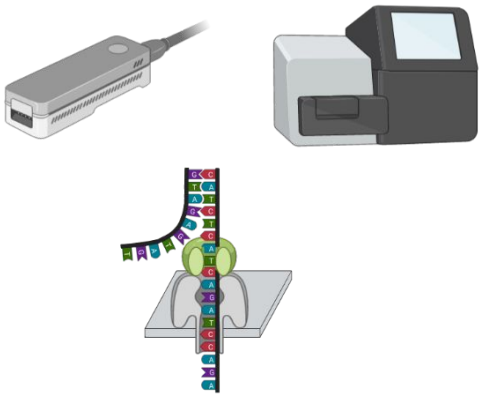
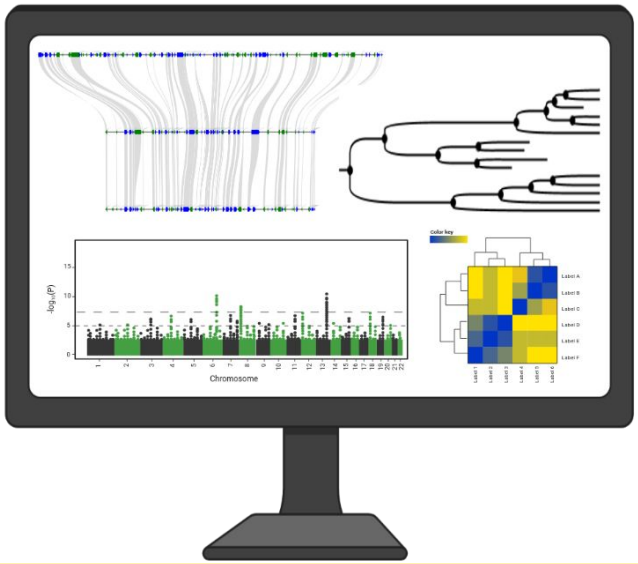
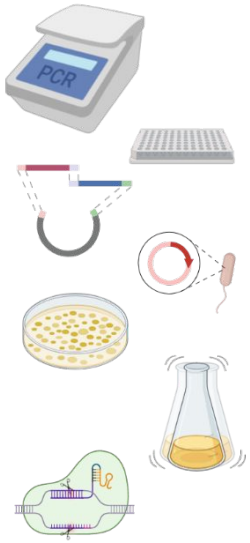
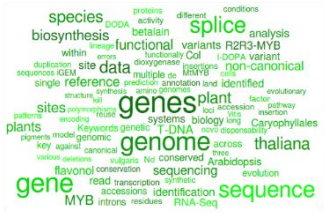
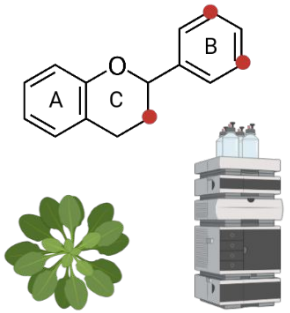
Technische Universität Braunschweig











Plant Biotechnology and Bioinformatics

RNA-seq (wet lab)

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

Availability of slides

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

- Questions: Feel free to ask at any time

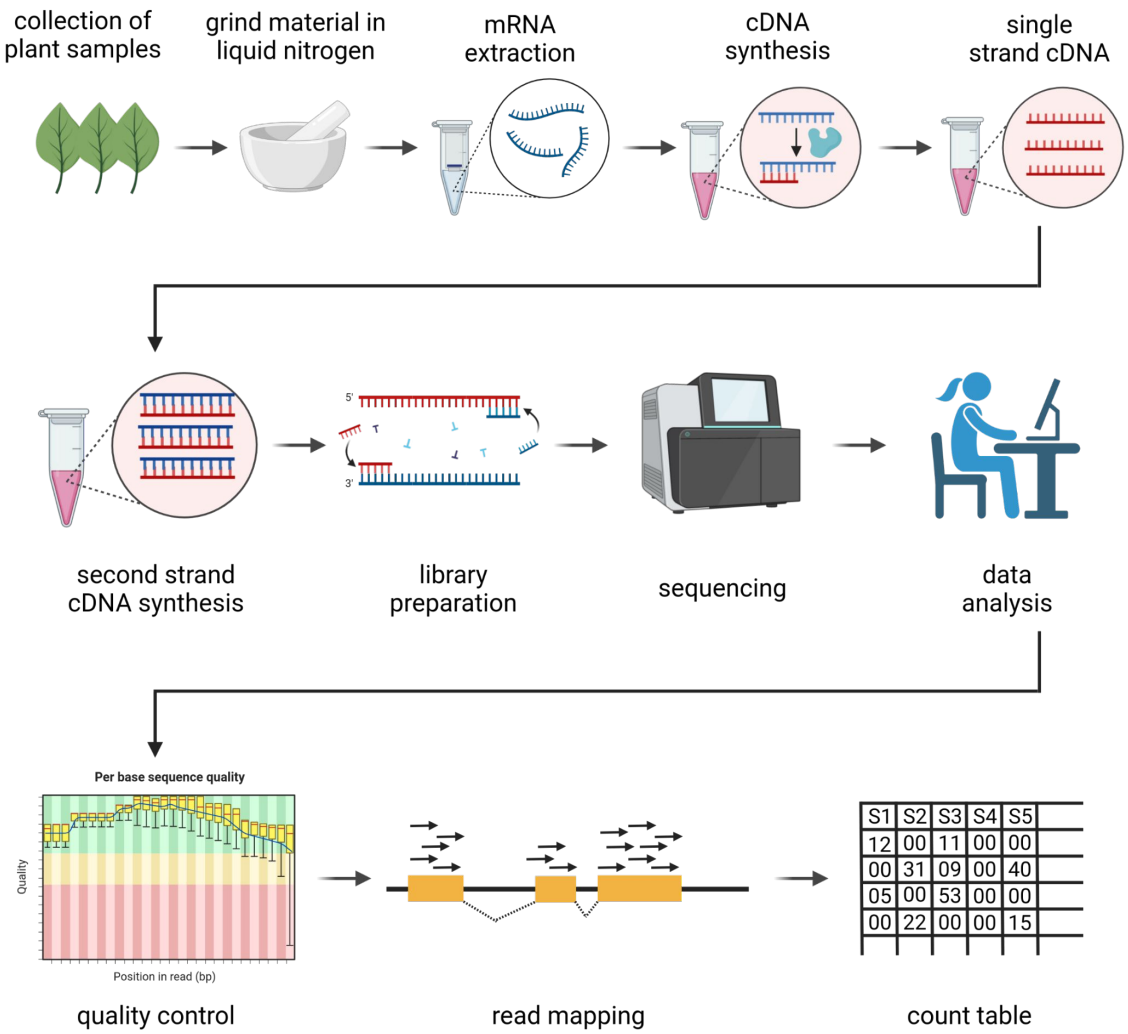
- Feedback, comments, or questions: b.pucker[a]tu-bs.de

What is RNA-seq?

What is RNA-seq?

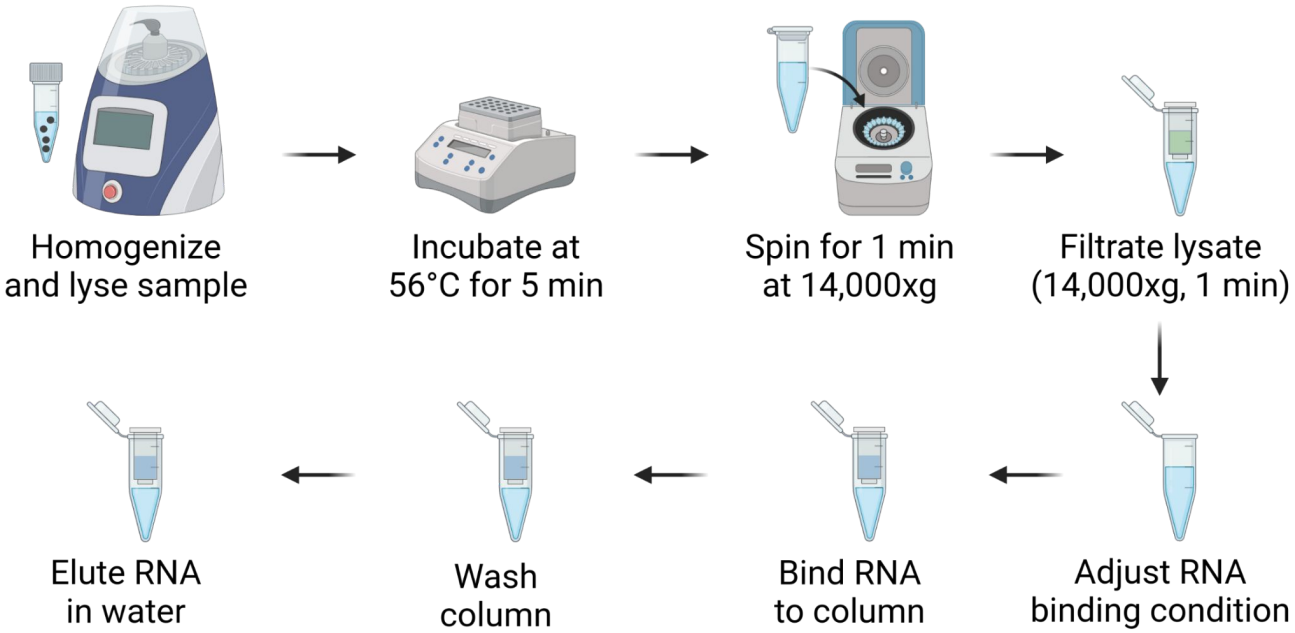
- High-throughput method to analyze gene expression
- Sequencing of cDNA fragments (NOT RNA)
- Number of reads proportional to number of transcripts (gene expression)

RNA-seq overview (simplified)



RNA extraction

- Test different protocols per species
- Effective homogenization of material is important
- Avoid contamination/activity of RNases



RNA extraction

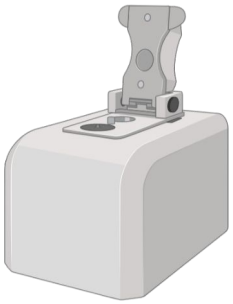
- Classical RNA extraction approaches:
  - Phenol-based methods: helpful to remove high polysaccharide contaminants
  - Trizol-based methods: commercial reagent combining phenol and guanidine isothiocyanate
  - CTAB-based methods: successive removal of polysaccharides, proteins, and specialized metabolites
- Kit-based RNA extraction methods

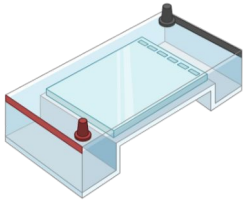
DNase treatment

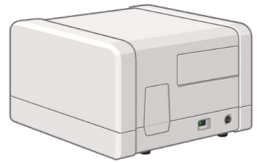
- Biochemical properties of DNA and RNA are very similar
- Extracts usually contain both nucleic acids
- Enzymatic removal of DNA with DNase treatment
- Inactivation/removal of DNase (on-column digest)

Quality assessment

- Evaluate quality through photometric measurement
- Check quality on gel
- Measure with fluorescence
- Check RNA integrity via Agilent chip





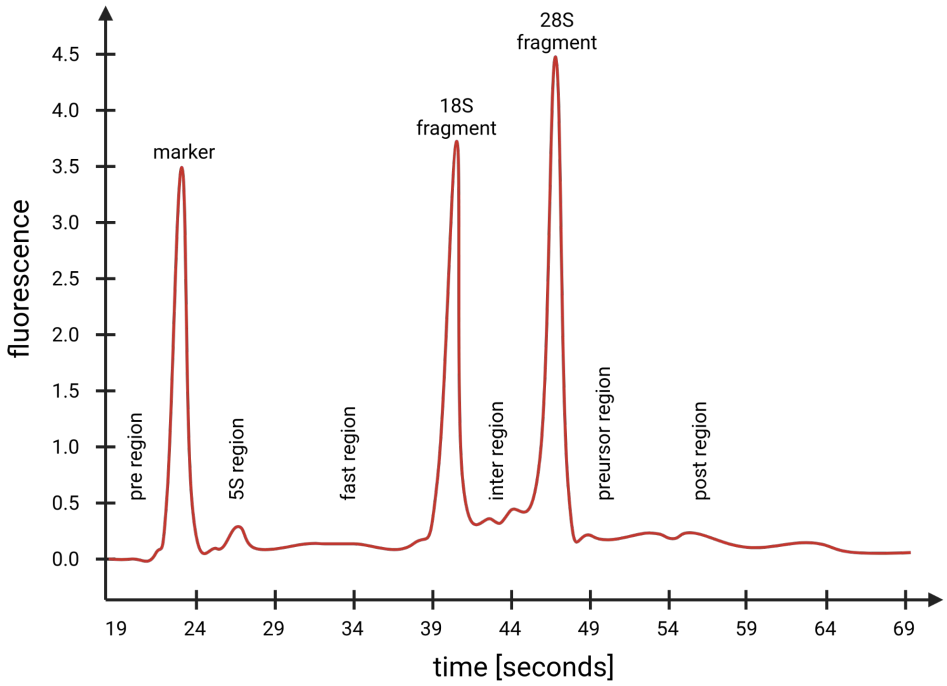


NanoDrop: photometric measurement

- Photometric measurement in tiny volumen
- No dilution required
- Analysis of RNA, DNA, and protein possible
- OD260/OD230 = contamination with small fragments or phenolic compounds
- OD260/OD280 = contamination with protein

RNA Integrity Number (RIN)

- Previously: ratio of 28S to 18S rRNA as indicator
- mRNA degrades faster than rRNA and in non-linear way
- RIN: inference of mRNA integrity based on overall RNA integrity
- Chip for analysis is like running a gel with higher resolution



Shipping

- Shipping to (international) service provider
- Dry ice (solid CO_2) is used to keep RNA samples frozen
- RNA-seq costs per sample: < $200

Depletion of rRNA

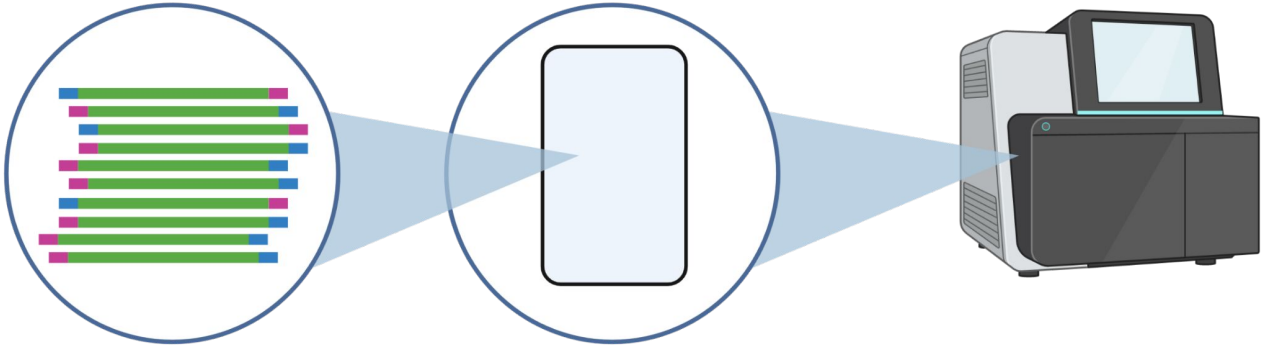
- rRNAs account for >80% of all RNAs
- rRNA probes are bound to magnetic beads
- Binding of probe to rRNA enables pull down and removal of rRNA
- Risk: pull down and removal might be incomplete

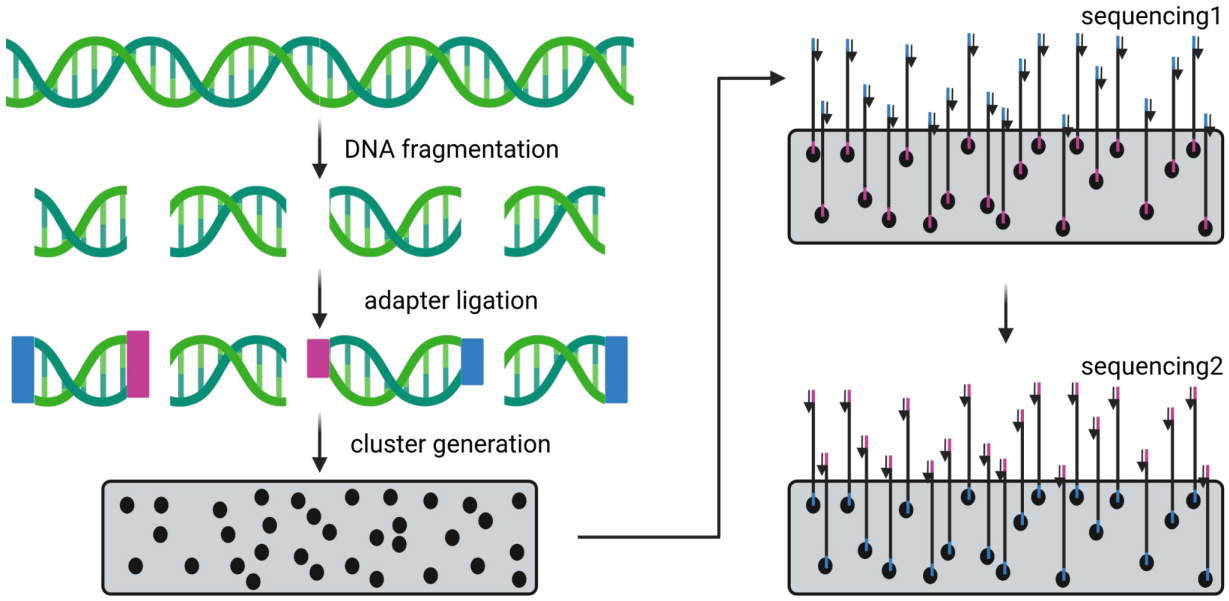
Enrichment of mRNA

- mRNAs are characterized by long poly-A tails
- Binding of mRNAs to oligo-T beads/columns
- Risk1: Degraded mRNAs (without poly-A) are lost; strong bias for 3'-end
- Risk2: Other RNAs with long A stretches might bind

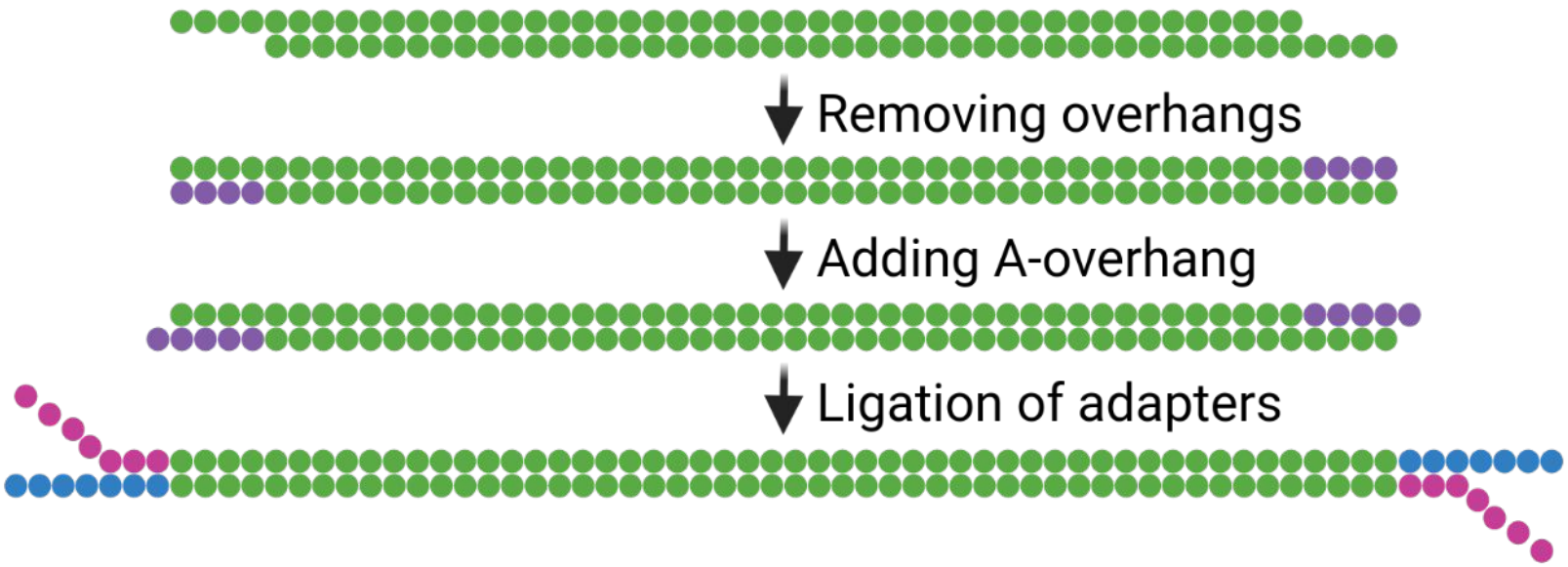
cDNA synthesis

Illumina sequencing of cDNAs

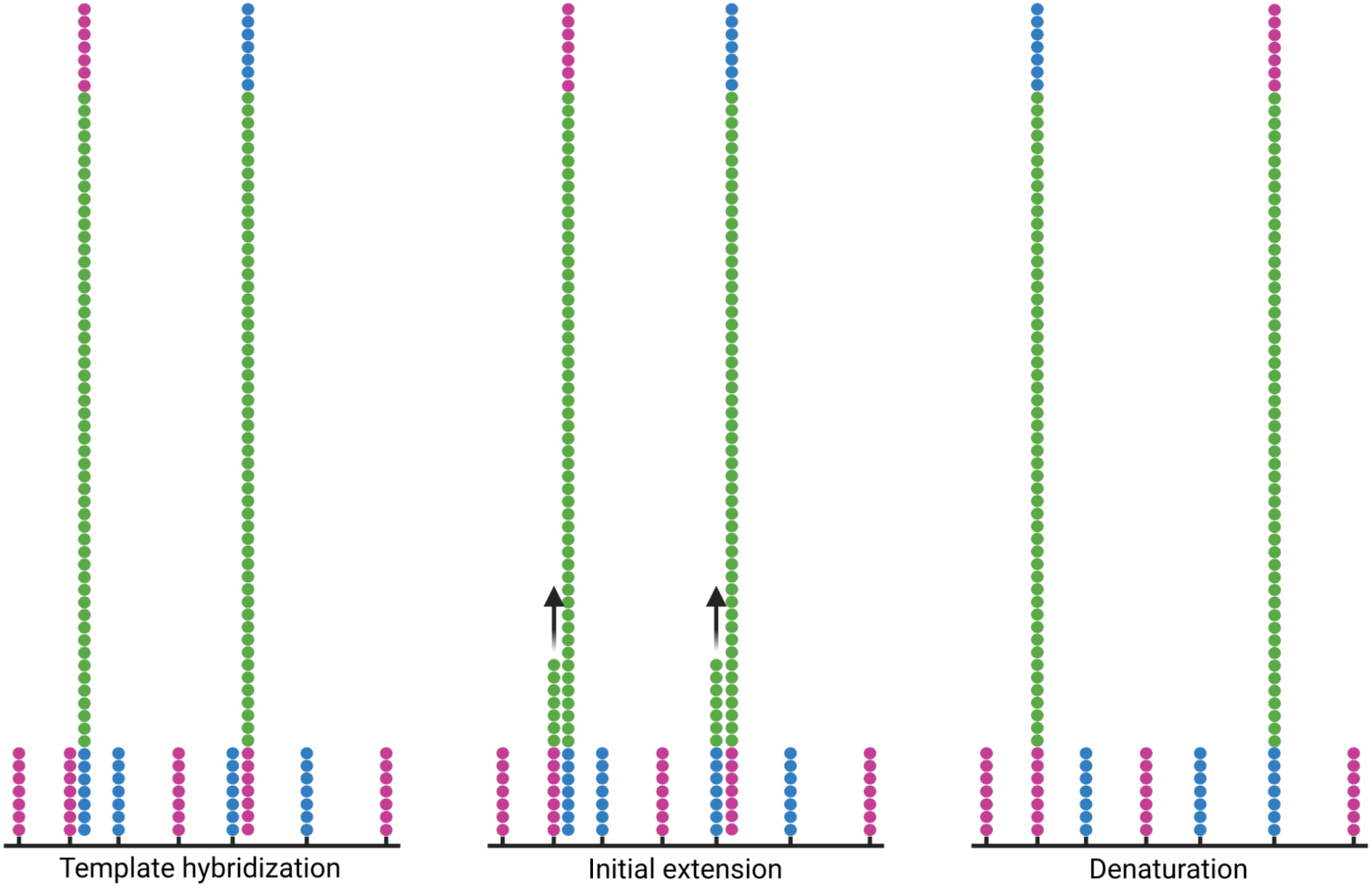




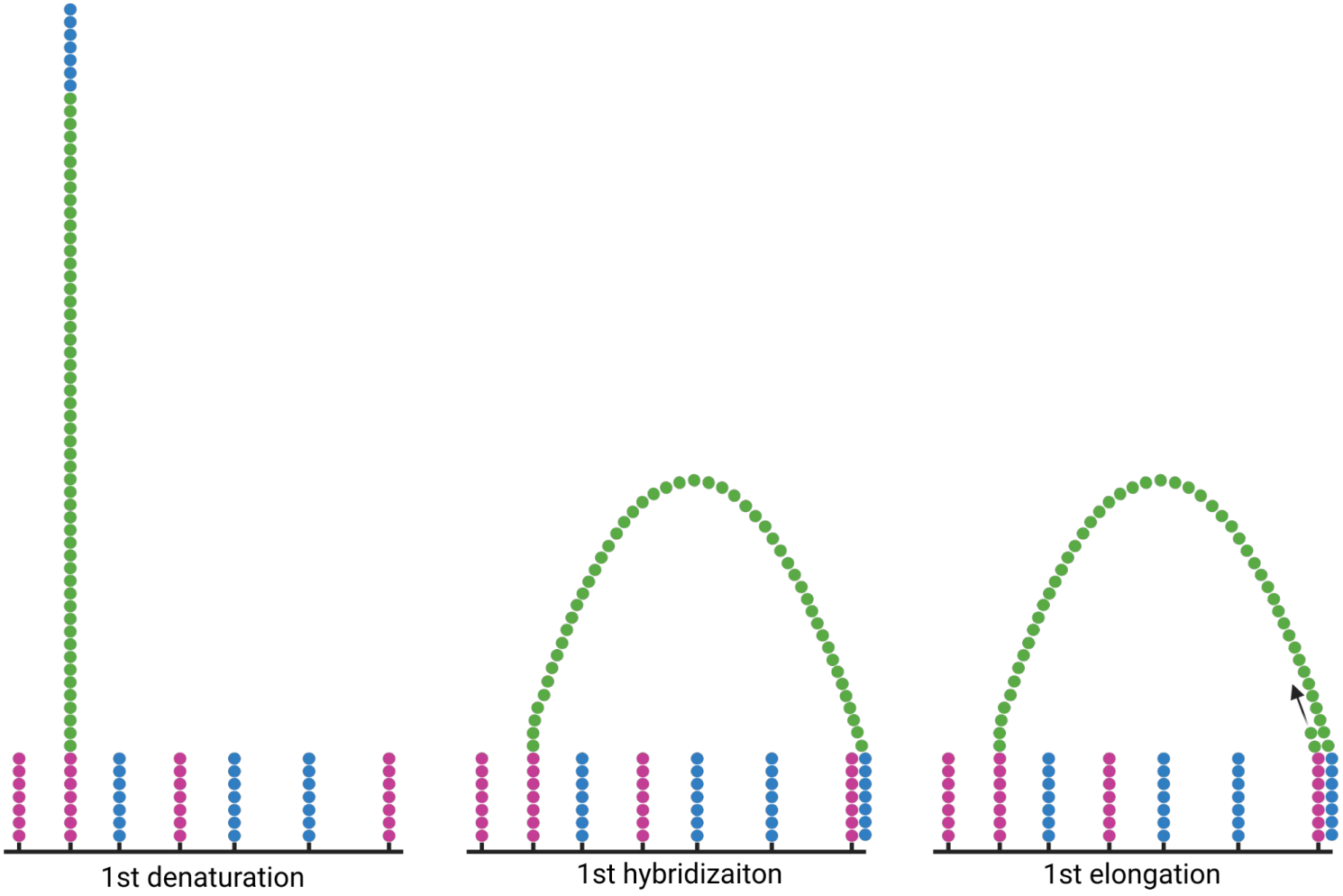
Adapter ligation (library preparation)



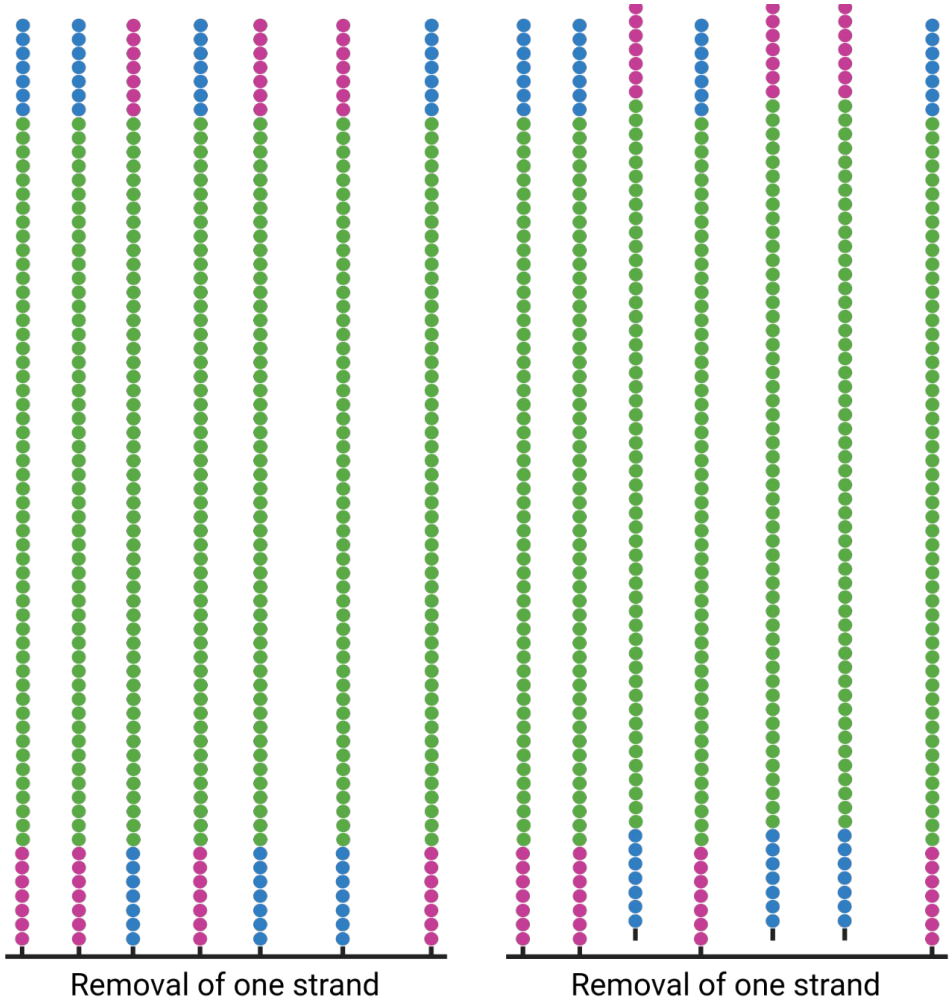
Bridge amplification - 1

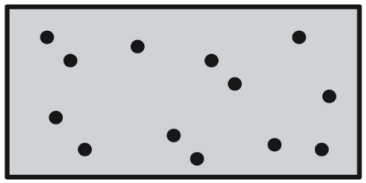


Bridge amplification - 2

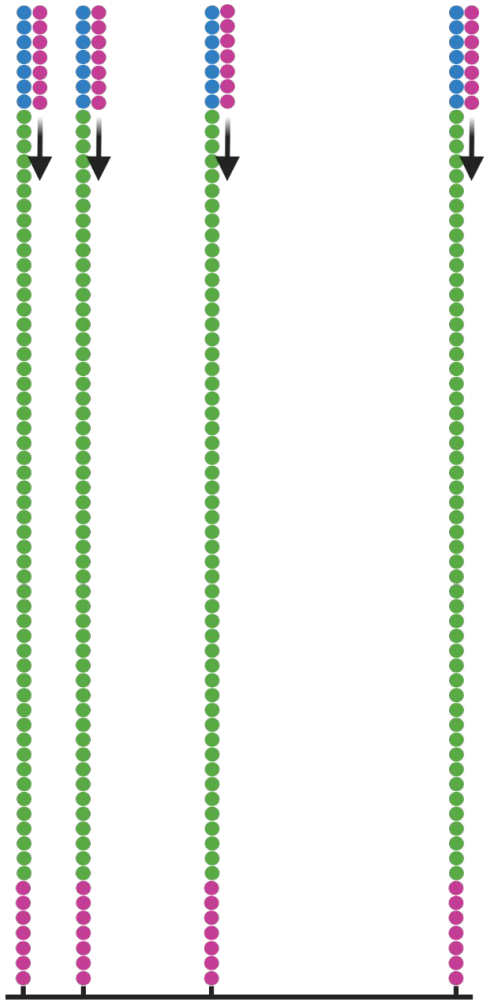


Bridge amplification - 3

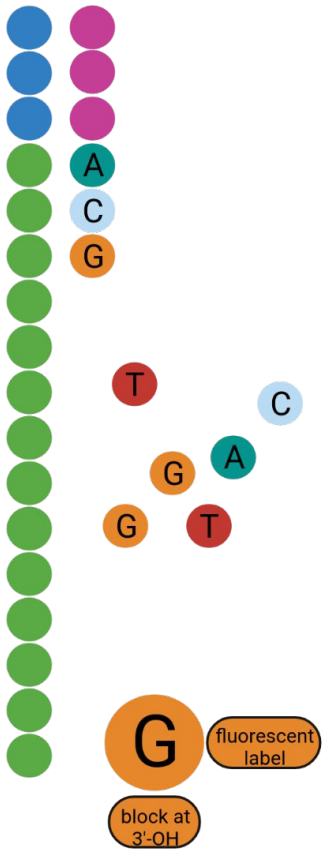



Clusters on flow cells

Bridge amplification - 4



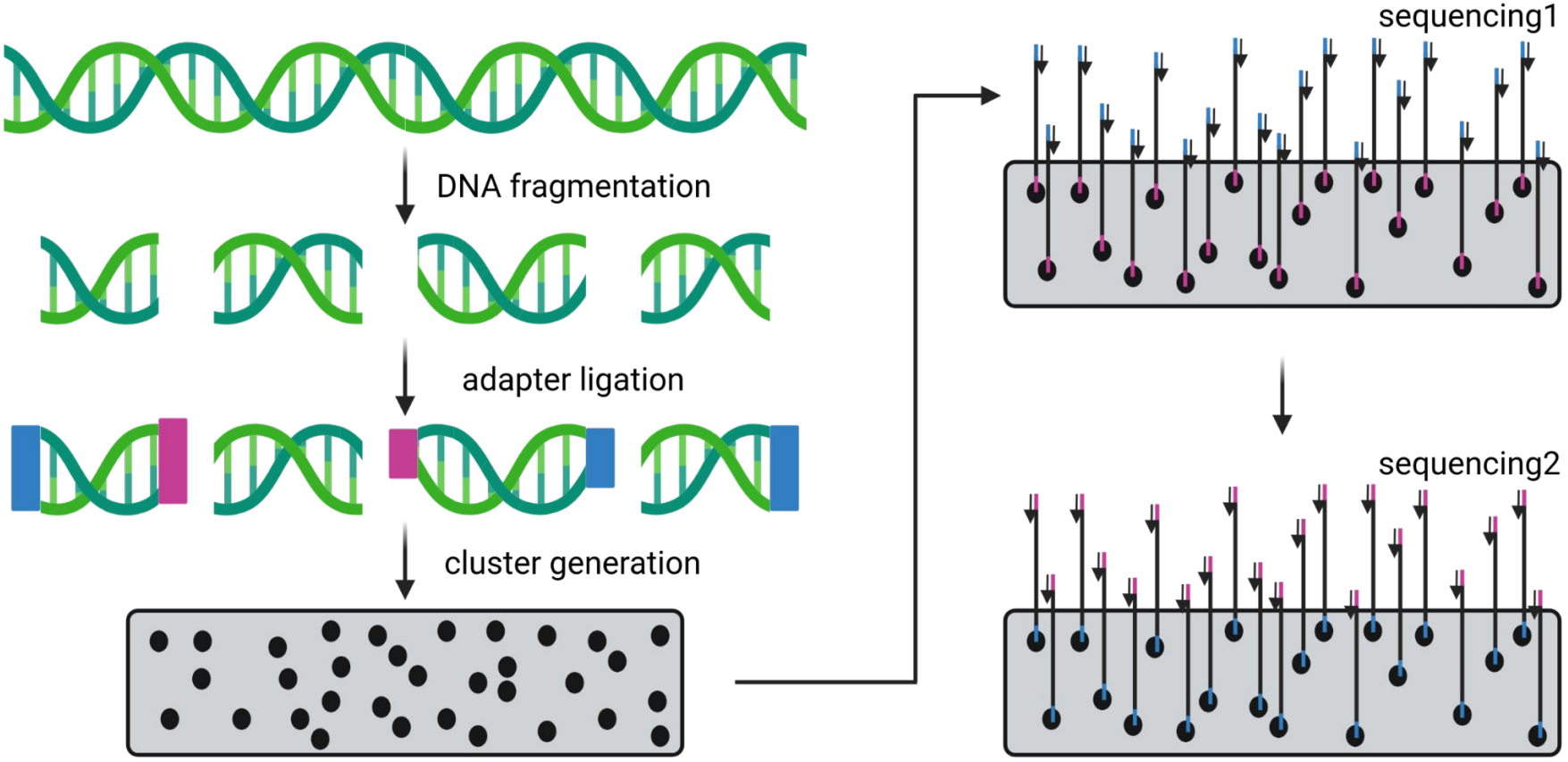
Sequencing



Sequencing chemistry

INCLUDE SLIDE ABOUT cluster picturing here

Illumina sequencing summary

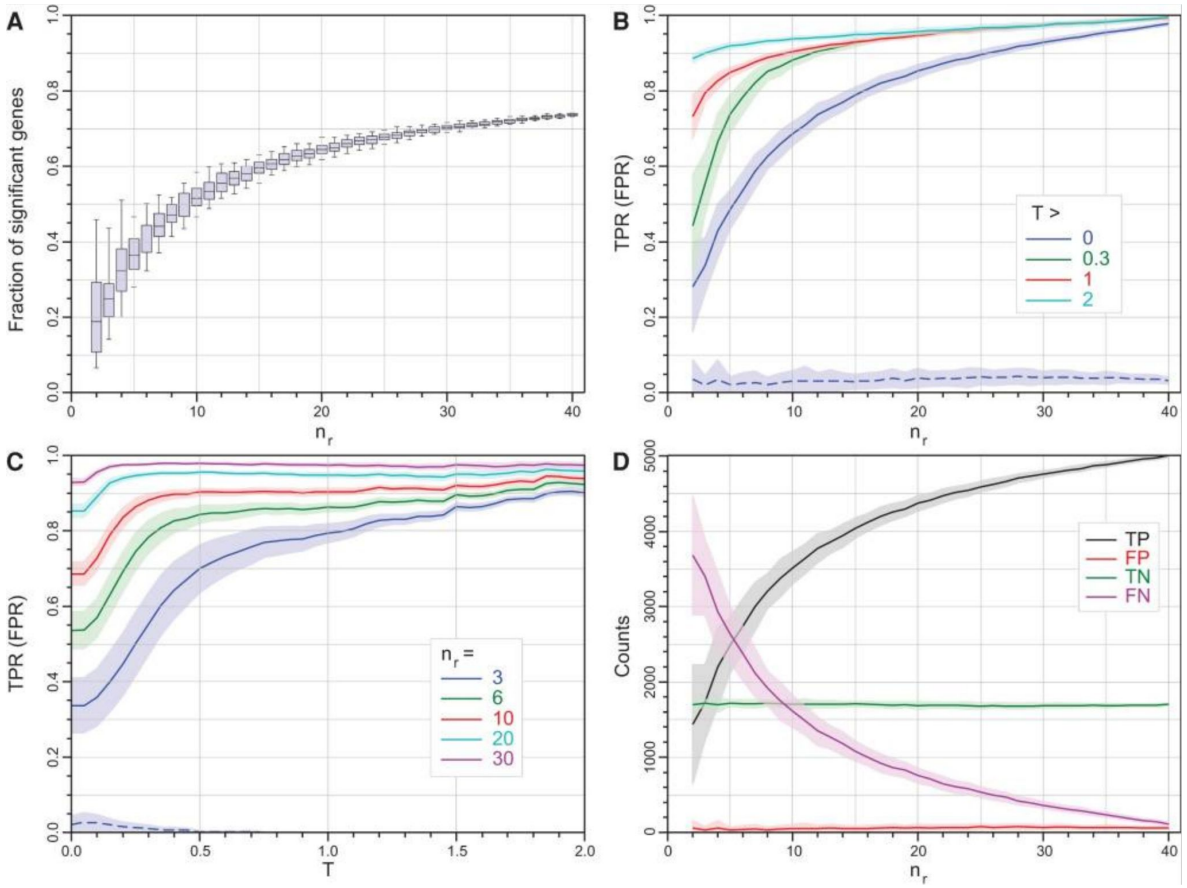


Experimental design

- Number of replicates determines power of analysis
- Experiments can have one or multiple factors (e.g. time, temperature, infection)
- Genotypes of individuals must not introduce a bias

Replicates vs. sequencing depth

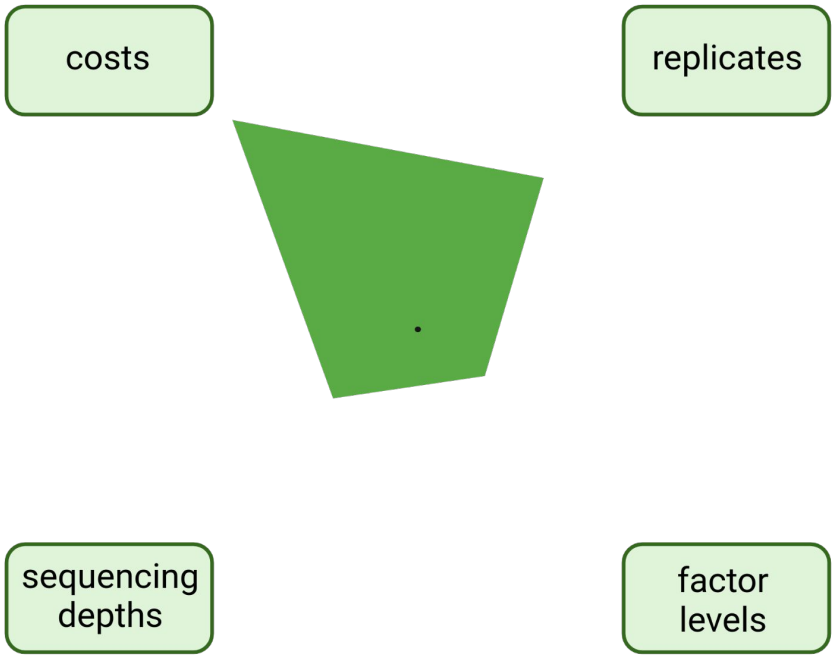
- Number of replicates determines power of analysis
- 3 biological replicates is considered minimum
- 12 biological replicates are recommended
- Technical replicates are not required for RNA-seq



Schurch et al., 2016: 10.1261/rna.053959.115

Trade offs

- Limited resources determine experimental setup
- Trade-offs between costs, factor levels, number of replicates, and sequencing depth



Importance of growth conditions

- Transcriptome responds to environmental conditions
- Development might be ongoing
- Conditions need to be the same for all replicates
- Regular rotation of study objectives avoids position bias; randomized positioning
- Precise documentation of temperature, humidity, provided substrate, infections, …

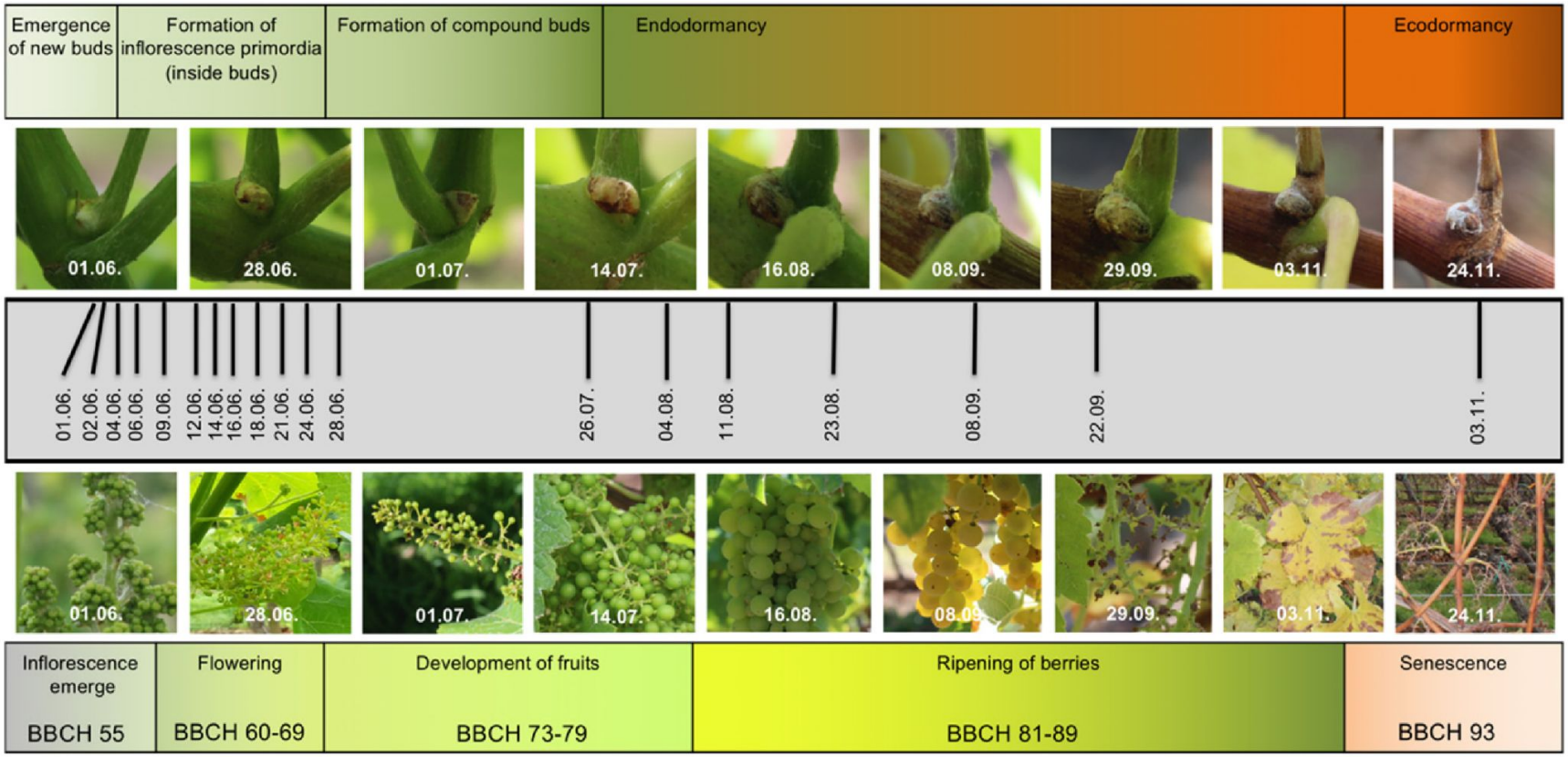
Nagoya protocol & ABS laws

- Original intention of Nagoya protocol was to protect genetic resources
- Currently one of the biggest obstacles to research projects
- German plants can be studied everywhere in the world
- Plants from abroad might require additional permissions and registration
- Getting permissions is often complicated
- Solution: only work with ‘free plants’

Phenotyping

- RNA-seq is often performed to investigate trait
- Phenotypic information about samples is important
- Phenotype examples:
  - presence of certain metabolite
  - visible color
  - morphological structure
  - resistance against pathogens

Example: time course experiments



https://doi.org/10.3390/plants9111548

Time for questions!

Questions

1. What are the important steps of an RNA-seq workflow?
2. Which methods are available for RNA extraction from plant samples?
3. How to check the quality of RNA?
4. What is ‘RIN’ and how is it used?
5. What are the important steps of Illumina sequencing?
6. Which factors should be considered when planning an RNA-seq experiment?