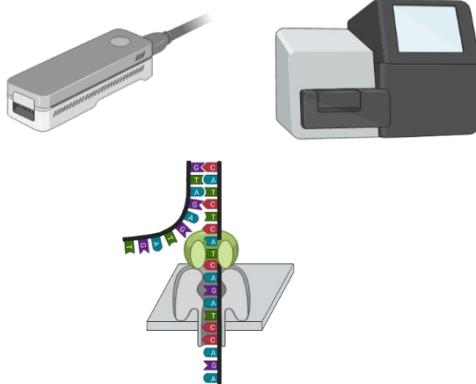
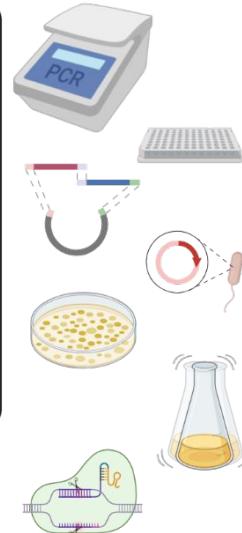
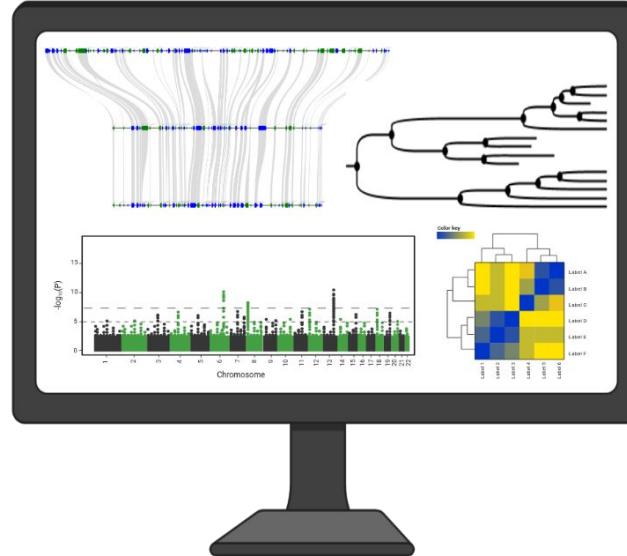




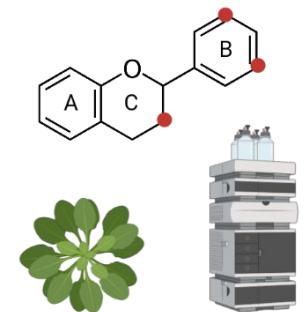
Technische  
Universität  
Braunschweig



Plant Biotechnology  
and Bioinformatics



species biosynthesis proteins analysis different conditions  
biosynthesis species activities within different variants H293-MYB  
within genes functional variants Col-0 Col-0 variant  
duplex site data divergently expressed non-canonical  
sequencer ICGM single reference protein annotation level identified  
sites synthesis gene structure evolutionary pathway  
single reference genes plant accessions  
sites evolution plants model systems biology long distance  
plants accessions Key words: genome across Canophylales  
pigments model genome key against canonical Arabidopsis  
flavonoid conservation sequencing evolution thaliana  
genes read transcription synthetic accessions identification sequence  
gene MYB introns residues RNA-Seq



# Polymerase Chain Reaction (PCR)

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

My figures and content can be re-used in accordance with CC BY 4.0, but this might not apply to all images/logos. Some figure were constructed using bioRender.com.

# Importance of PCR in diagnostics

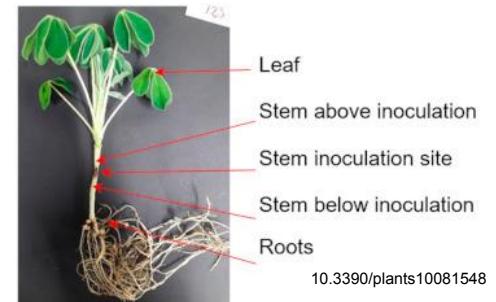
- Illegal logging of tropical wood (Dipterocarpus)
- SMART breeding (sugar beet)
  - Selection with Markers and Advanced Reproductive Technologies
- Pathogen detection (e.g. *Colletotrichum lupini* in *Lupinus alba*)



[https://commons.wikimedia.org/wiki/File:Sugar\\_beets.jpg](https://commons.wikimedia.org/wiki/File:Sugar_beets.jpg) CC BY SA 4.0



[https://commons.wikimedia.org/wiki/File:Dipterocarpus\\_baudii\\_01.JPG](https://commons.wikimedia.org/wiki/File:Dipterocarpus_baudii_01.JPG) CC BY SA 4.0

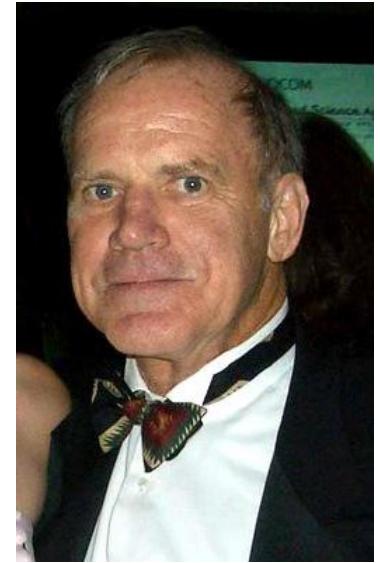


# Who invented the polymerase chain reaction (PCR)?



# Invention of the PCR

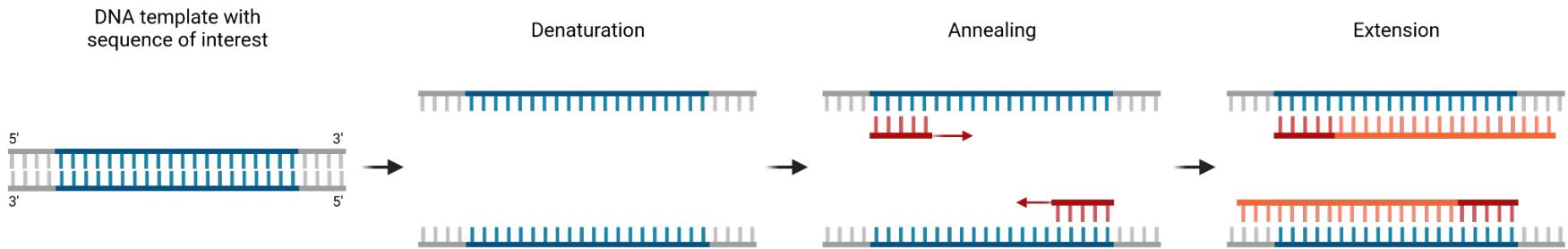
- Kary Mullis invented the PCR in 1985
- Nobel prize in 1993 (shared with Michael Smith)
- Objective: exponential amplification of DNA



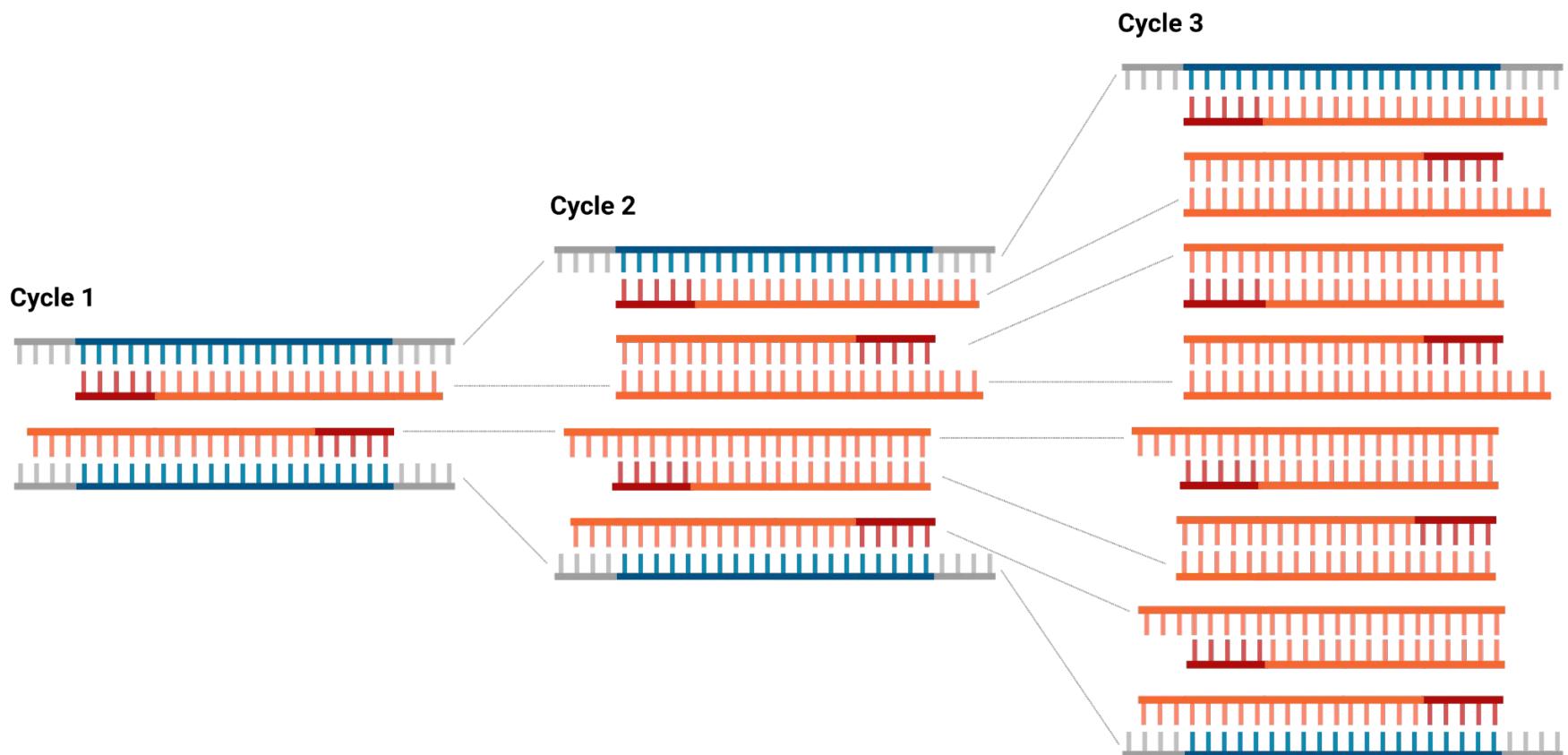
(photo credit: Dona Mapston, CC BY-SA 3.0  
[https://en.wikipedia.org/wiki/Kary\\_Mullis#/media/File:Kary\\_Mullis.jpg](https://en.wikipedia.org/wiki/Kary_Mullis#/media/File:Kary_Mullis.jpg))

# Concept of the PCR (1)

- Objective: amplification of sequence of interest
- Denaturation (step1): heating DNA to separate strands
- Annealing (step2): binding of primers to DNA template
- Extension (step3): synthesis of complementary strand

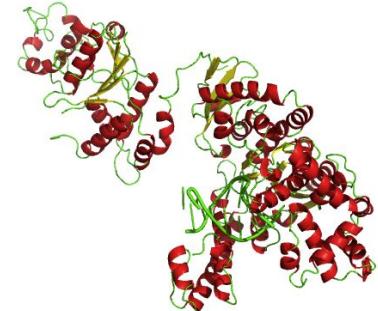
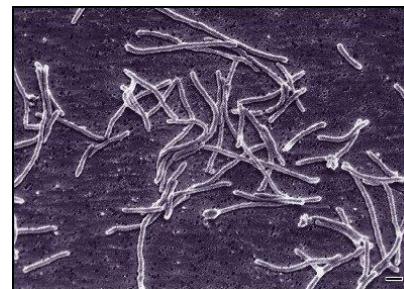


# Concept of PCR (2)



# Thermostable polymerases - *Thermophilus aquaticus*

- Thermotolerant bacterial species isolated from hot springs in Yellowstone national park
- Conditions: 50-80°C
- *Thermophilus aquaticus* is source of a thermostable polymerase: Taq



<https://commons.wikimedia.org/wiki/File:Taq.png> CC BY-SA 3.0

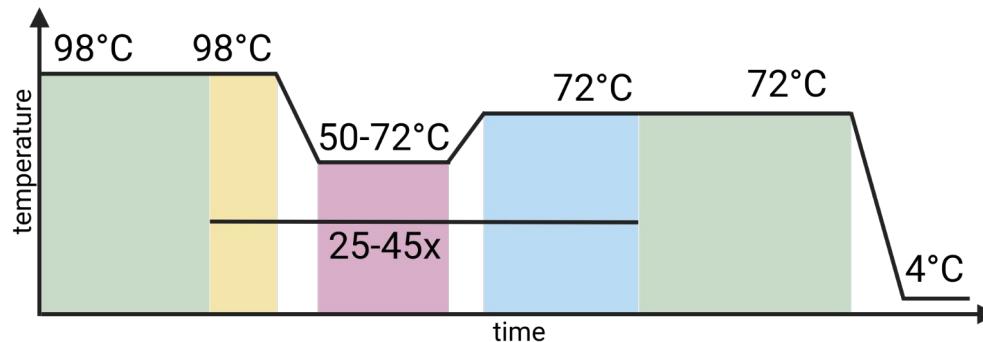
# Thermocycler

- Quick temperature change poses technical challenge
- Heating the lid prevents condensation of evaporated solution
- Cooling function preserves PCR product after end of run
- Numerous programs can be saved for various applications
- Device also suitable for other incubation steps



# PCR program

- Initial denaturation: thoroughly separating DNA strands
- Denaturation: separating DNA strands
- Annealing: binding of primers to template
- Elongation: synthesis of complementary DNA strand
- Final elongation: completion of started syntheses



# PCR components

- DNA as template for synthesis of novel strand
- Oligonucleotides to initiate the synthesis of complementary strands
- Deoxyribonucleotides serve as material for strand synthesis
- Buffer ensures correct reaction conditions
- Polymerase catalyzes the synthesis of a complementary strand

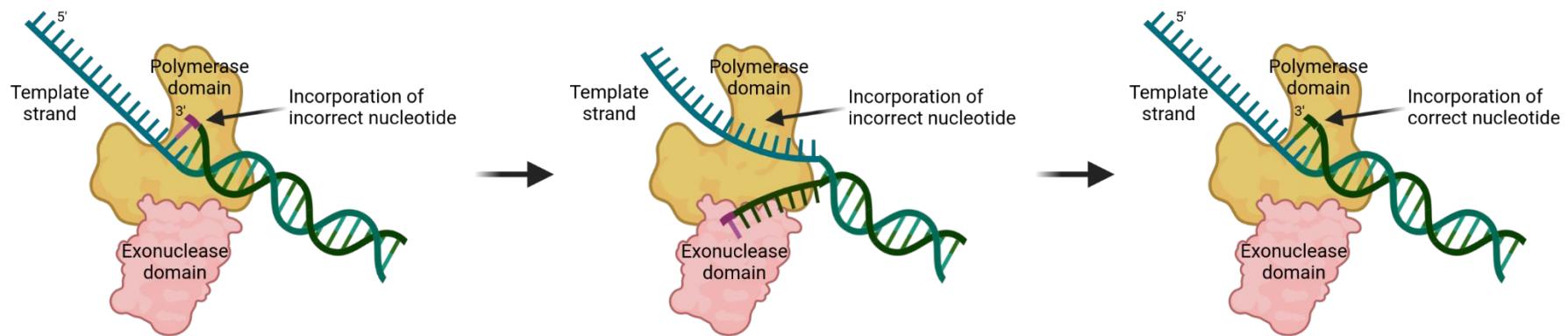
# Properties of DNA polymerases

- Thermostability: stable at high temperatures for a long time
- Extension rate: fast polymerases speed up experiments
- Fidelity: low error rate desired for specific applications
- Processivity: generation of large amplicons required for certain applications



# Fusion DNA polymerases and proofreading

- Taq polymerase lacks proofreading activity (error rate:  $10^{-6}$ )
- Fusion polymerase offers proofreading activity (error rate:  $10^{-9}$ )



# Types of DNA polymerases

- phi29 DNA polymerases (high processivity, *Bacillus subtilis* phage Phi29)
- pfu DNA polymerases (proofreading, *Pyrococcus furiosus*)
- Taq DNA polymerases (no proofreading, *Thermophilus aquaticus*)

# Primer/oligonucleotide design rules

- Primer length determines specificity (about 20nt)
- GC clamp: 2xG/C at the 3' end
- Avoid self complementarity
- Avoid long homopolymers
- Primers must bind specifically to desired position on template

# Primer design tools: Primer-BLAST

- Online tool for the design of primer pairs at the NCBI
- Design of exon junction spanning primers possible
- Integration with NCBI databases

**Primer-BLAST**  
A tool for finding specific primers  
Finding primers specific to your PCR template (using Primer3 and BLAST).

**Primers for target on one template** Primers common for a group of sequences  
PCR Template Enter accession, gi, or FASTA sequence (A refseq record is preferred)   Primers common for a group of sequences  
Or, upload FASTA file  No file chosen  
Range   Forward primer  Reverse primer   
**Primer Parameters**  
Use my own forward primer (5->3 on plus strand)   Use my own reverse primer (5->3 on minus strand)    
PCR product size Min  Max   
# of primers to return   
Primer melting temperatures (T<sub>m</sub>) Min  Opt  Max  Max T<sub>m</sub> difference  ?  
**Exon/intron selection** A refseq mRNA sequence as PCR template input is required for options in the section  
Exon junction span No preference  
Exon junction match Min 5' match  Min 3' match  Max 3' match   
Minimal and maximal number of bases that must anneal to exons at the 5' or 3' side of the junction  
Intron inclusion  Primer pair must be separated by at least one intron on the corresponding genomic DNA  
Intron length range Min  Max  ?  
**Primer Pair Specificity Checking Parameters**  
Specificity check  Enable search for primer pairs specific to the intended PCR template  
Search mode Automatic  
Database Refseq mRNA  
Exclusion  Exclude predicted Refseq transcripts (accession with XM, XR prefix)  Exclude uncultured/environmental sample sequences  
Organism Homo sapiens   
Enter an organism name (or organism group name such as enterobacteriaceae, rodents), taxonomy id or select from the suggestion list as you type.  
Entrez query (optional)  
Primer specificity stringency Primer must have at least  total mismatches to unintended targets, including at least  mismatches within the last  bps at the 3' end  
Ignore targets that have  or more mismatches to the primer  
Max target amplicon size   
Allow splice variants  Allow primer to amplify mRNA splice variants (requires refseq mRNA sequence as PCR template input)  
  Show results in a new window  Use new graphic view

<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>



# Primer design tools: Primer3Plus

- Online and command line tool for the design of primer pairs
- Standard algorithm incorporated into many other tools
- Detailed control over numerous parameters

**Primer3Plus**  
pick primers from a DNA sequence

Load server settings: Default  
Activate Settings  
Task: generic

Select primer pairs to detect the given template sequence. Optionally targets and included/excluded regions can be specified.

Pick Primers Reset Default

Main General Settings Advanced Settings Internal Oligo Penalties Advanced Seq.

Sequence Id:

Paste template sequence below or upload sequence file:  No file chosen

Mark selected region: < Excluded > [ Target ] { Included } Clear Regions from Seq. Save Sequence

Excluded Regions: <  >  
Targets: [  ]  
Included Region: {  }  
Primer overlap positions: -   
Pair OK Region List:

Pick left primer  Pick hybridization probe  Pick right primer  
or use [left primer](#) below. (internal oligo) or use [oligo](#) below.  
[right primer](#) below (5'→3' on opposite strand).

5' Overhang:  5' Overhang:

Primer3Plus - [Primer3-GitHub](#) · [GEAR-Genomics](#) · [Contact](#) · [Datenschutzerklärung](#) · [Impressum](#)

Supported by [EMBL](#)

# *In silico* PCR

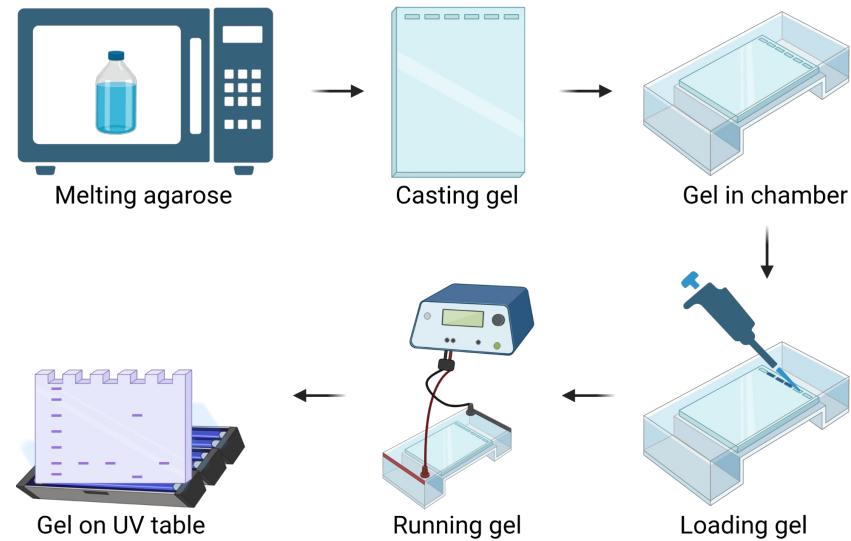
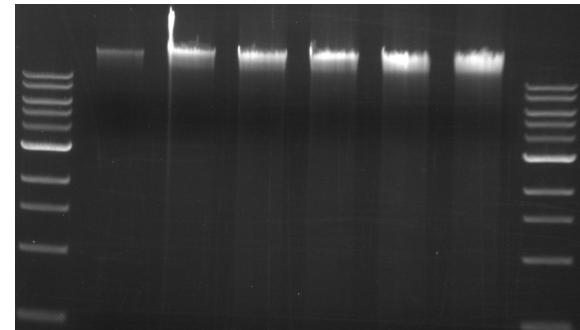
- Simulate PCR with software to ensure that primers would work as expected
  - Most cloning tools offer functions to validate the PCR/cloning strategy

<https://genome.ucsc.edu/cgi-bin/hgPcr>



# Endpoint PCR and agarose gel

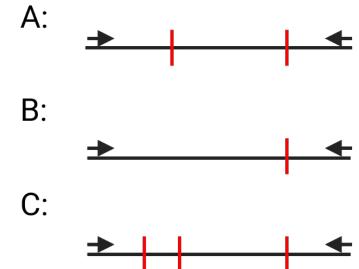
- Classical type of a PCR
- Running 25-45 cycles
- PCR is only exponential during the initial phases, but runs into saturation
- Result of an endpoint PCR is usually assessed on an agarose gel



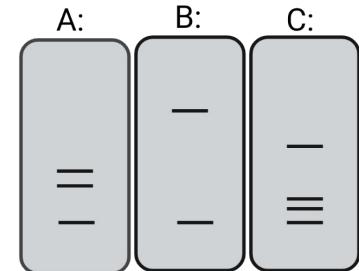
# Genetic markers - CAPS

- Many genetic markers rely on PCR
- CAPS = Cleaved Amplified Polymorphic Sequences
- Sequence difference causes presence/absence of restriction enzyme recognition site
- PCR is required to amplify the signal intensity
- Analysis is performed on an agarose gel

CAPS: Cleaved  
Amplified Polymorphic  
Sequences



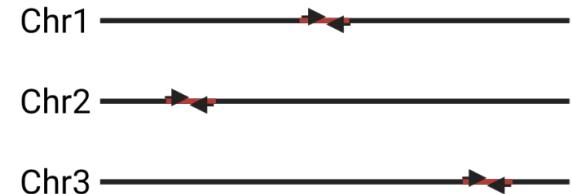
PCR & restriction digest



# Nested PCR

- (Unspecific) amplification of a large region followed by specific amplification of a subregion
- PCR products of first run serve as template in the second run
- Example1: studying a large gene family
- Example2: adding specific overhangs

PCR1:

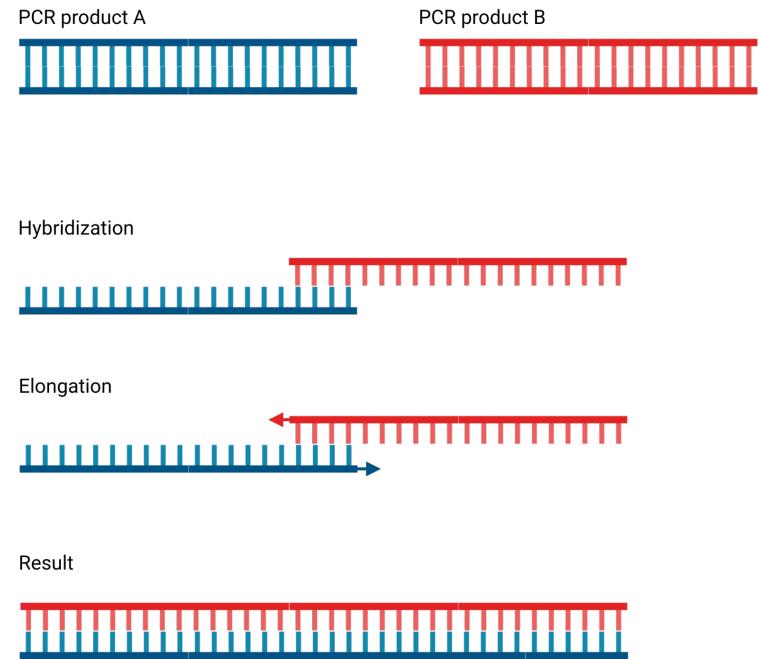


PCR2:



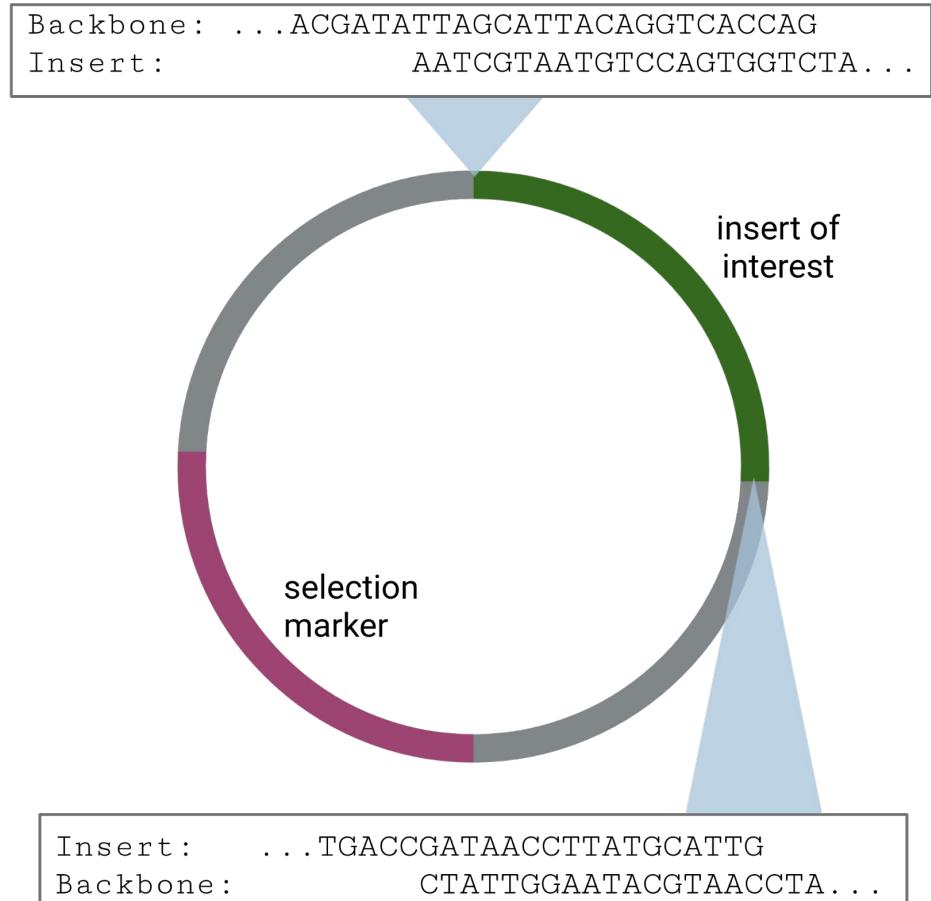
# Overlap extension PCR

- Alternative name: crossover PCR
- Objective: combination of different DNA molecules through a PCR
- Annealing temperature of fragments need to be considered



# Example: construction of plasmid

- Combination of DNA sequences based on homologous regions
- Overlap needs to stabilize interaction at reaction temperature (e.g. 50°C)



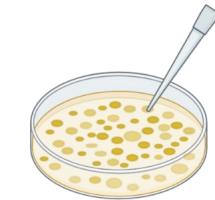
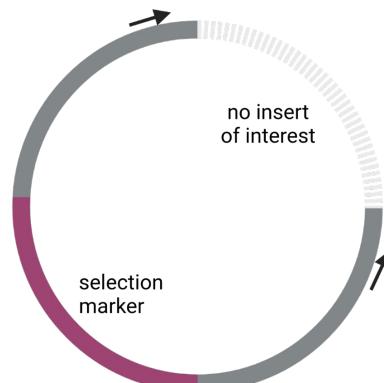
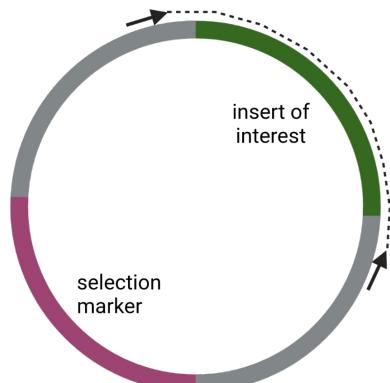
# Multiplexing

- Combining multiple PCR objectives in one tube
- Multiple primer combinations are mixed in one tube
- Different PCR products need to differ in properties (e.g. size, fluorescence)

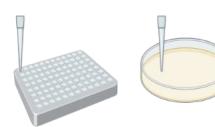


# Colony PCR

- Objective: identify correct plasmids in bacterial clones
- Bacterial cells are given into reaction; lysis of cells releases template for PCR
- Positive clones are identified based on agarose gel results



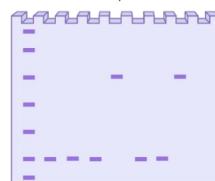
picking bacterial colonies from plate



transfer cells into PCR master mix and onto new plate



running PCR releases template from bacterial cells



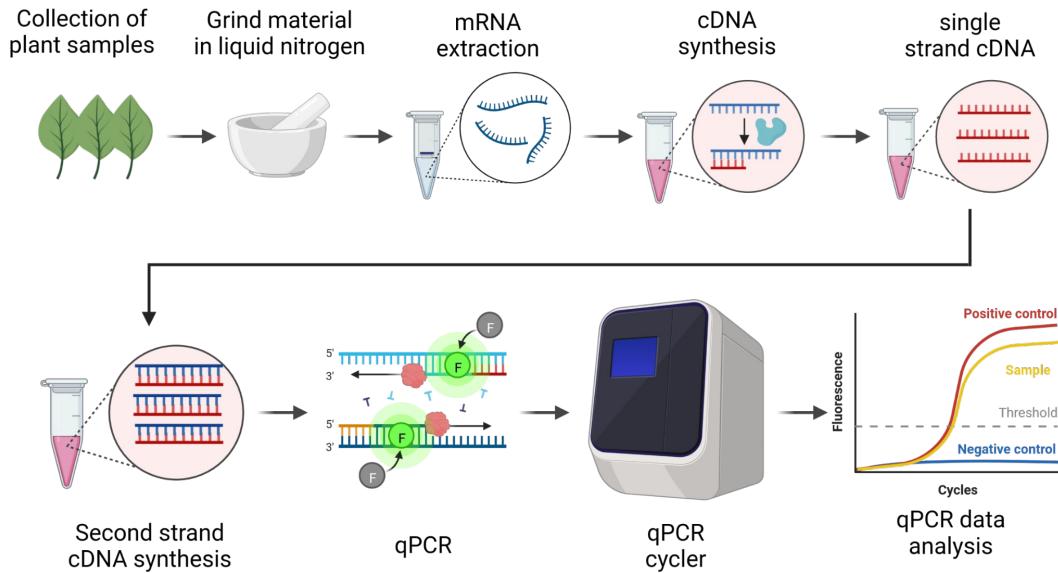
analysis on agarose gel

# What is a qPCR?

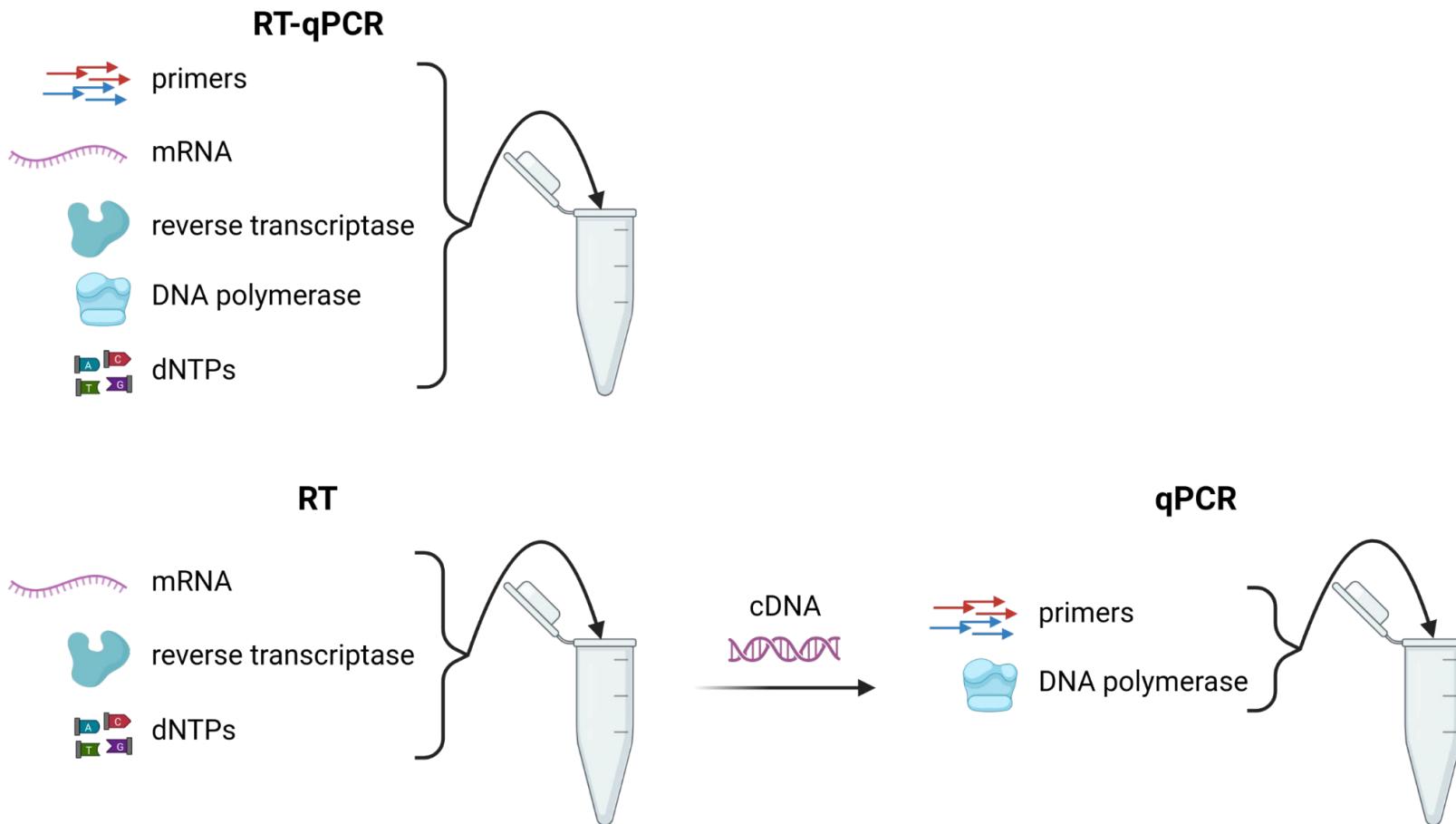


# RT-qPCR

- RT-qPCR = Reverse Transcriptase quantitative PCR
- Objective: quantification of gene expression (mRNA abundances)
- Real time tracking of PCR product formation

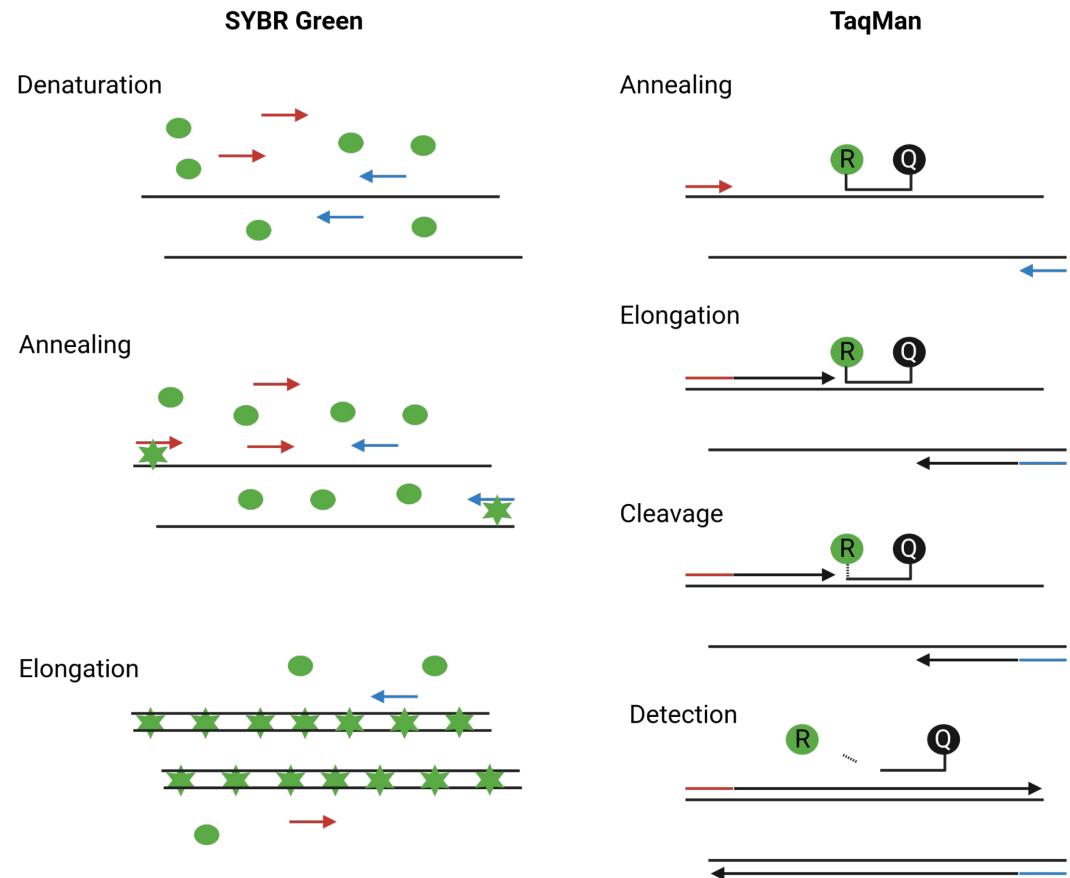


# One step vs. two step RT-qPCR



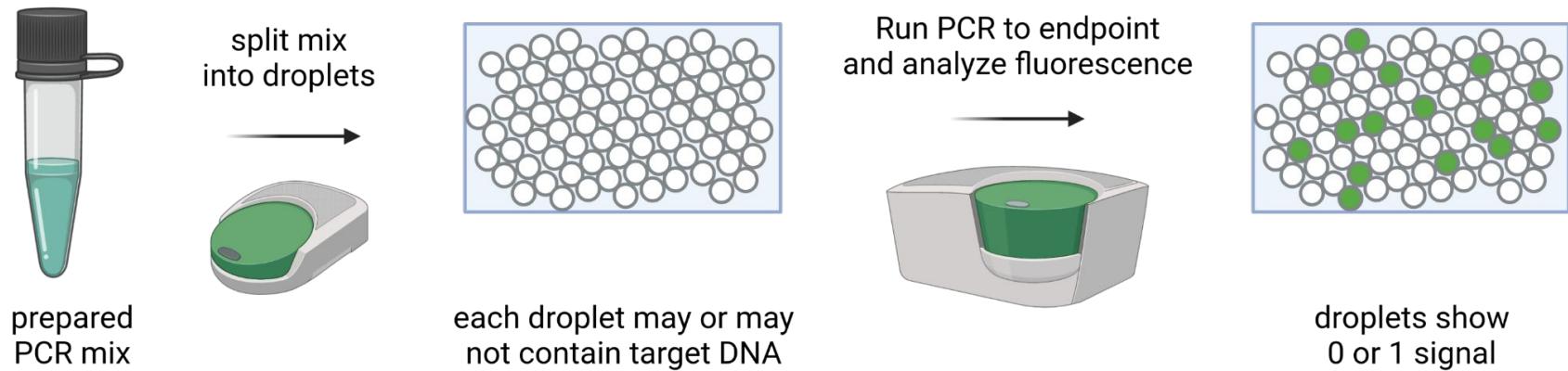
# qPCR: SYBR Green vs. TaqMan

- SYBR Green is universally applicable
- TaqMan requires specific design for each analysis
- TaqMan allows a more specific analysis



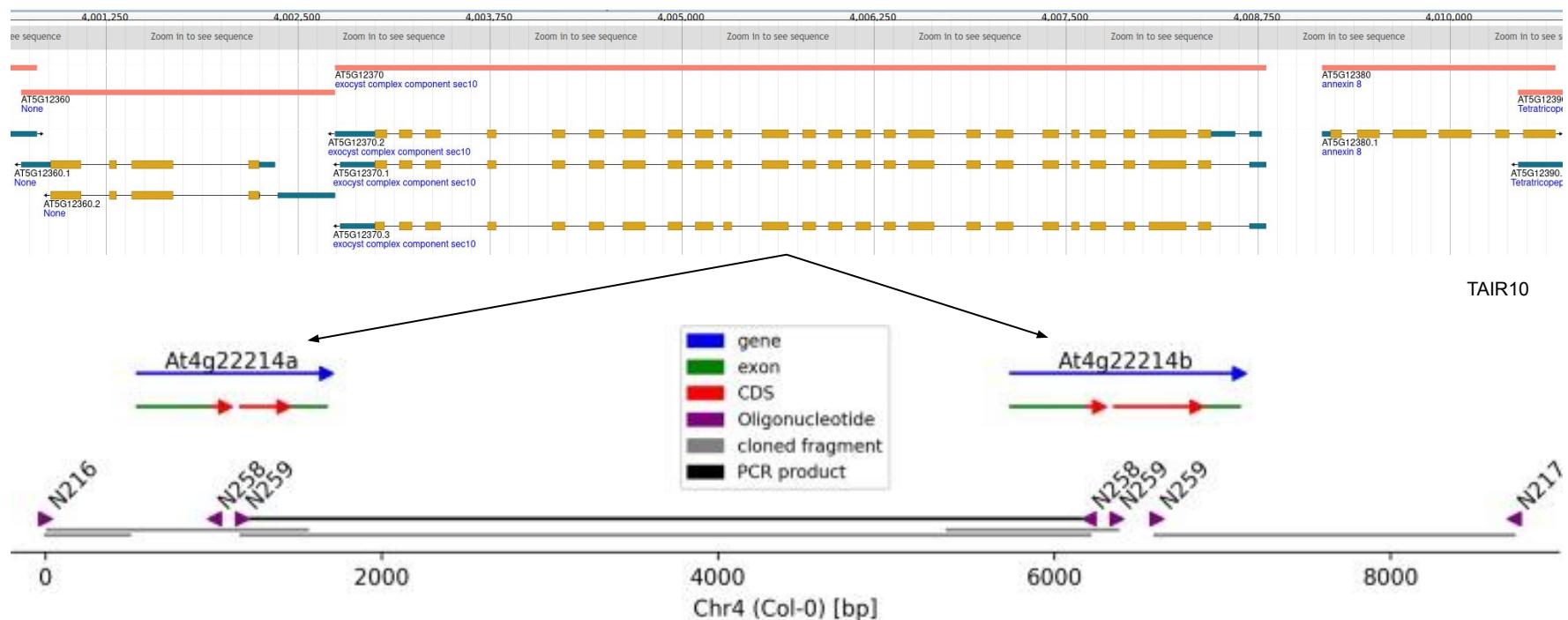
# Droplet digital PCR (ddPCR)

- Objective: quantification of input DNA concentration
- Digital: each droplet shows 0 or 1 result



# Example: detection of gene copy numbers

- Sample needs to be compared against a standard (single copy)
- Small difference in copy number can be quantified



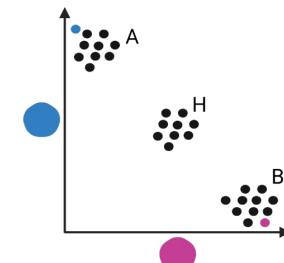
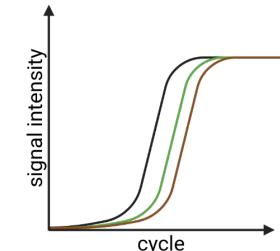
10.1371/journal.pone.0216233

# Genetic markers - KASP

- KASP = Kompetitive Allele Specific PCR
- Differently labeled alternative primers bind at variant position
- Fluorescent signal of product reveals frequency of variants
- AHB = different genotypes:
  - A = homozygous for allele 1
  - H = heterozygous
  - B = homozygous for allele 2



qPCR with  
fluorescently labeled  
primers



# Summary

- Concept of PCR
- Primer design
- Types of PCR and applications

# Time for questions!



# Questions

1. What is the concept of PCR?
2. Which components are required for a PCR?
3. What is a typical PCR program?
4. What are important rules to consider for primer design?
5. What are important properties of a DNA polymerase for a PCR?
6. Which different types of DNA polymerases are suitable for a PCR?
7. Which types of PCR can be applied to quantify DNA concentrations?