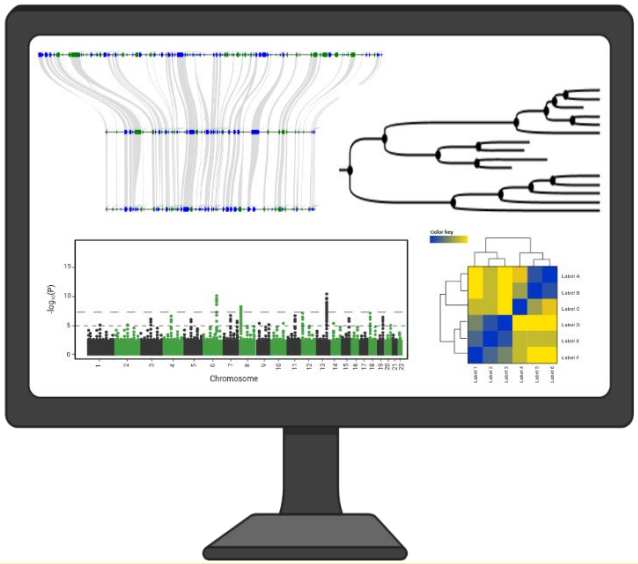
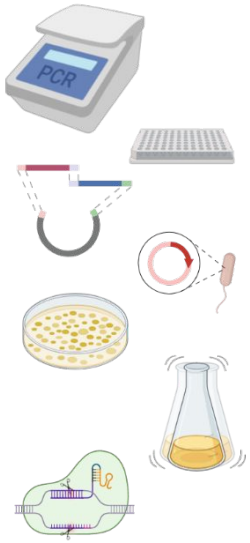
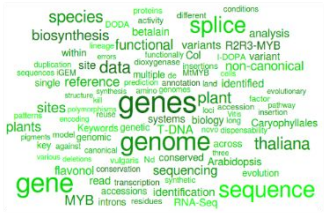
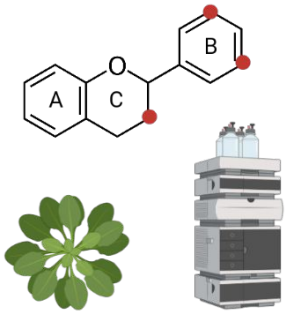
Technische Universität Braunschweig






Plant Biotechnology and Bioinformatics

# Biotechnology

Prof. Dr. Boas Pucker (Plant Biotechnology and Bioinformatics)

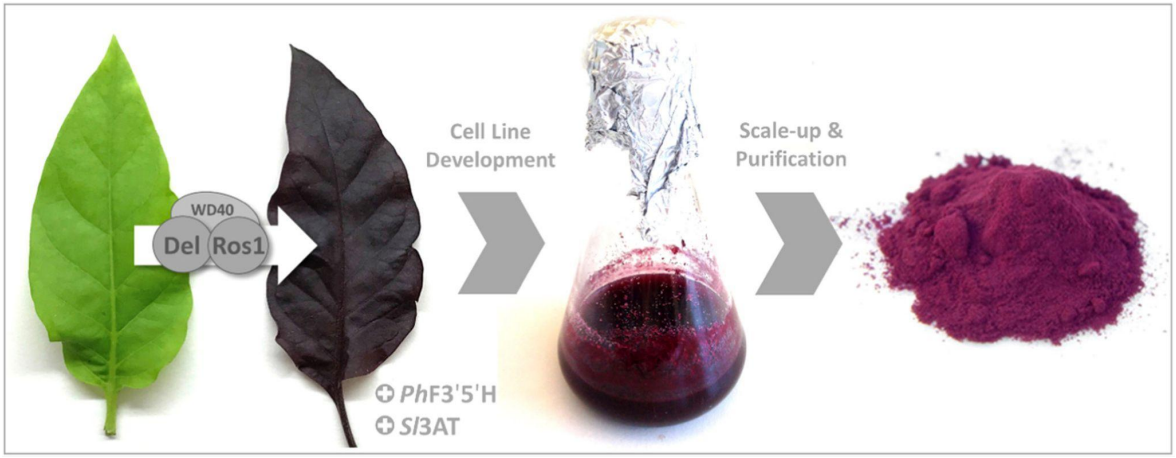
# Availability of slides

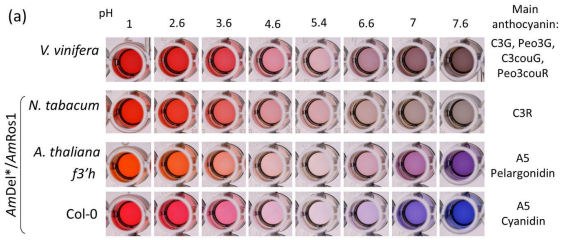
- All materials are freely available (CC BY) - after the lectures:
  - StudIP: LMChemBSc12
  - GitHub: https://github.com/bpucker/teaching

- Questions: Feel free to ask at any time

- Feedback, comments, or questions: b.pucker[a]tu-bs.de

# Producing food colorants

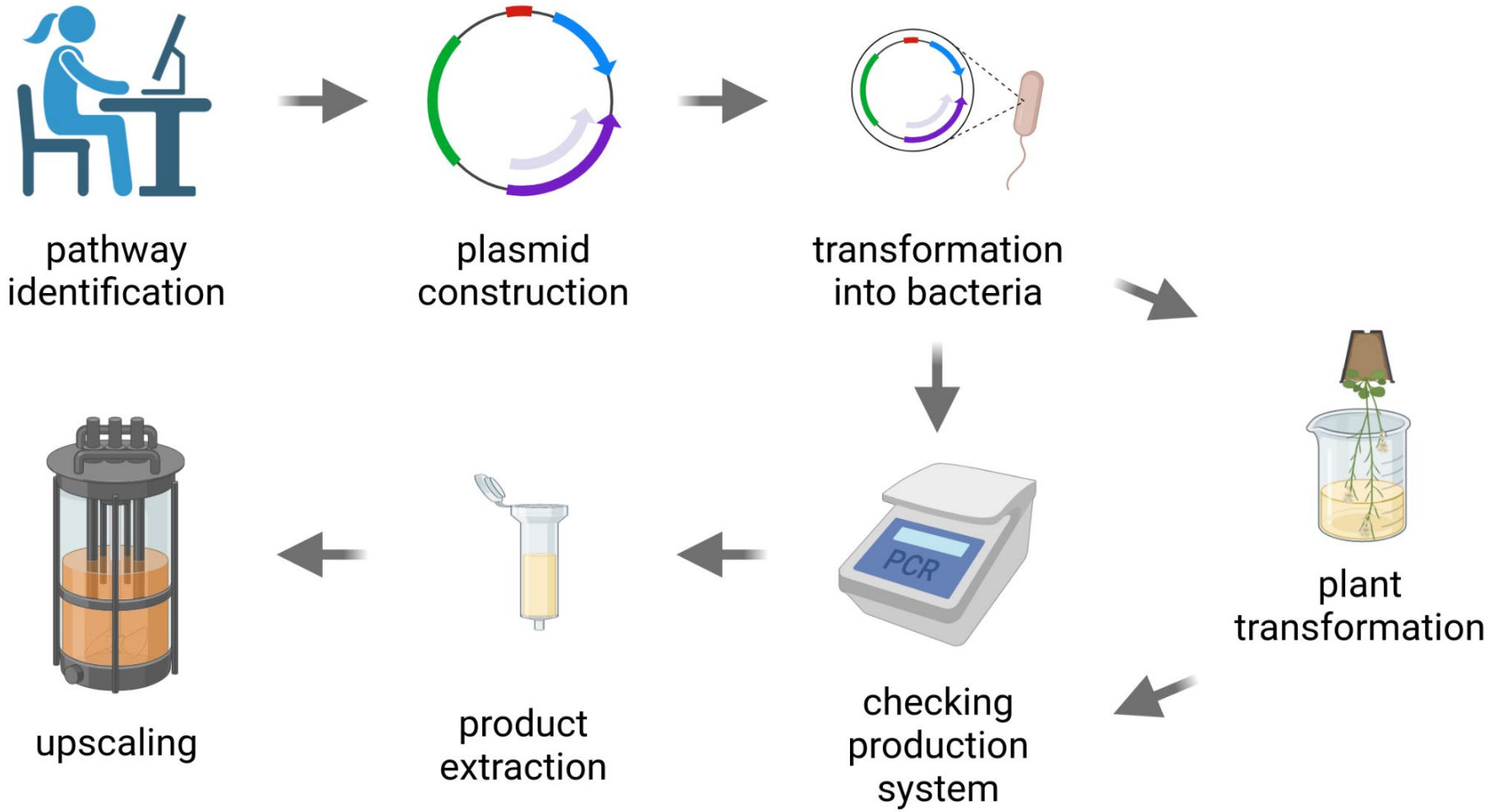


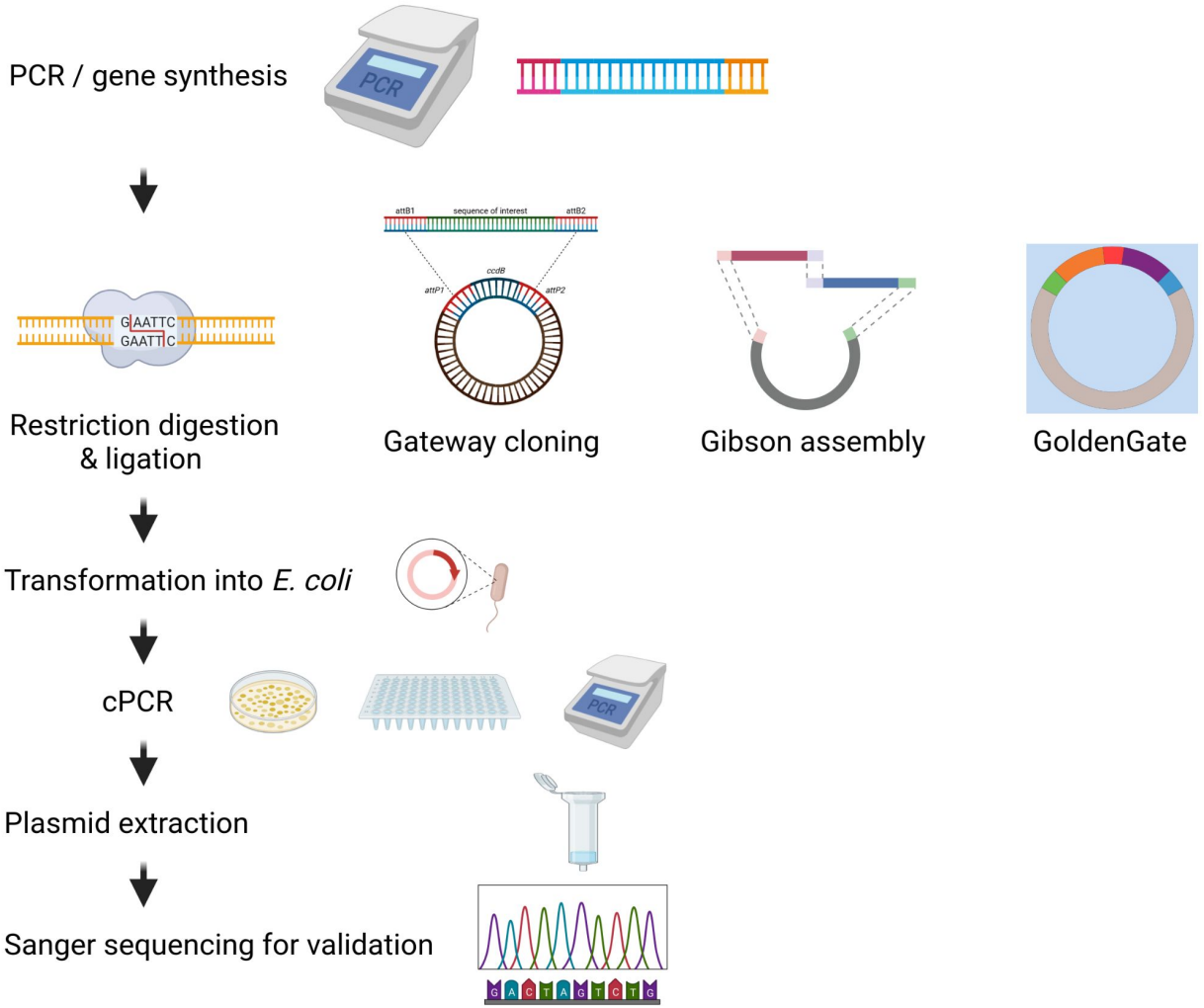

(b)

Appelhagen, 2018: 10.1016/j.ymben.2018.06.004

# Cloning genes for heterologous expression



# Plasmid construction

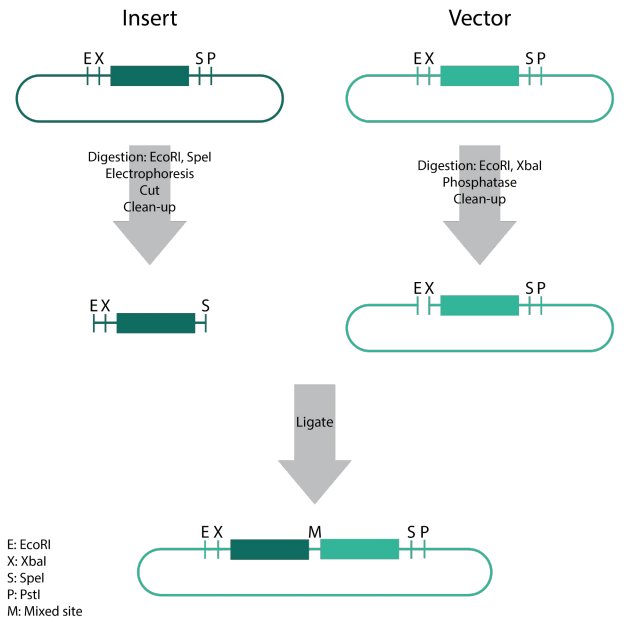


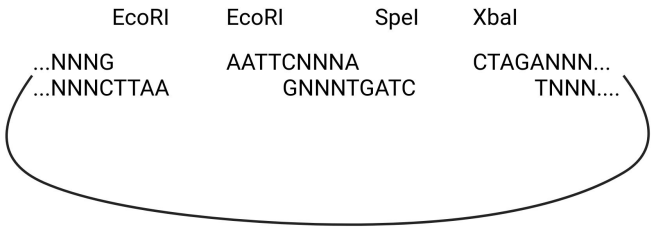
# Plasmid construction: planning the process

- Tools exist for the design of primers (e.g. Primer3Plus)
- Check if PCR can work with primers
- Cloning steps are simulated *in silico*
- Cloning workflow can be visualized
- Frequently used tools: geneious, SnapGene, CloneManger, benchling

# Plasmid construction: restriction enzymes & ligation

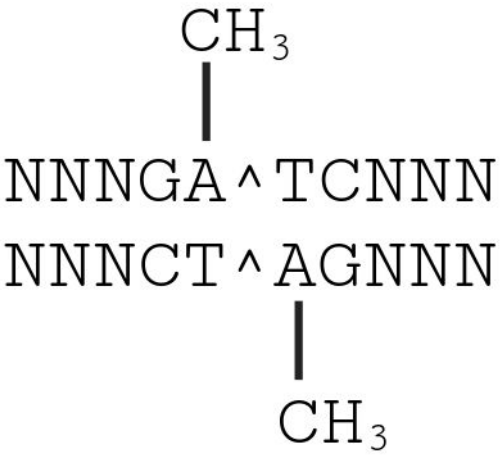
- Type II restriction enzymes (often palindromic recognition sites)
  - EcoRI: G^AATTC
  - SpeI: A^CTAGT
  - XbaI: T^CTAGA
- Oriented DNA ligation possible
- Prevention of relegation through smart combination of restriction enzymes
- Dephosphorylation of DNA fragments prevents undesired ligation



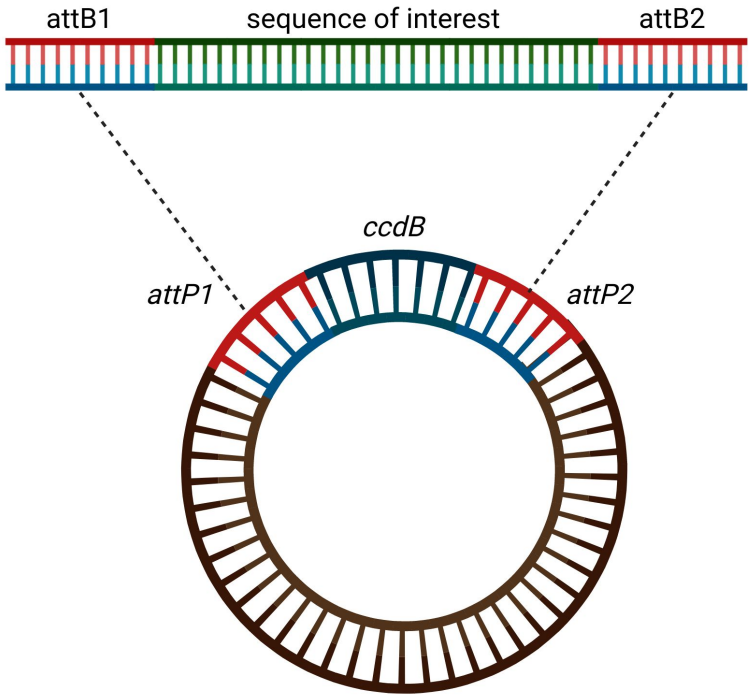
# Plasmid construction: degrading template plasmid

- Degradation of template DNA desired in many applications
- DpnI is specifically recognizing methylated DNA
- Template DNA (plasmid) is methylated; PCR product is not



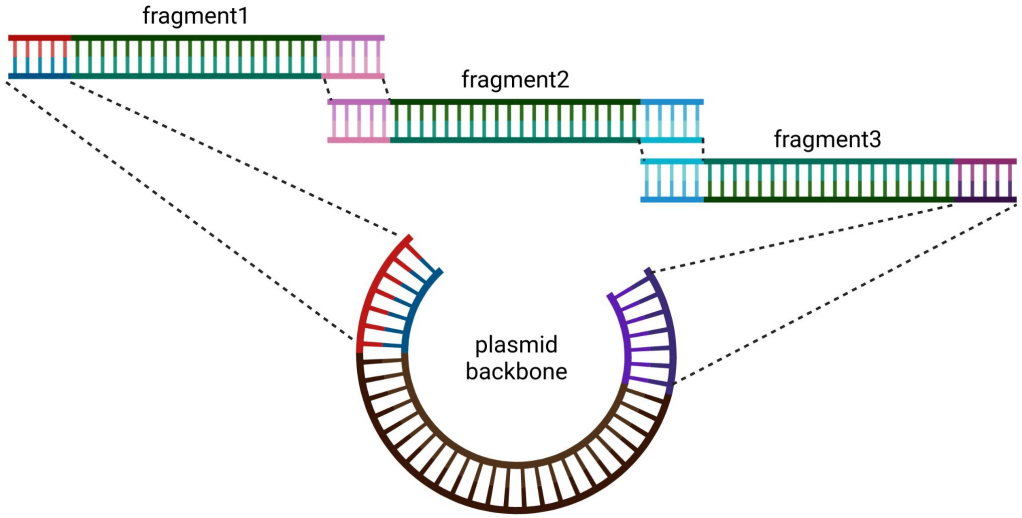
# Plasmid construction: Gateway system

- Integration of attB sites through PCR
- Homologous recombination between PCR product and plasmid
- Transfer of target sequence between plasmids
- Highly reliable system (very specific)
- Use of suizide gene (*ccdB*) to ensure high success rate



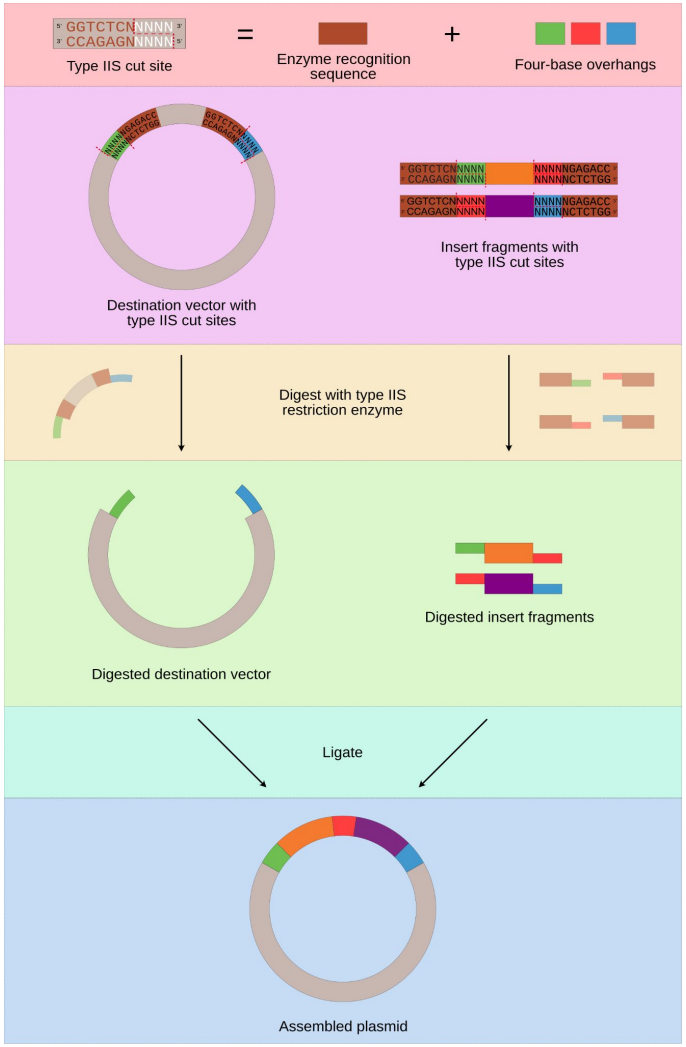
# Plasmid construction: Gibson assembly

- Combination of DNA fragments based on matching sequences
- Overlapping DNA parts can be introduced through PCR
- Advantage: no scars at combination points
- Highly flexible system
- Up to 7 fragments
- 20-30bp overlap (50°C)



# Plasmid construction: Golden Gate

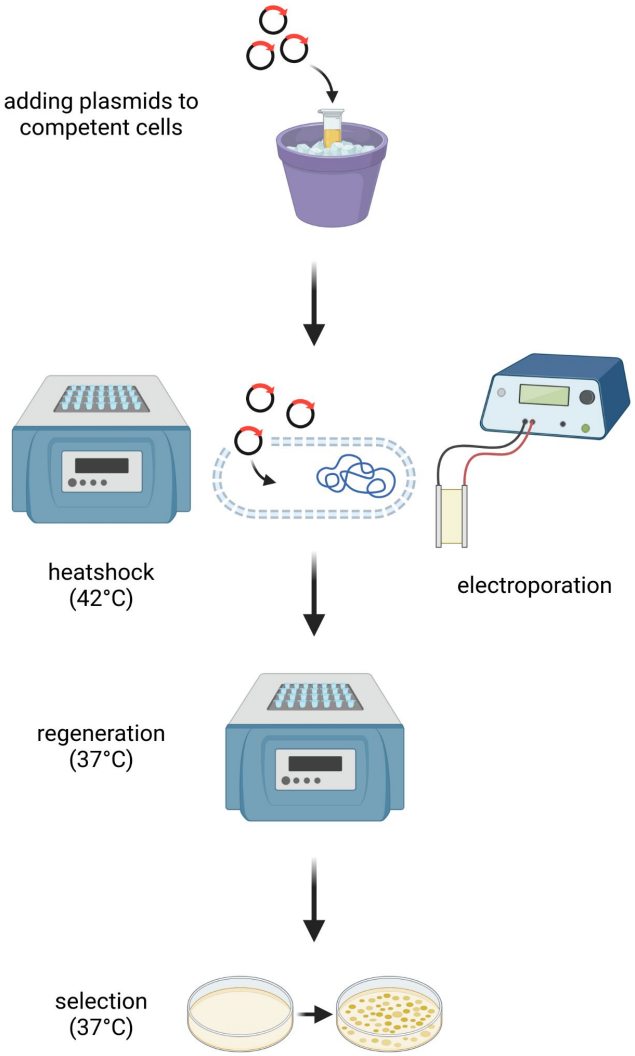
- Restriction enzymes cut outside there binding site (no scars)
- Highly reliable and efficient system
- Combination of multiple DNA parts in one reaction




Rob Hurt, CC BY-SA 4.0, https://commons.wikimedia.org/wiki/File:Golden_Gate_assembly.svg

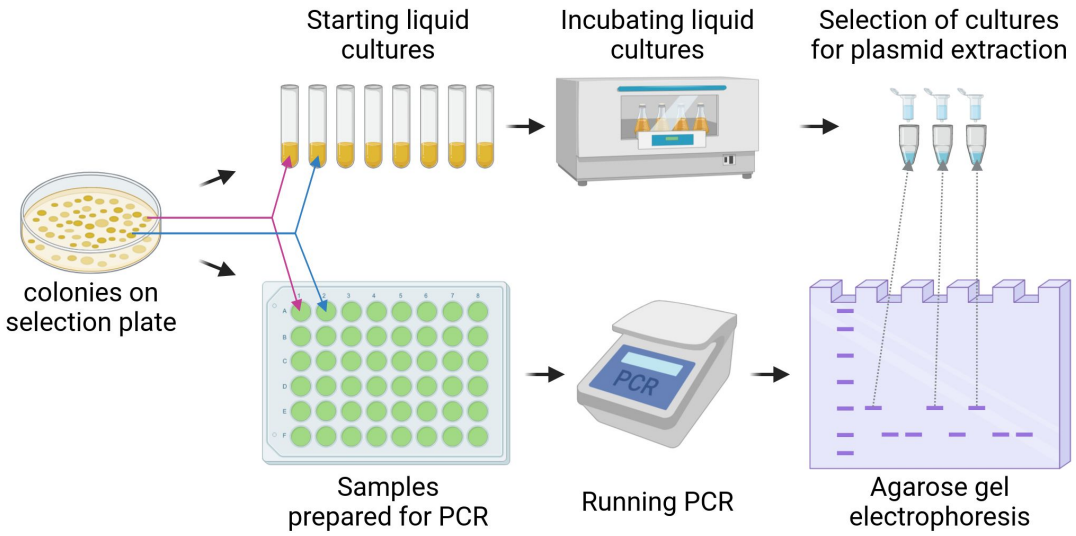
# Transformation of *Escherichia coli*

- Transfer of plasmid into bacterial cell
- Heatshock and electroporation are frequently used methods
- Electroporation is about 10x more efficient
- Preparation of competent cells required
- Cultivation at 37°C



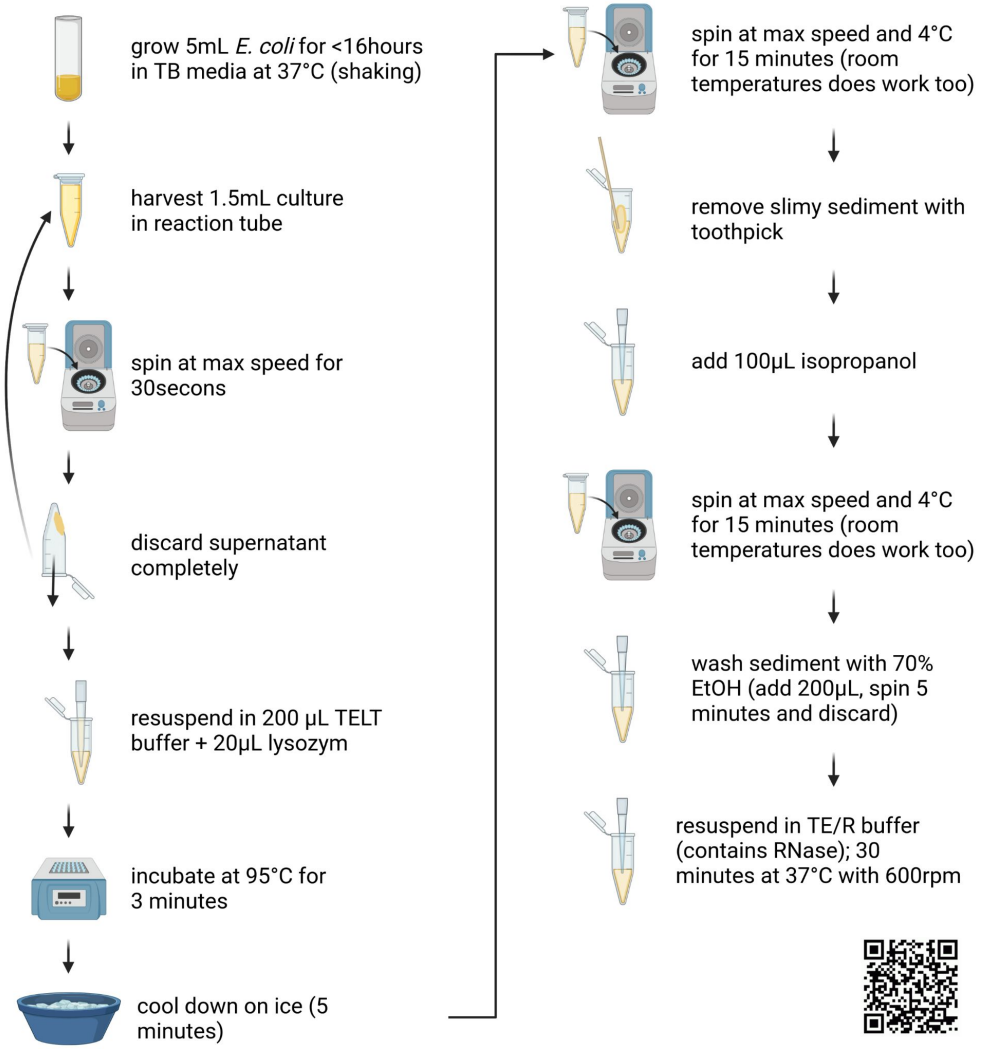
# Colony PCR (cPCR)

- Checking colonies for desired plasmid
- Bacterial cells are used as template in PCR (plasmid is released through cell lysis)
- Agarose gel electrophoresis results are connected to colonies
- Picking colonies for new plate or to start liquid cultures
- cPCR only indicates potentially positive colonies (error prone)



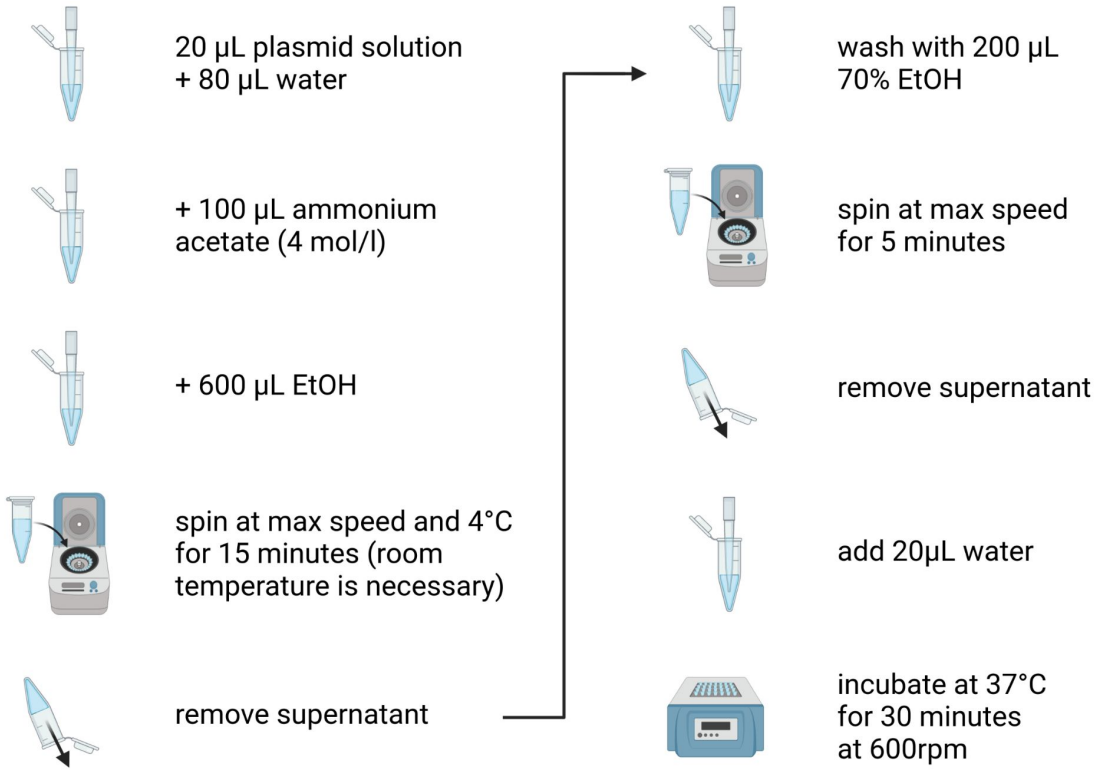
# Plasmid extraction

- Growing culture for 12-16h (over night)
- Spinning down cells of 1-5mL of sample
- Cell lysis and plasmid extraction
- Alkaline lysis vs. TELT
- Separation of plasmids from chromosomal DNA
- Removal of RNA, proteins and other contaminants



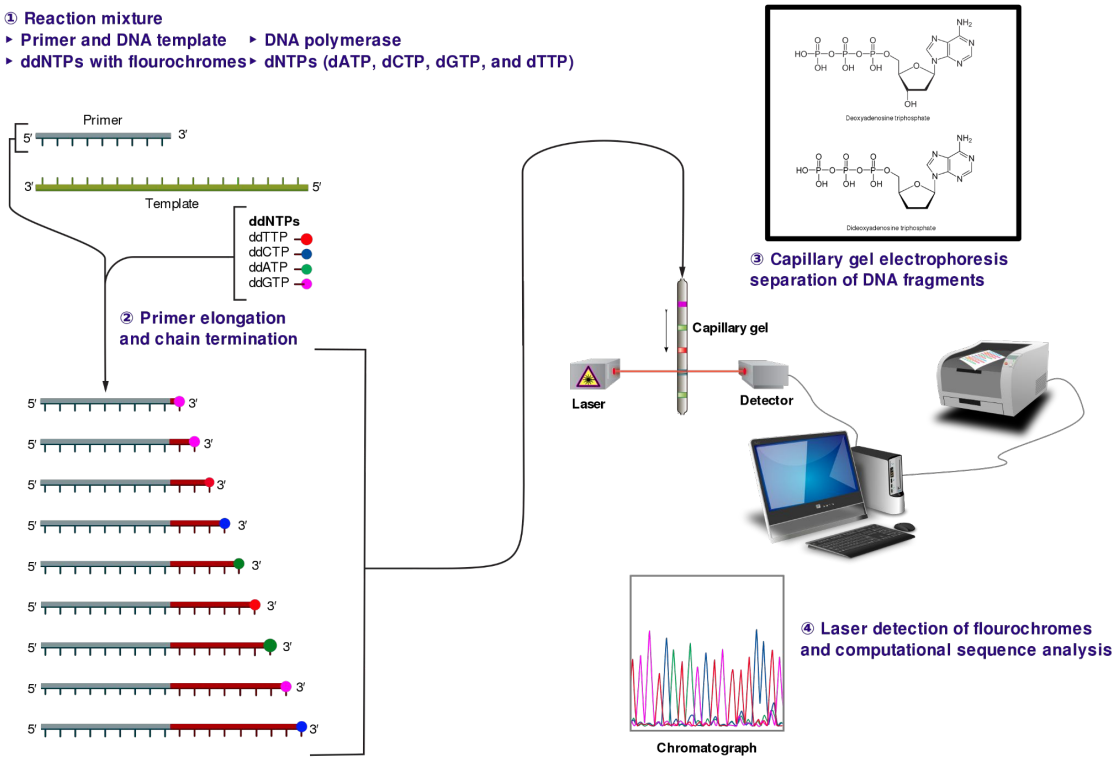
# Plasmid DNA purification for sequencing

- Sanger sequencing is important method for validation of plasmids
- Successful sequencing requires the removal of contaminants
- DO NOT use TE buffer in the final resuspension step, because EDTA will block the sequencing reaction



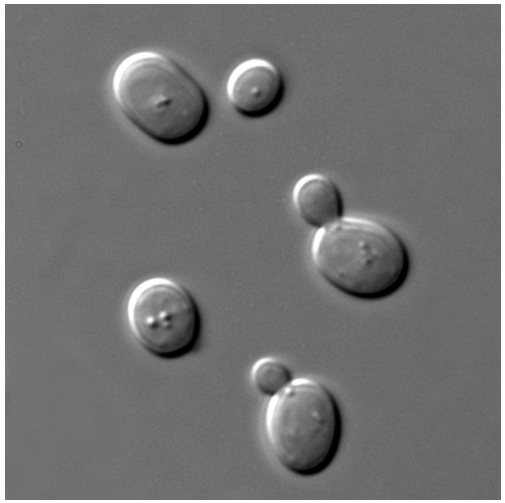
# Sanger sequencing validation

- Synthesis of DNA strand until ddNTP integration
- Different ddNTPs are fluorescently labeled
- Separation of generated DNA fragments by length
- Electropherogram reveals DNA sequence



# Transformation of yeast

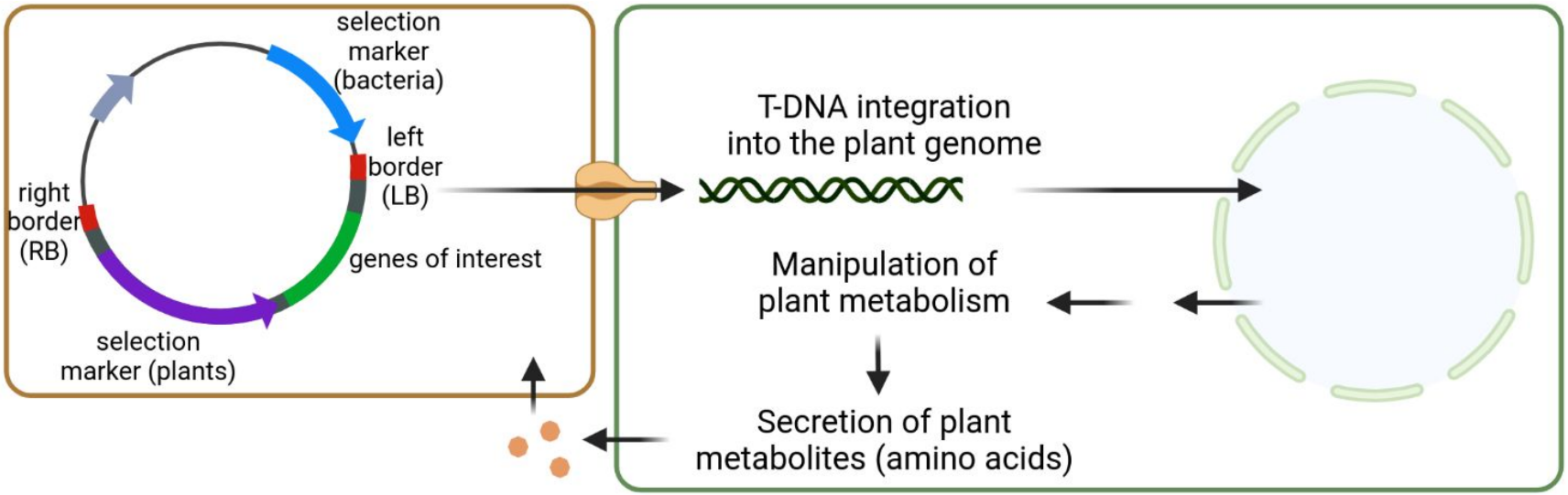
- Preparation of competent cells required
- Electroporation of cells for transformation
- Eukaryotic cells require different selection markers: complementing auxotrophy
- Cultivation at 30°C



https://en.wikipedia.org/wiki/Yeast#/media/File:S_cerevisiae_under_DIC_microscopy.jpg

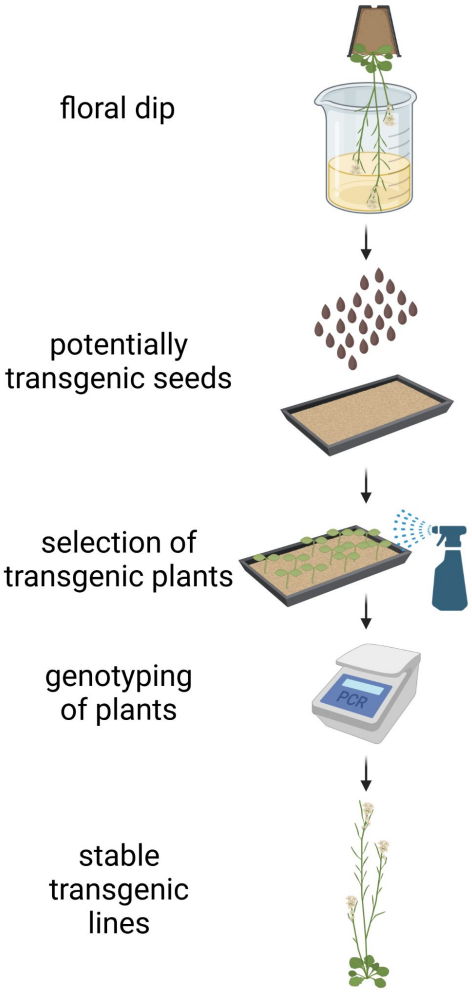
# Transformation of *Agrobacterium tumefaciens*

- Agrobacterium serves a shuttle for DNA into plants
- Preparation of competent cells for electroporation (or heat shock)
- Cultivation at 28°C; Multiple selection markers for plasmids and strain



# Transformation of *Arabidopsis thaliana*

- Floral dip: dipping *A. thaliana* flowers into *A. tumefaciens* suspension
- T-DNA transfer from *A. tumefaciens* into *A. thaliana* genome
- Selection of transgenic seeds based on herbizide or antibiotic resistance (stable transgenic lines)
- Genotyping by PCR or long read sequencing



# Transformation of *Nicotiana benthamiana*

- Leaf disk transformation allows high throughput
- *A. tumefaciens* transfers T-DNA into leaf cells
- Only transient transfection; no stable transgenic lines

https://en.wikipedia.org/wiki/Nicotiana_benthamiana#/media/File:Nicotiana_benthamiana_plant.jpg

# Heterologous protein production

- Producing plant proteins in *E. coli* is easiest approach (does not always work)
  - Low cultivation temperature reduces protein biosynthesis and supports folding

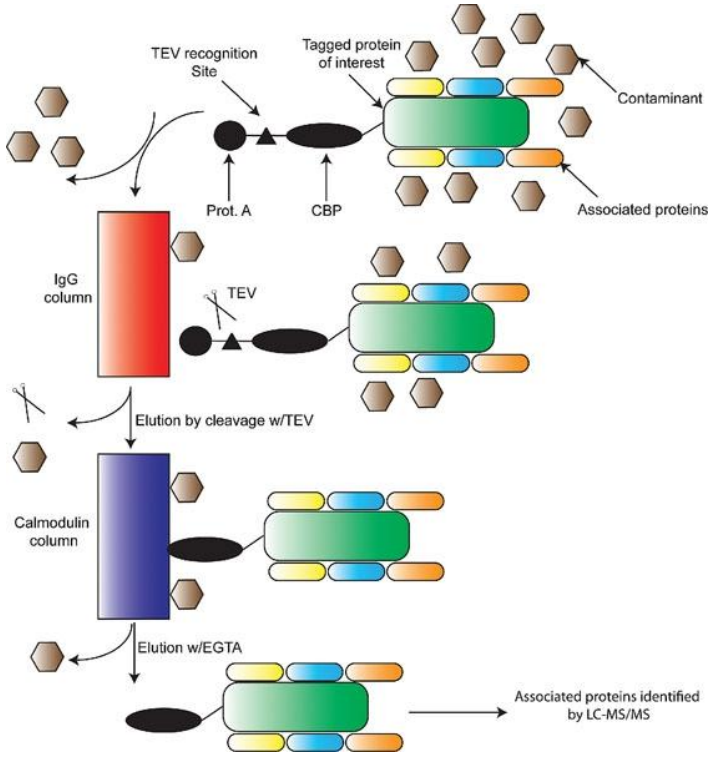
- Yest would be the next complex expression host
  - Eukaryotic modifications possible
  - Eukaryotic compartments available

- Production in plant suspension cultures / transgenic lines more challenging

# Protein extraction

- Breaking cells carefully without destroying protein of interest

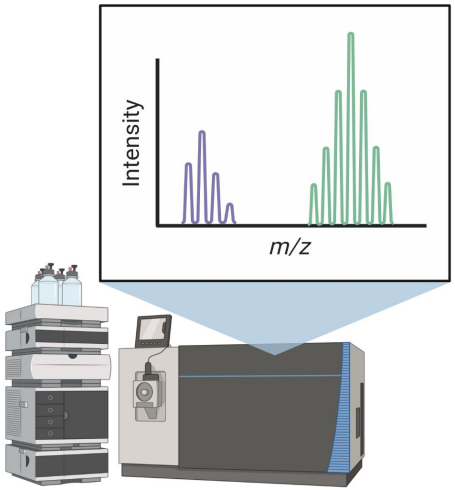
- Protein purification via tags
  - His-tag: multiple His bind to nickel on column
  - Intein: tag can be cleaved off to elute only clean protein
  - TEV protease can cleave off tags

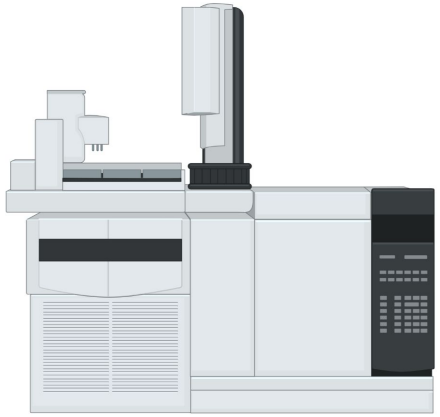



https://commons.wikimedia.org/wiki/File:Schematic_depiction_of_two_affinity_purification_approaches..jpg

# Product purification

- Extract metabolites from production system
- Example: flavonoid extraction with 80% methanol
- Analysis of compounds via HP(T)LC/GC-MS/MS
- Optimizing conditions for product extraction



# Upscaling

- Optimizing cultivation conditions for higher production
- Flask to bioreactor results in fundamentally different conditions
- Plant cultivation in field completely different from controlled conditions
- Research required to understand upscaling issues

# Summary

- Cloning / plasmid construction
- Transformation protocols
- Heterologous production
- Upscaling

# Time for questions!

# Questions

1. What are the important steps of a cloning workflow for heterologous expression?
2. Which methods can be used to construct a plasmid?
3. How is DNA transformed into *E. coli*?
4. How does a colony-PCR work?
5. Which steps are important for plasmid extraction from *E. coli*?
6. How does Sanger sequencing work?
7. How can you transform DNA into a plant genome?
8. Which methods can be applied to purify protein?