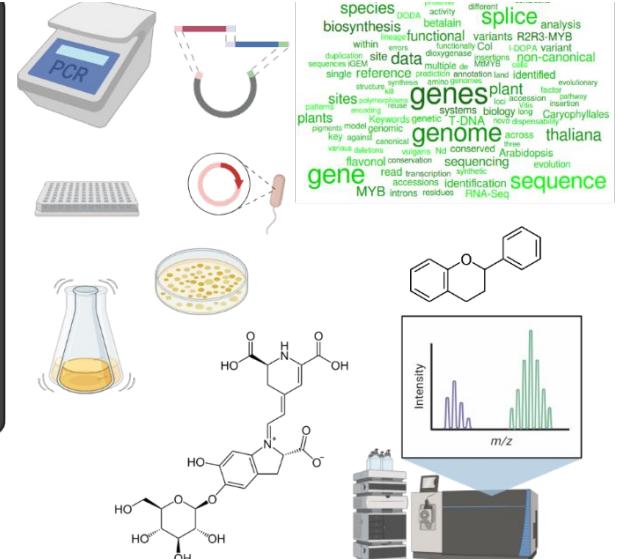
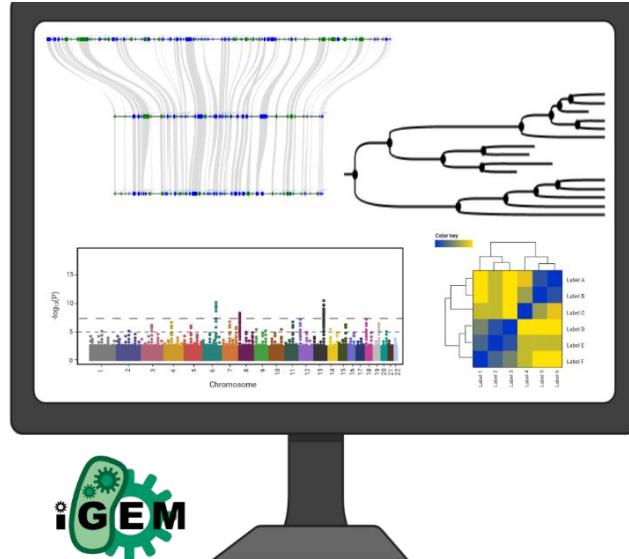
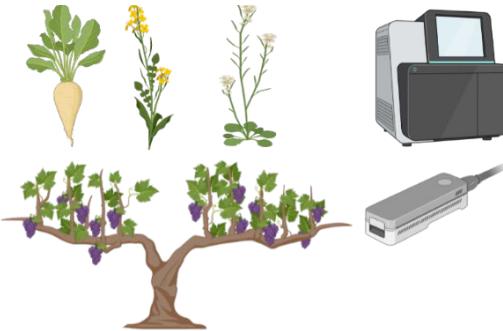




Technische
Universität
Braunschweig



Gene expression analysis

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

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What causes these pigmentation patterns?



What is the transcriptome?



What is the transcriptome?

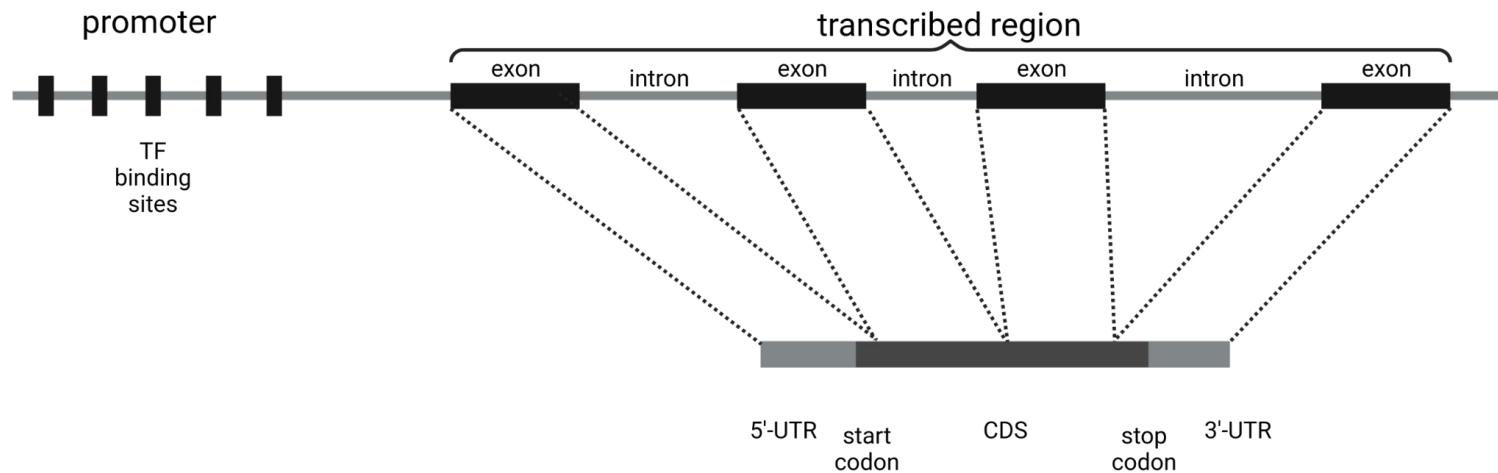
- “The set of all transcripts and their abundances in a defined cell/tissue/organism under defined conditions at a certain time point.”
- Transcriptome is highly variable over time
- Transcriptome responds to various stimuli

What is a gene? What are the features of a gene?

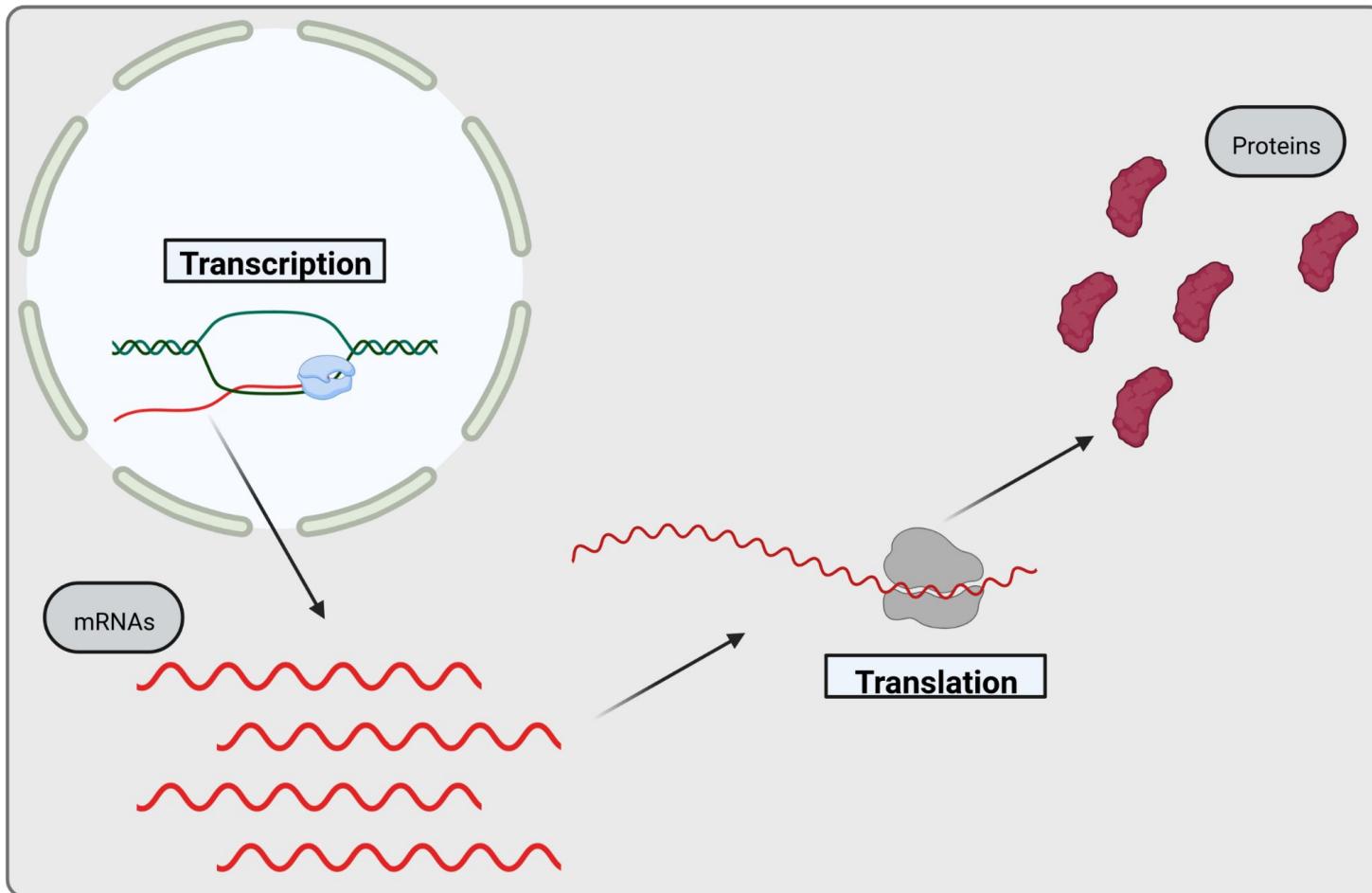


What is a gene?

- No perfect and universal definition
- Restrict to protein coding gene in plants:
 - promoter region
 - UTRs
 - coding sequence
 - introns



Protein biosynthesis (overview)



Types of RNA

- rRNA (about 80%)
- tRNA (about 10-15%)
- mRNA (about 1-5%)
- Various regulatory RNAs



RNA extraction



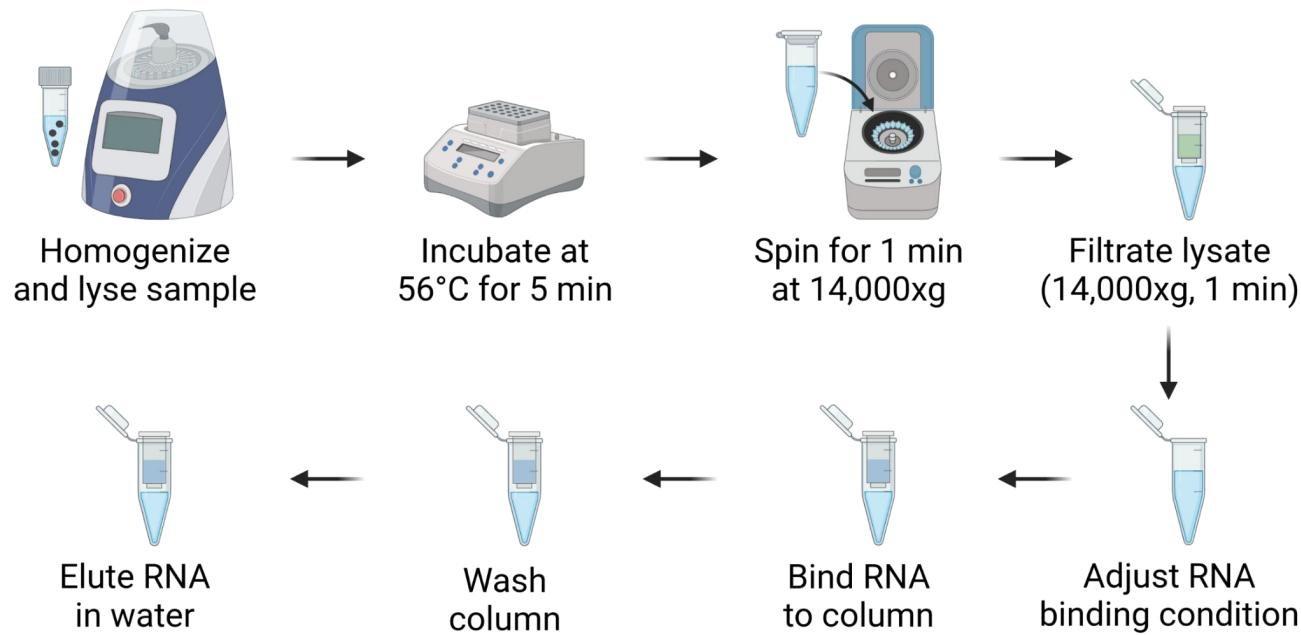
RNA extraction

- 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 (silica spin columns)



RNA extraction

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

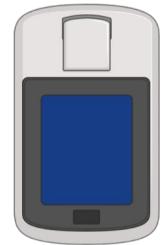
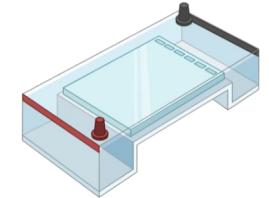
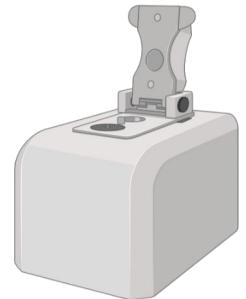


DNase treatment

- 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



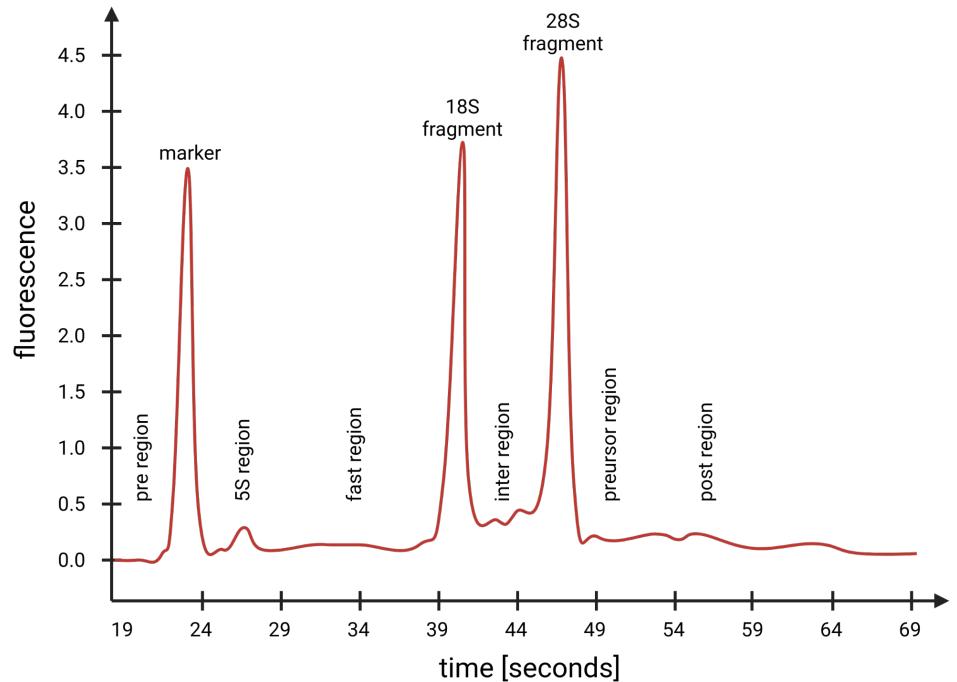
NanoDrop: photometric measurement

- 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



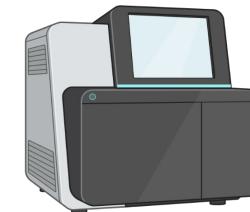
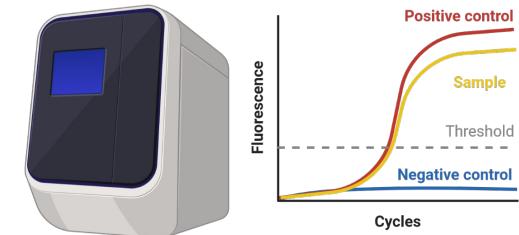
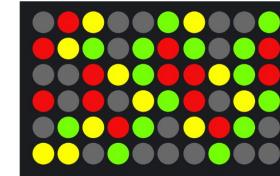
Gene expression quantification

How to measure gene expression?



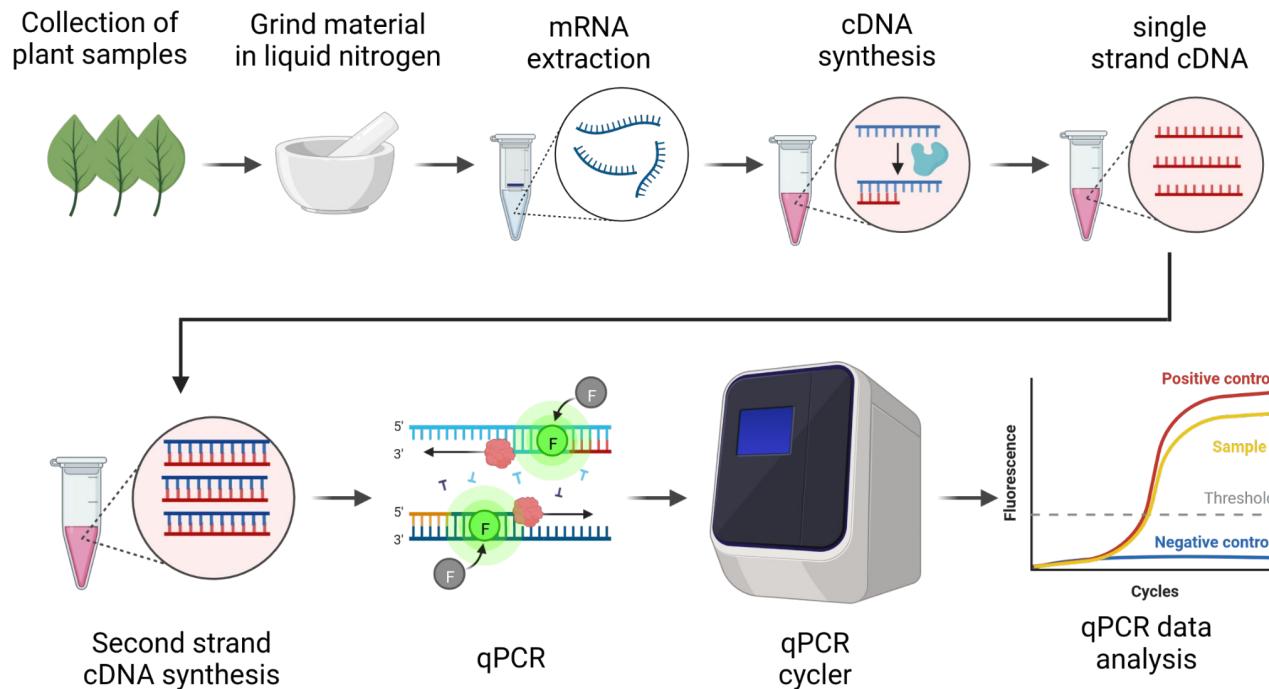
How to measure gene expression?

- Northern blot
- Reverse transcription quantitative PCR (RT-qPCR)
- Microarray
- Expressed Sequence Tags (ESTs)
- Serial Analysis of Gene Expression (SAGE)
- RNA-seq (not RNA sequencing!!)
- Droplet digital PCR (ddPCR)
- Direct RNA sequencing

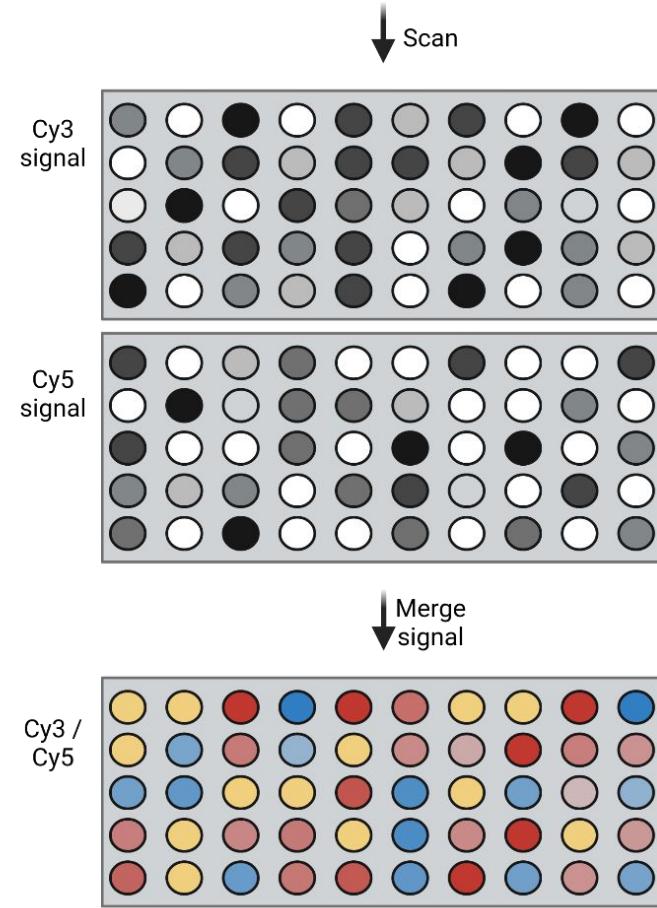
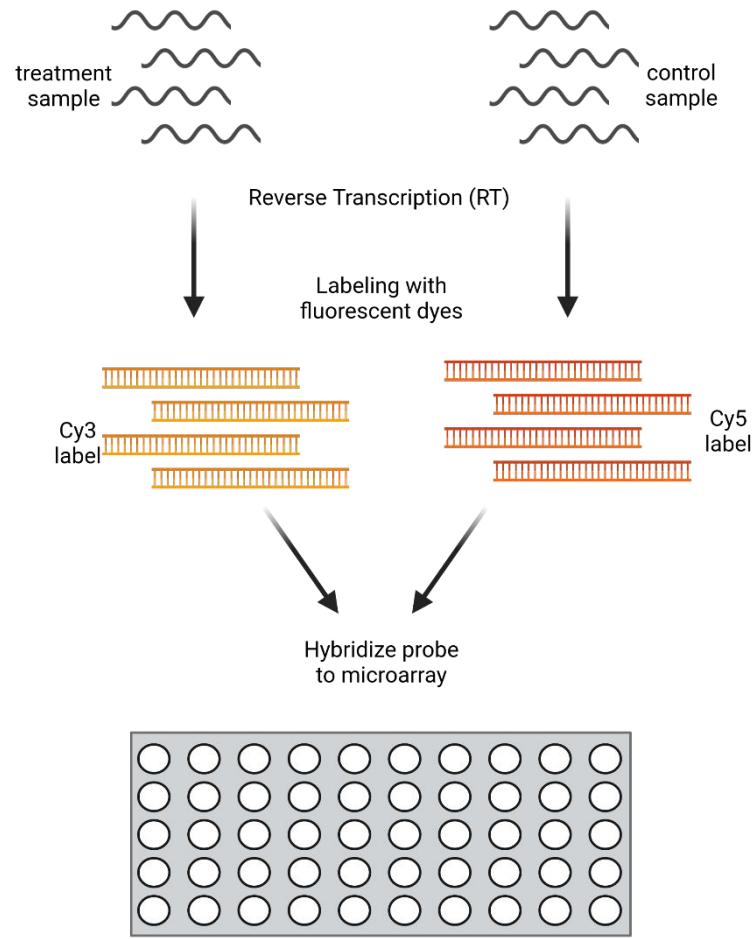


Reverse transcription quantitative PCR (RT-qPCR)

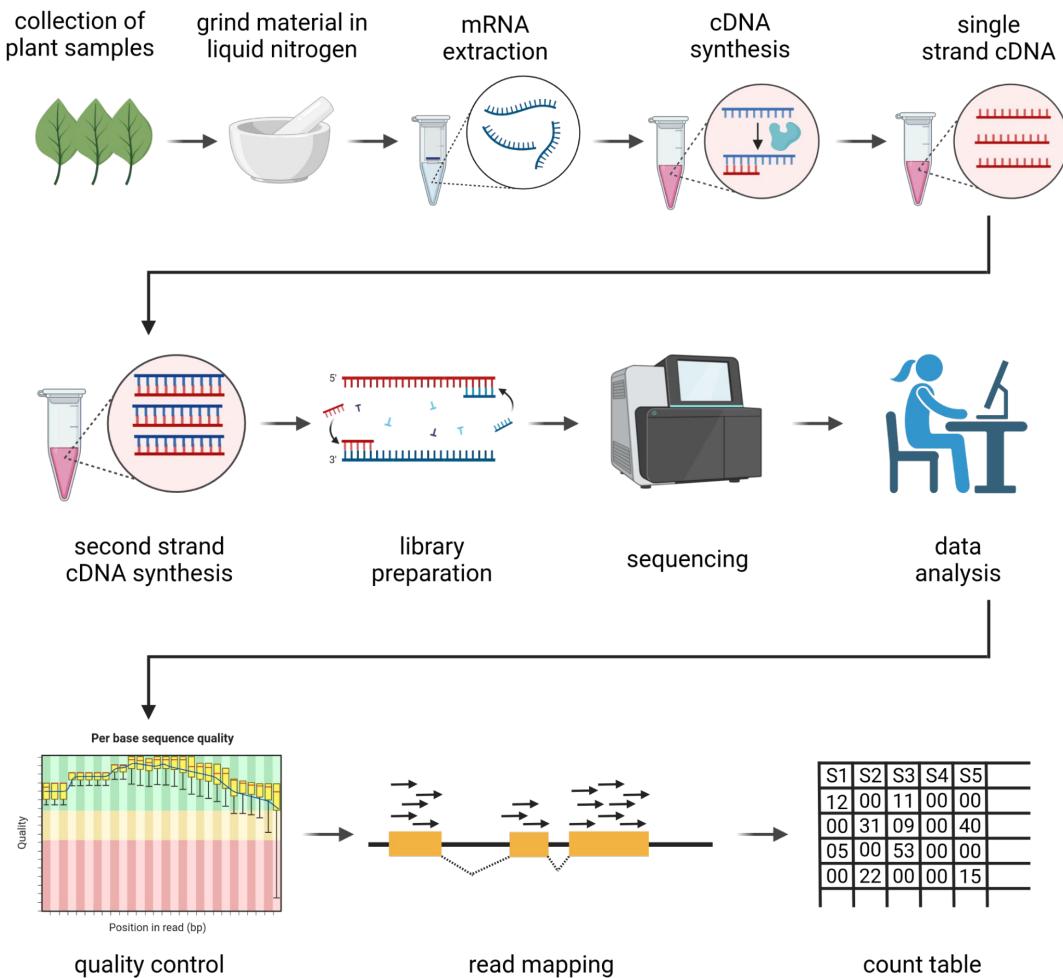
- Reverse transcription = conversion of mRNA into cDNA
- qPCR = quantitative PCR, real time detection of product formation



Microarray

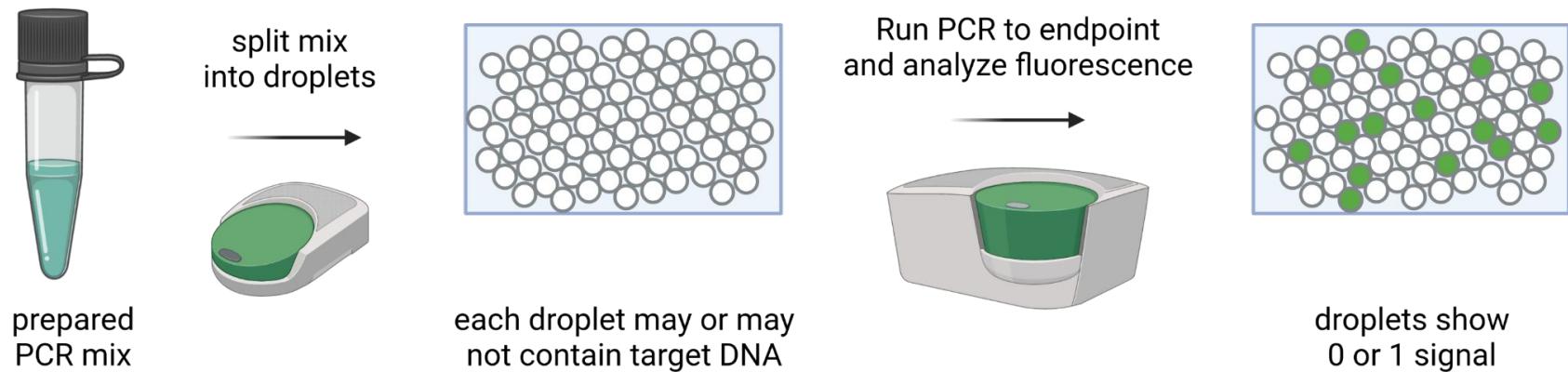


RNA-seq (not RNA sequencing!)

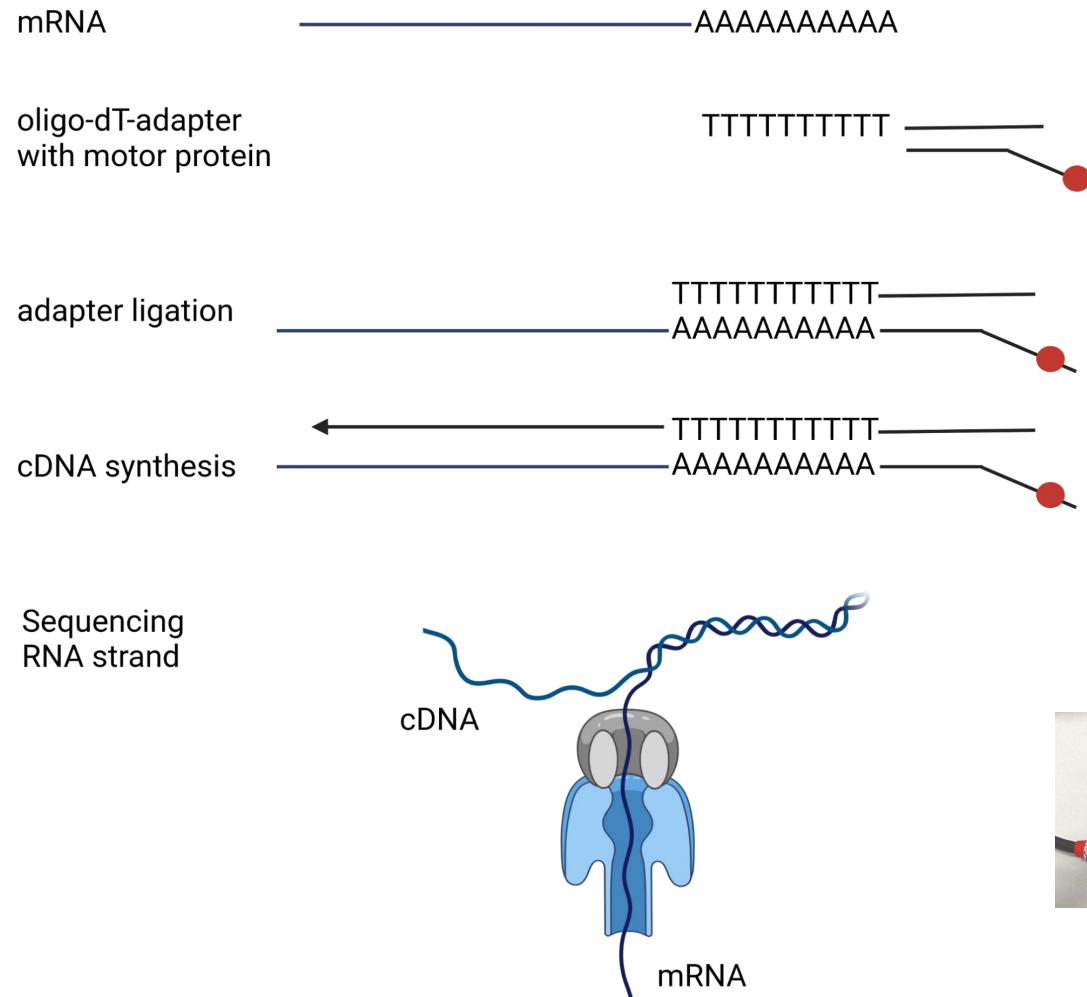


Droplet digital PCR (ddPCR)

- Digital = individual droplets show 0 or 1 signal
- Superior to qPCR in certain applications
- Requires specific hardware (droplet generation and analysis)



Direct RNA sequencing



(photo credit: Melina Nowak)

RNA-seq



Shipping

- Shipping RNA to (international) service provider
- Dry ice (solid CO₂) is used to keep RNA samples frozen
- RNA-seq costs per sample: < \$200



https://commons.wikimedia.org/wiki/File:Dry_Ice_Vapor_%2817490553041%29.jpg; CC-BY 2.0

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

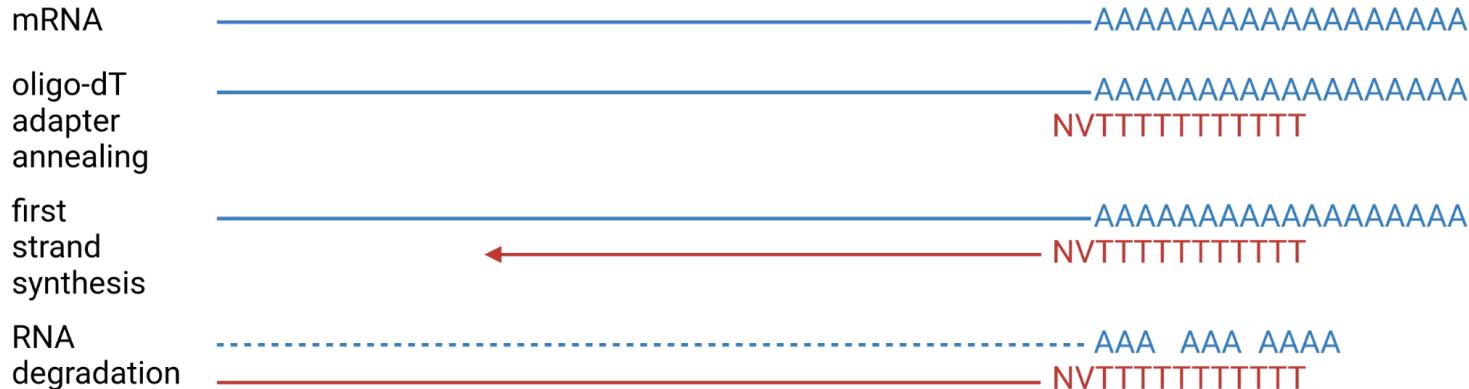


Enrichment of mRNA

- 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

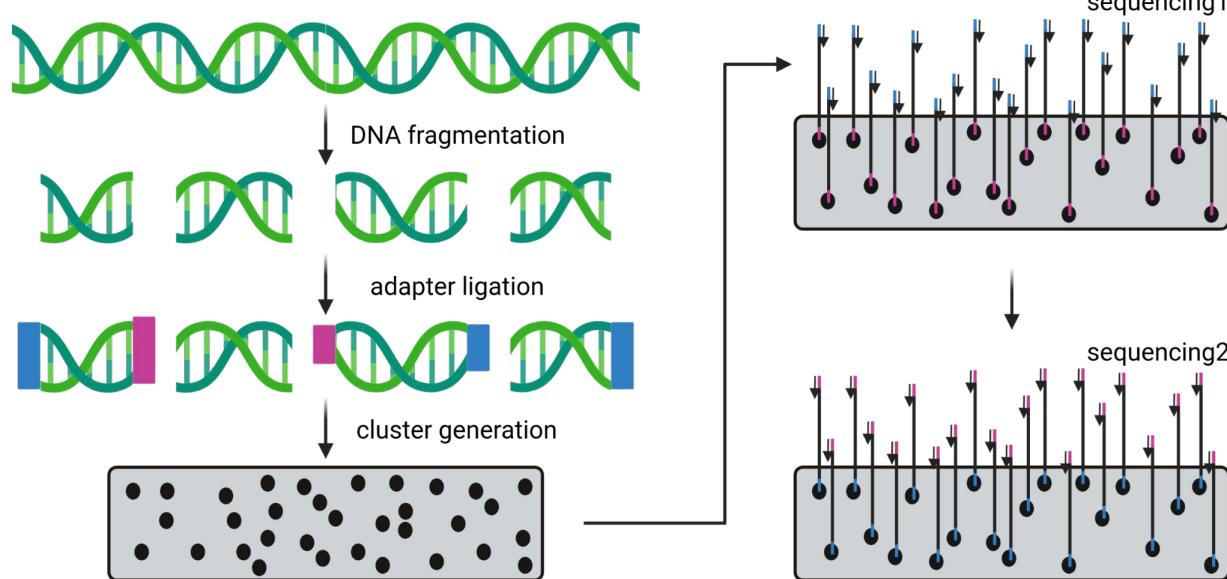
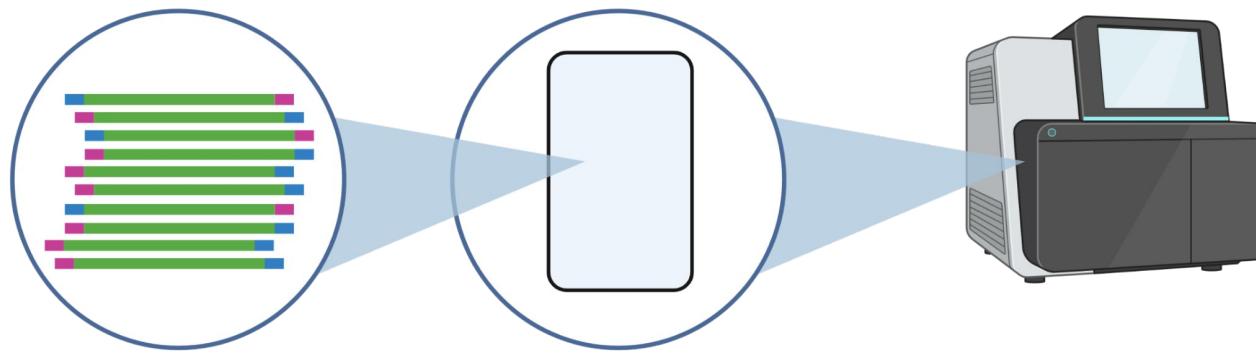
oligo-dT priming



random hexamer priming

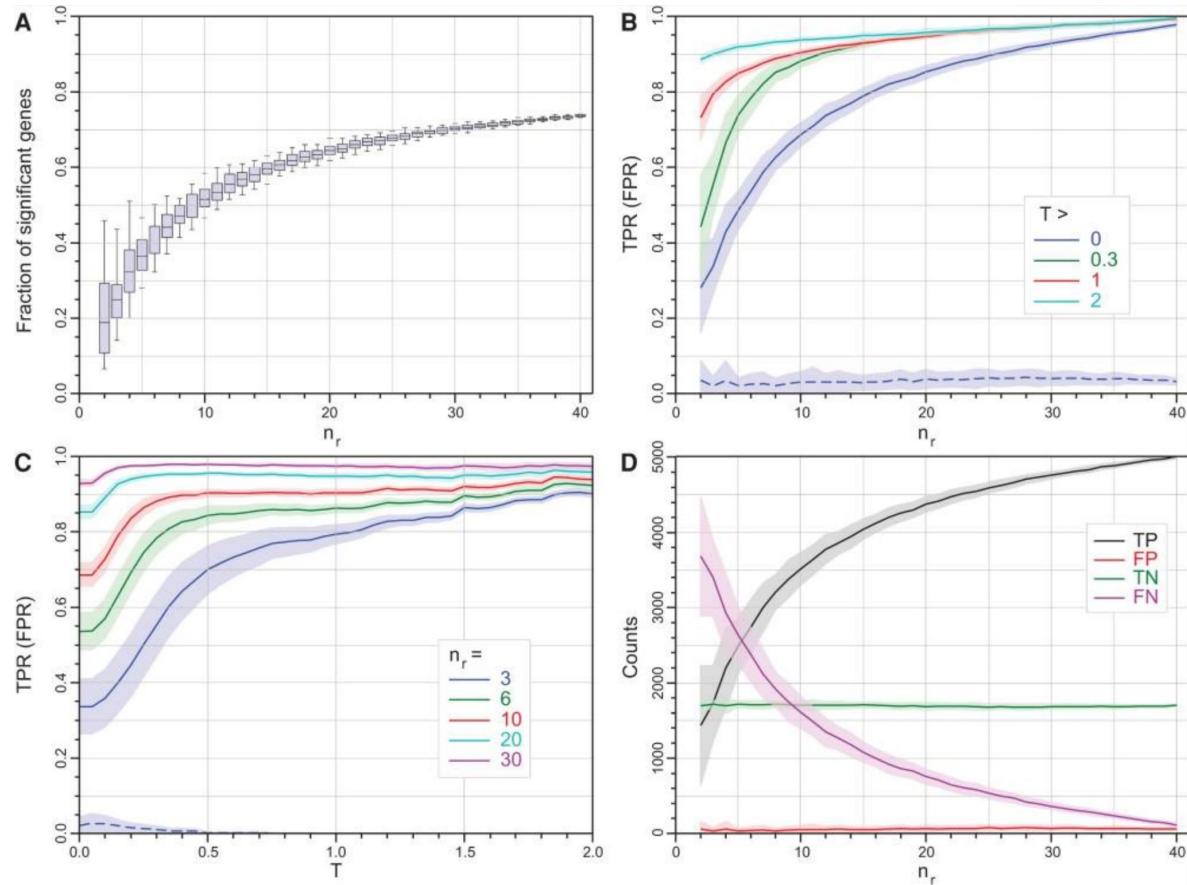


Illumina sequencing of cDNAs



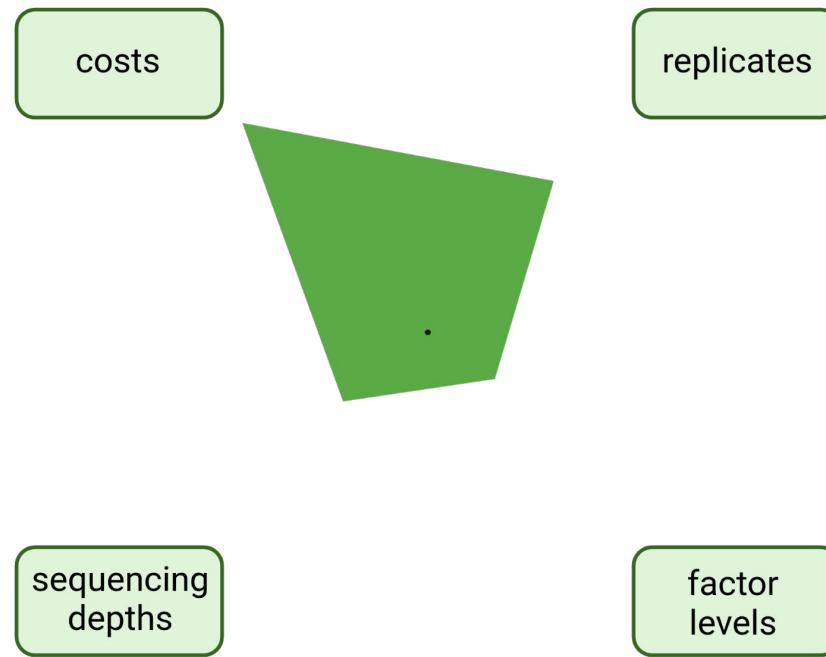
Replicates vs. sequencing depth

- 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



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
- Precise documentation of temperature, humidity, provided substrate, infections, ...

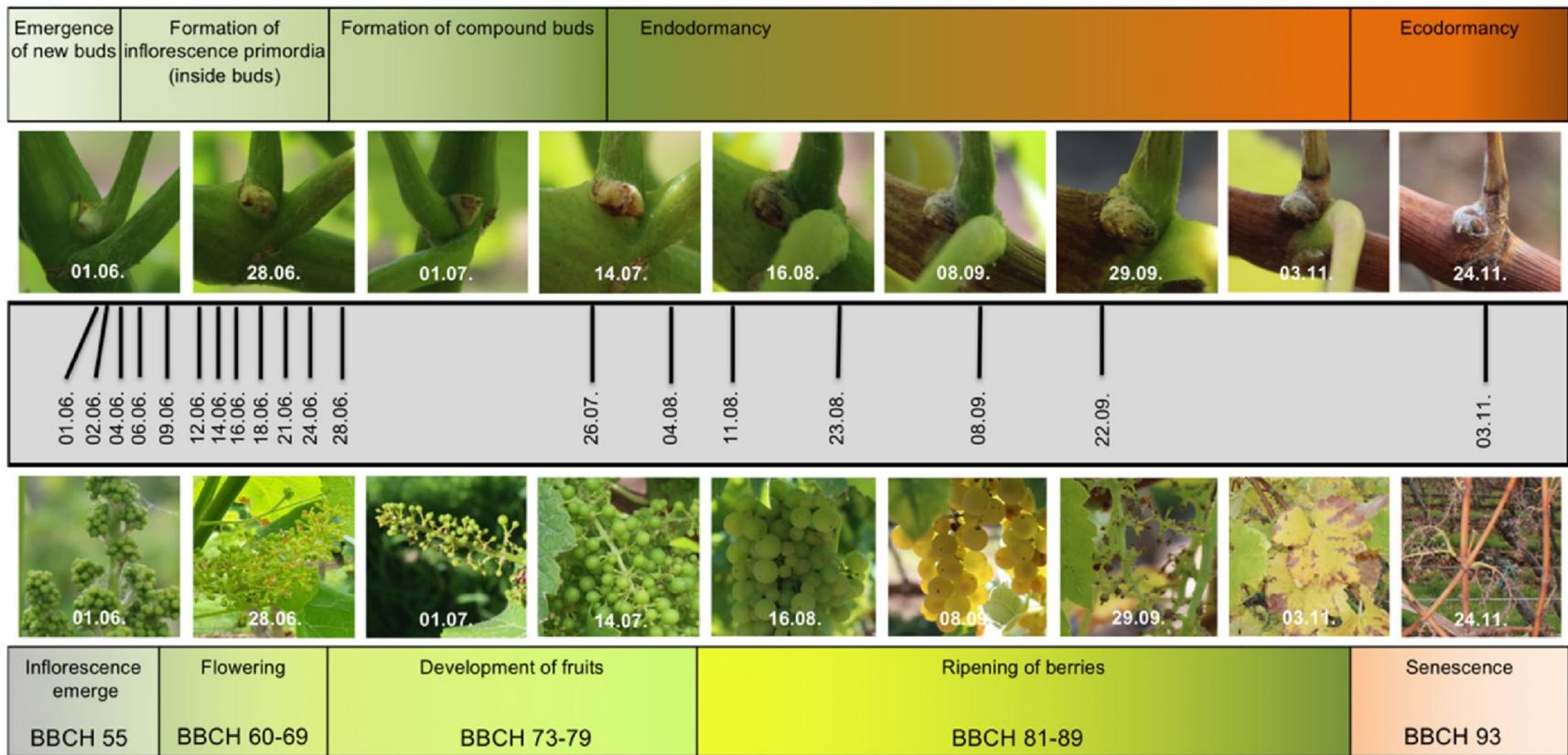
Nagoya protocol & ABS laws

- 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>

Summary

- RNA biology
- RNA extraction methods
- Method for gene expression quantification
- RNA-seq workflow

Time for questions!



Questions

1. What is a transcriptome?
2. Which elements form a eukaryotic gene?
3. Which are the three most abundant RNA types?
4. Which methods can be used to measure gene expression?
5. How does RT-qPCR work?
6. What are the important steps of an RNA-seq workflow?
7. How does ddPCR work?
8. How to check the quality of RNA?
9. What is ‘RIN’ and how is it used?
10. Which factors should be considered when planning an RNA-seq experiment?