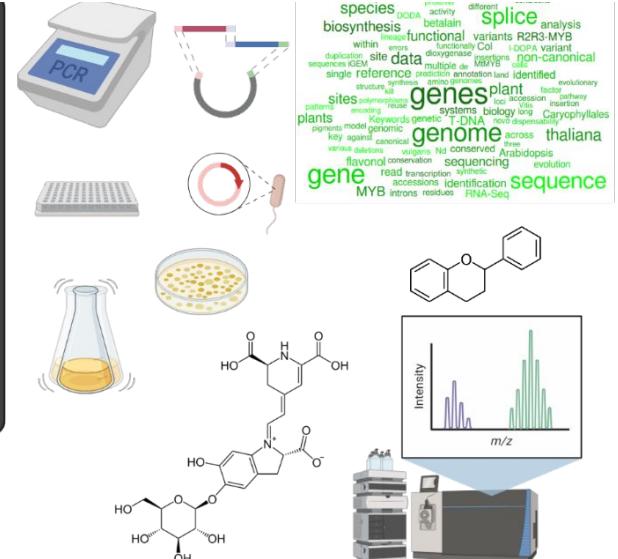
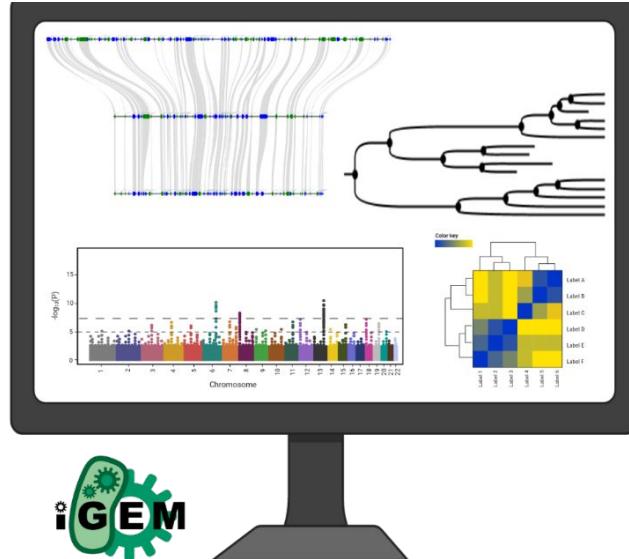
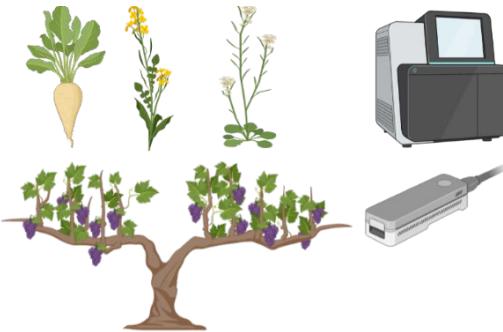




Technische
Universität
Braunschweig



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

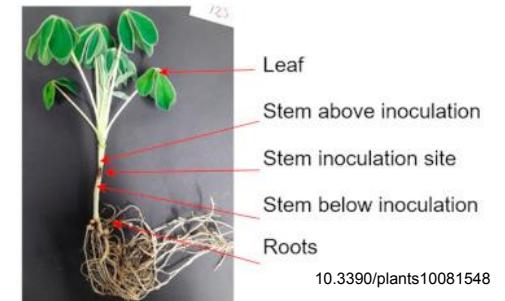
- Illegal logging of tropical wood
(*Dipterocarpus*)



- SMRT breeding (sugar beet)



- Pathogen detection (e.g.
Colletotrichum lupini in
Lupinus alba)

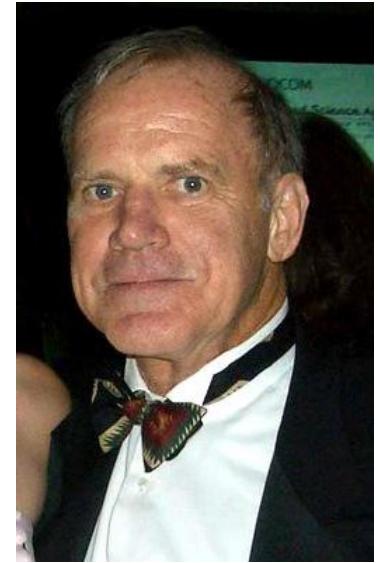


Who invented the polymerase chain reaction (PCR)?



Invention of the PCR

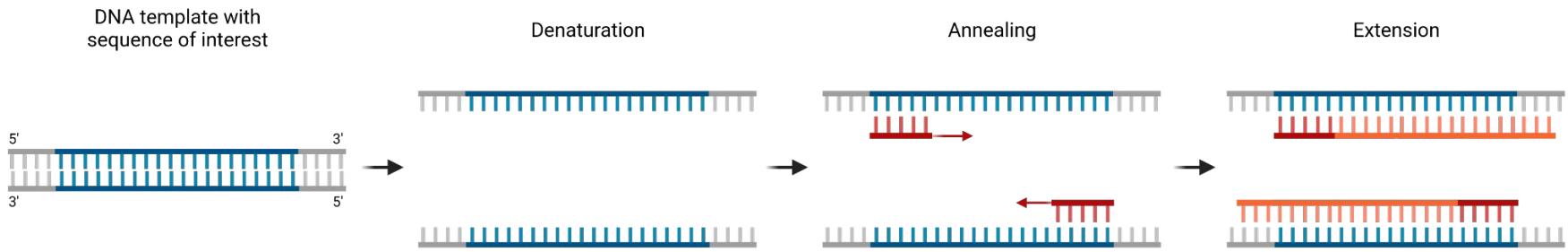
- Kary Mullis invented the PCR in 1985
- Nobel prize in 1993 (shared with Michael Smith)
- Objective: exponential amplification of DNA



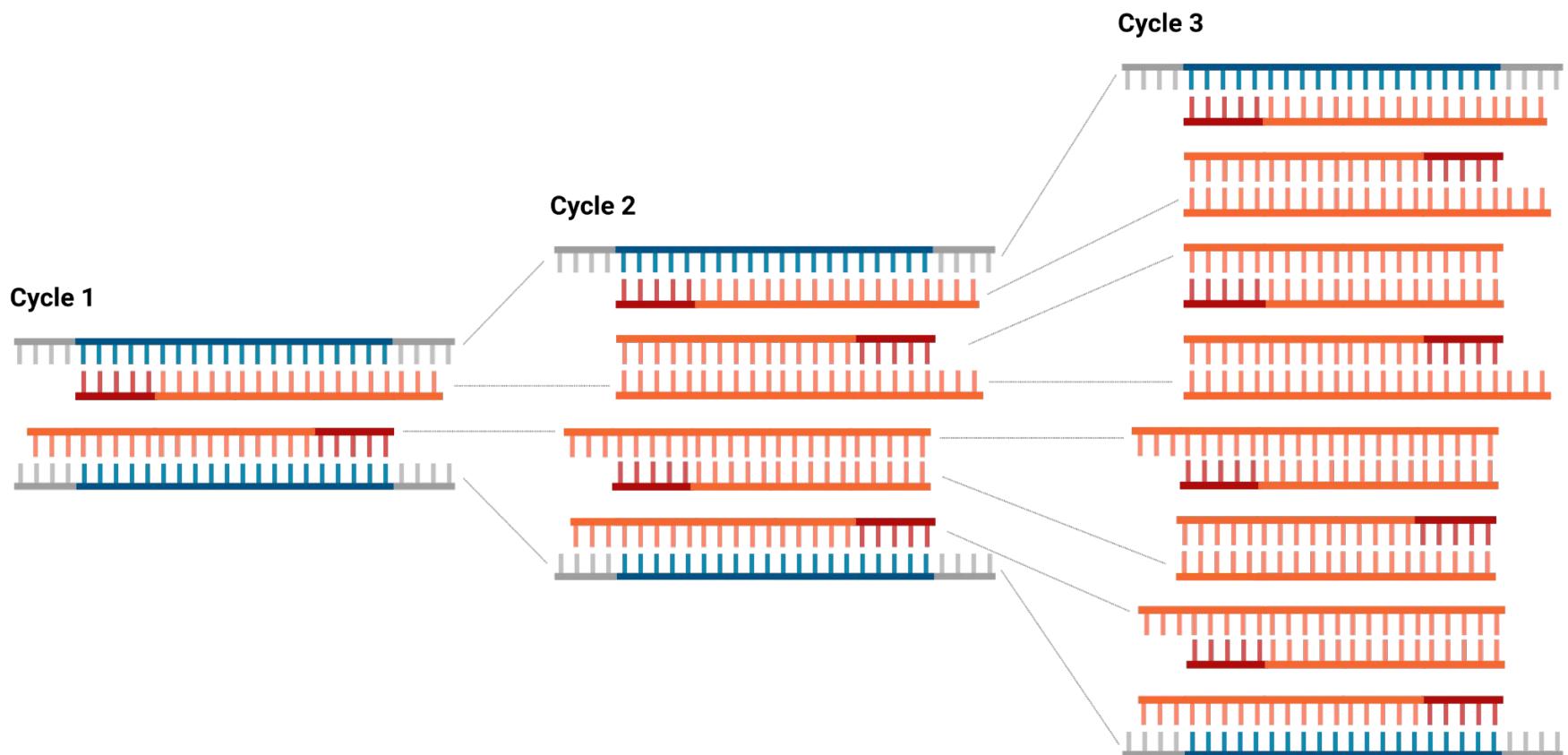
(photo credit: Dona Mapston, CC BY-SA 3.0
https://en.wikipedia.org/wiki/Kary_Mullis#/media/File:Kary_Mullis.jpg)

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

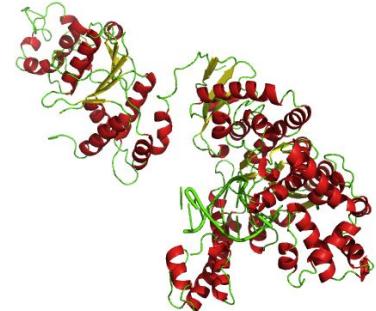
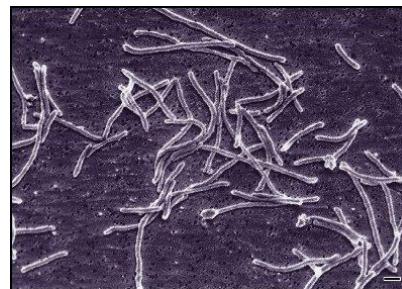


Concept of PCR (2)



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

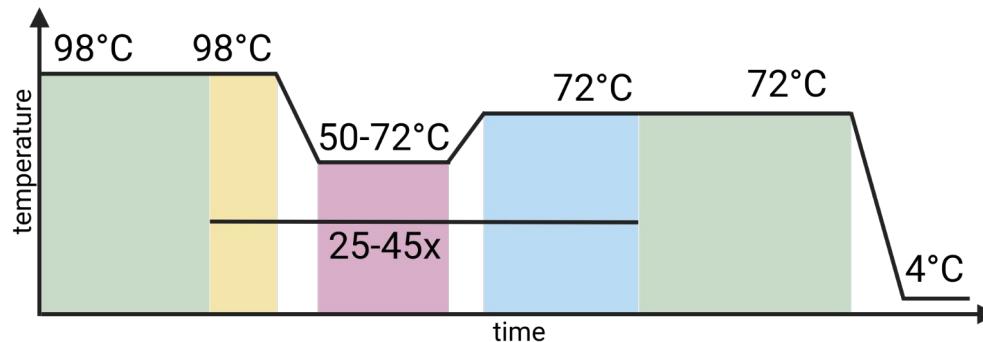
Thermocycler

- 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

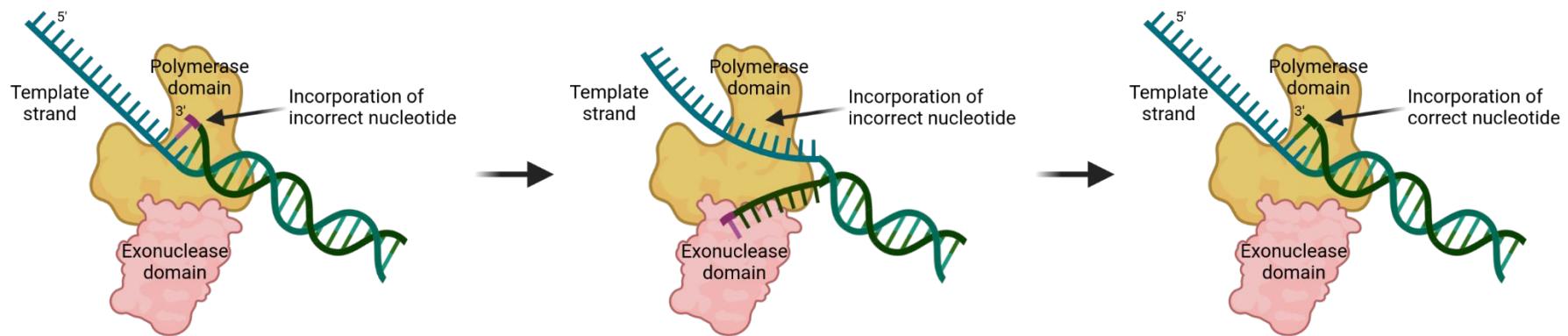


PCR components

- 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

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) Range
Or, upload FASTA file No file chosen
Forward primer From To
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
of primers to return
Primer melting temperatures (T_m)
Min: 70 Max: 1000
Min: 10 Opt: 60.0 Max: 63.0 Max T_m difference: 3

Exon/intron selection
A refseq mRNA sequence as PCR template input is required for options in the section
Exon junction span
Exon junction match
Min 5' match: 7 Min 3' match: 4 Max 3' match: 8
Minimal and maximal number of bases that must anneal to exons at the 5' or 3' side of the junction
Intron inclusion
Intron length range
Min: 1000 Max: 10000

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 2 total mismatches to unintended targets, including at least 2 mismatches within the last 5 bps at the 3' end.
Ignore targets that have 6 or more mismatches to the primer.
Max target amplicon size: 4000
Allow splice variants
 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

UCSC In-Silico PCR

Genome: Human Assembly: Dec. 2013 (GRCh38/hg38) Target: genome assembly Forward Primer: Reverse Primer:

Max Product Size: 4000 Min Perfect Match: 15 Min Good Match: 15 Flip Reverse Primer:

About In-Silico PCR

In-Silico PCR searches a sequence database with a pair of PCR primers, using an indexing strategy for fast performance. See an example [video](#) on our YouTube channel.

Configuration Options

Genome and Assembly - The sequence database to search.
Target - If available, choose to query transcribed sequences.
Forward Primer - Must be at least 15 bases in length.
Reverse Primer - On the opposite strand from the forward primer. Minimum length of 15 bases.
Max Product Size - Maximum size of amplified region.
Min Perfect Match - Number of bases that match exactly on 3' end of primers. Minimum match size is 15.
Min Good Match - Number of bases on 3' end of primers where at least 2 out of 3 bases match.
Flip Reverse Primer - Invert the sequence order of the reverse primer and complement it.

Output

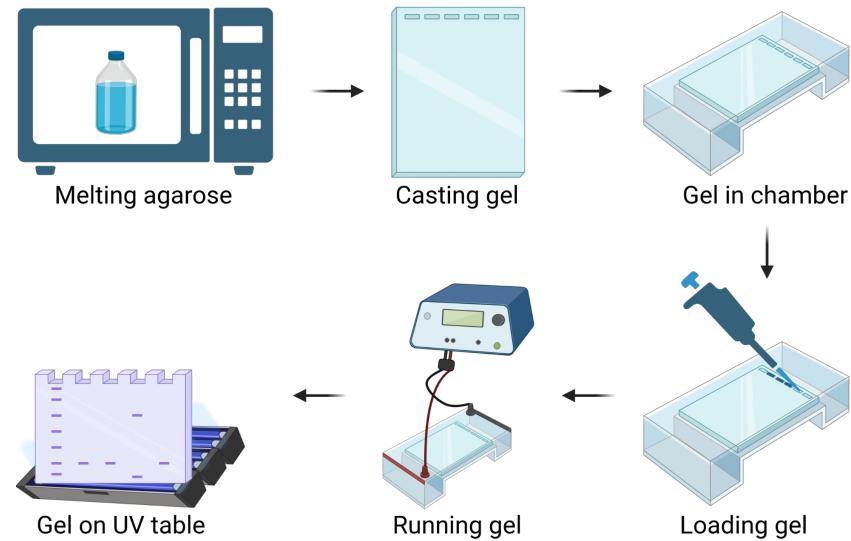
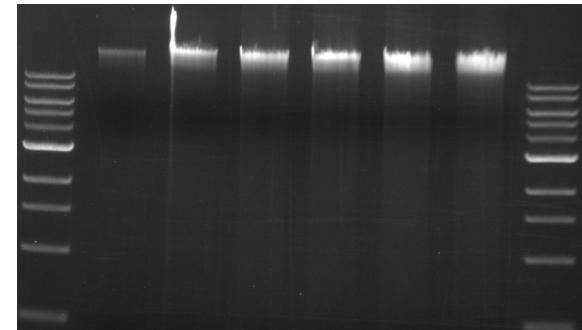
When successful, the search returns a sequence output file in fasta format containing all sequence in the database that lie between and include the primer pair. The fasta header describes the region in the database and the primers. The fasta body is capitalized in areas where the primer sequence matches the database sequence and in lower-case elsewhere. Here is an example from human:

```
>chr22:31000551+31001000 TAACAGATTGATGATGCATGAAATGGG CCCATGAGTGGCTCTAAAGCAGCTG  
TTACAGATTGATGATGCATGAAATGGGgggtggccagggtgggggtga  
gactgcagagaaggcaggctgtttcataacaagcttgcgtccaa  
tatgcacatcgtaagtttccaggggctgtatgttgcggcgtggtaag  
tacacagaacatcttagagaaacccttatttcctaaagataaaaaaa  
gacttgctgttaaggattgttgcatttgcggatattctgtta
```

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

Endpoint PCR and agarose gel

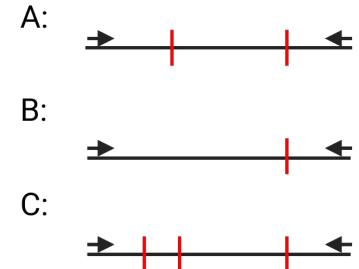
- Classical type of a PCR
- Running 25-45 cycles
- PCR is only exponential during the initial phases, but runs into saturation
- Result of an endpoint PCR is usually assessed on an agarose gel



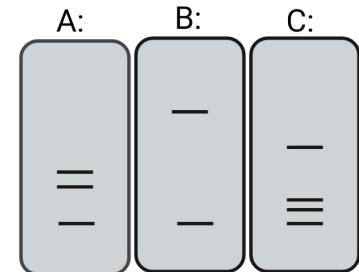
Genetic markers - CAPS

- 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



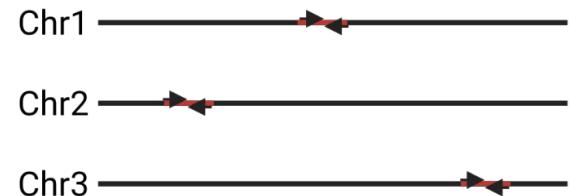
PCR & restriction digest



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:

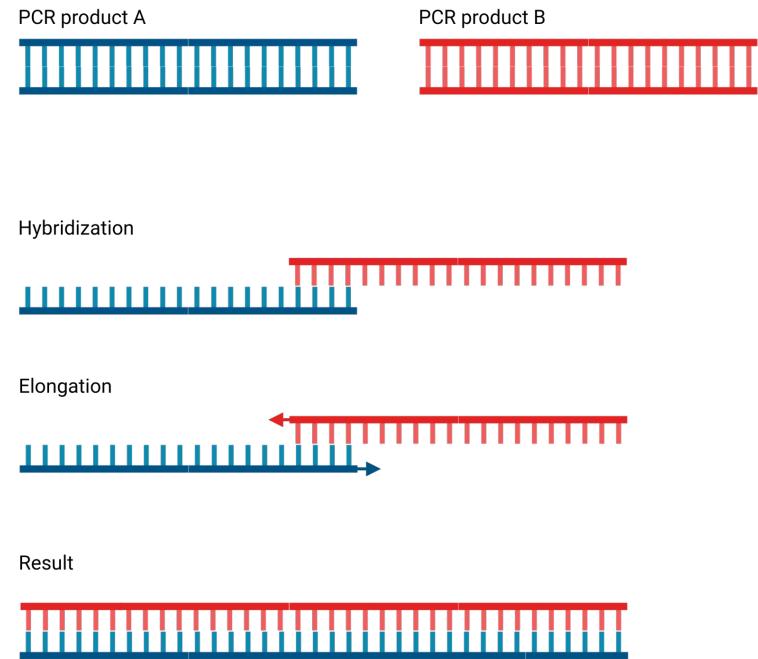


PCR2:



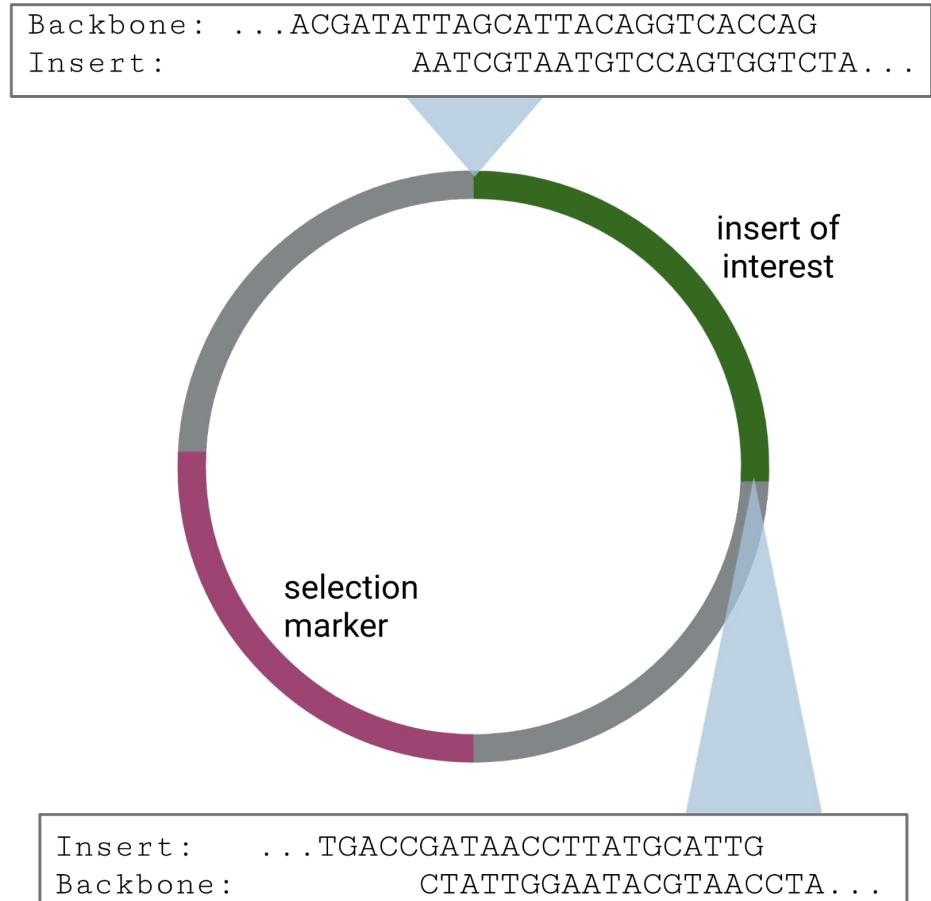
Overlap extension PCR

- 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 homolog regions
- Overlap needs to stabilize interaction at reaction temperature (e.g. 50°C)



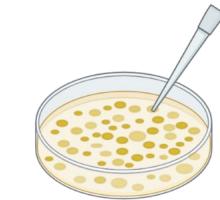
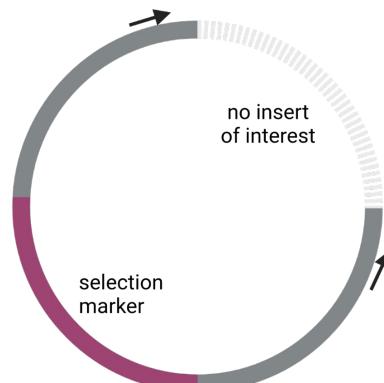
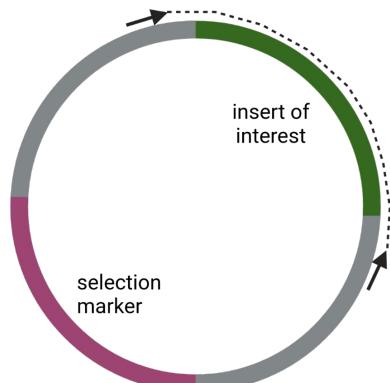
Multiplexing

- 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)

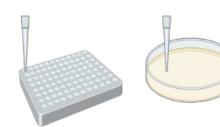


Colony PCR

- 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



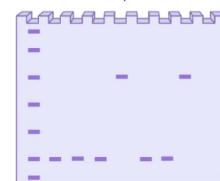
picking bacterial colonies from plate



transfer cells into PCR master mix and onto new plate



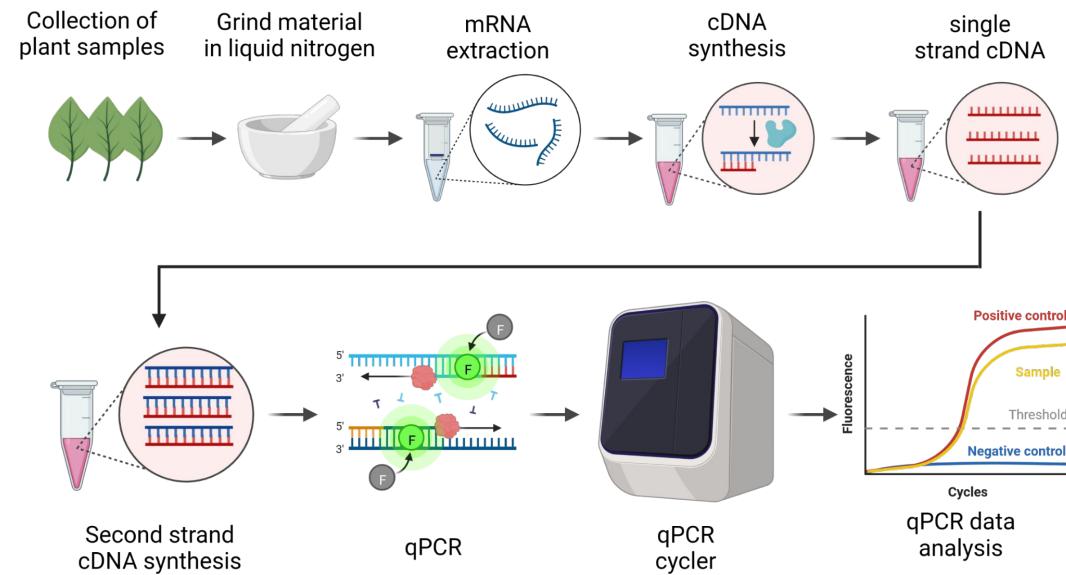
running PCR releases template from bacterial cells



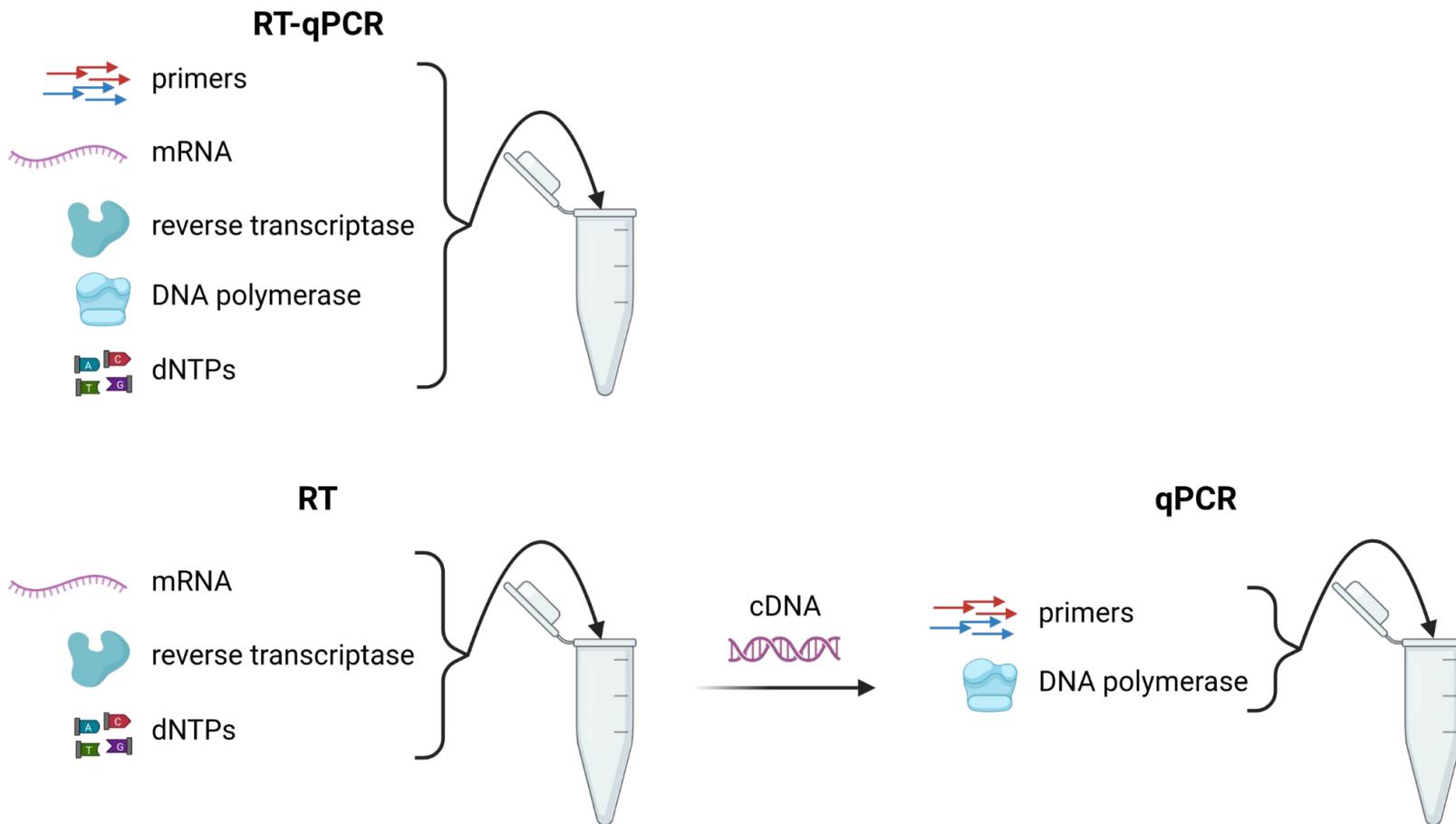
analysis on agarose gel

RT-qPCR

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

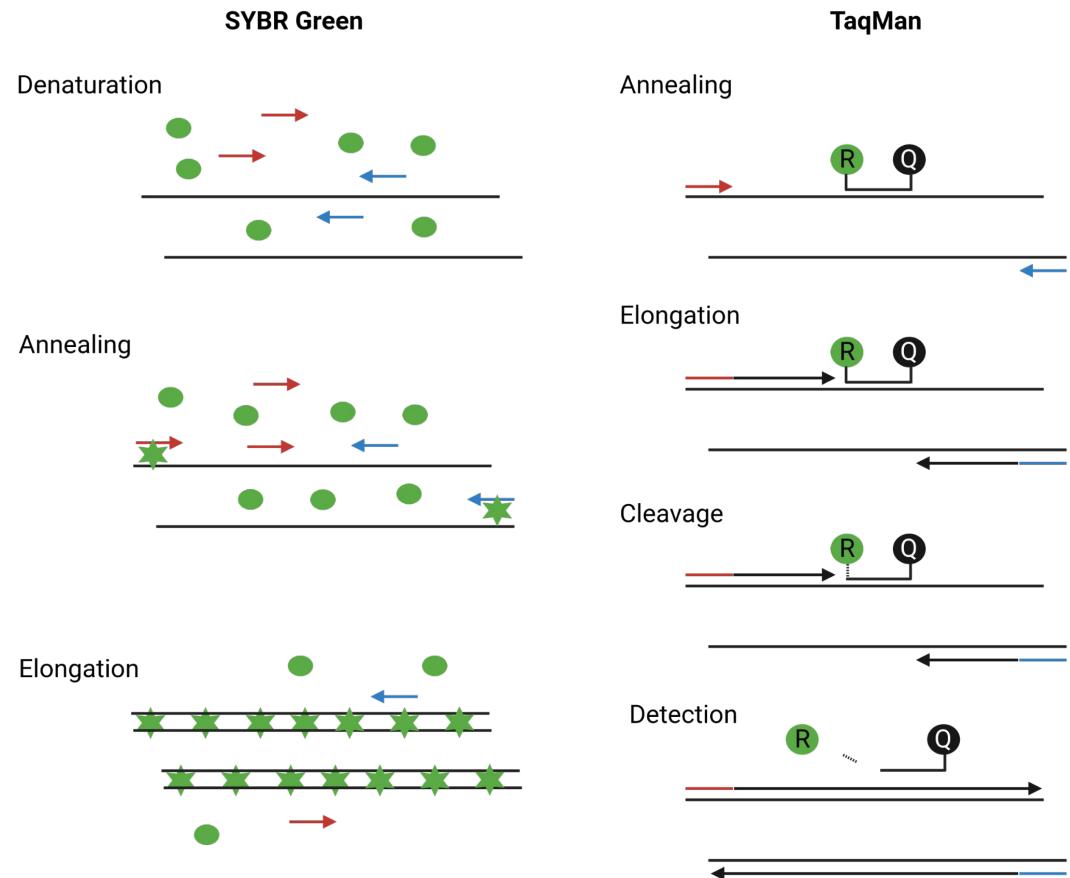


One step vs. two step RT-qPCR



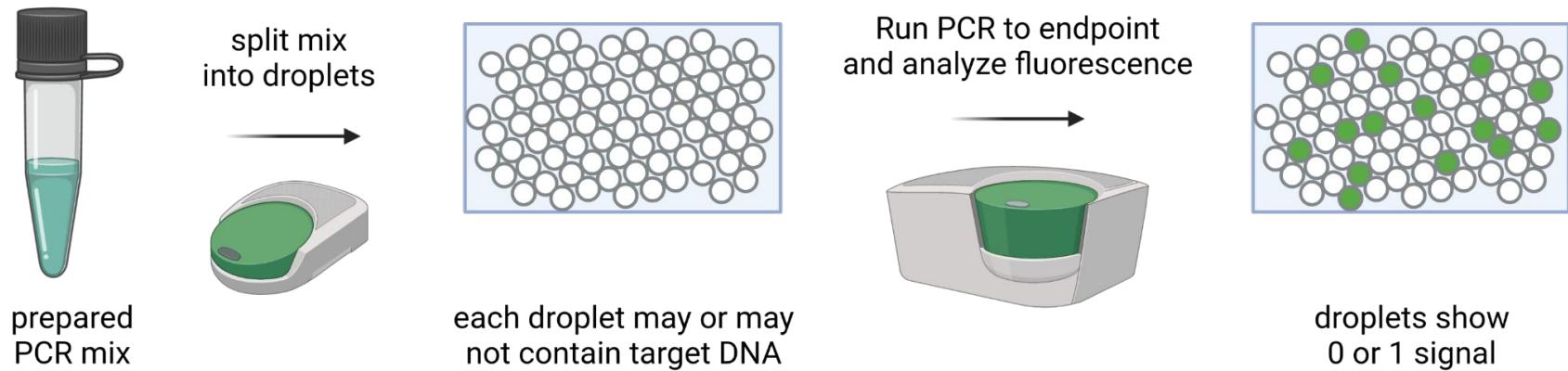
qPCR: SYBR Green vs. TaqMan

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



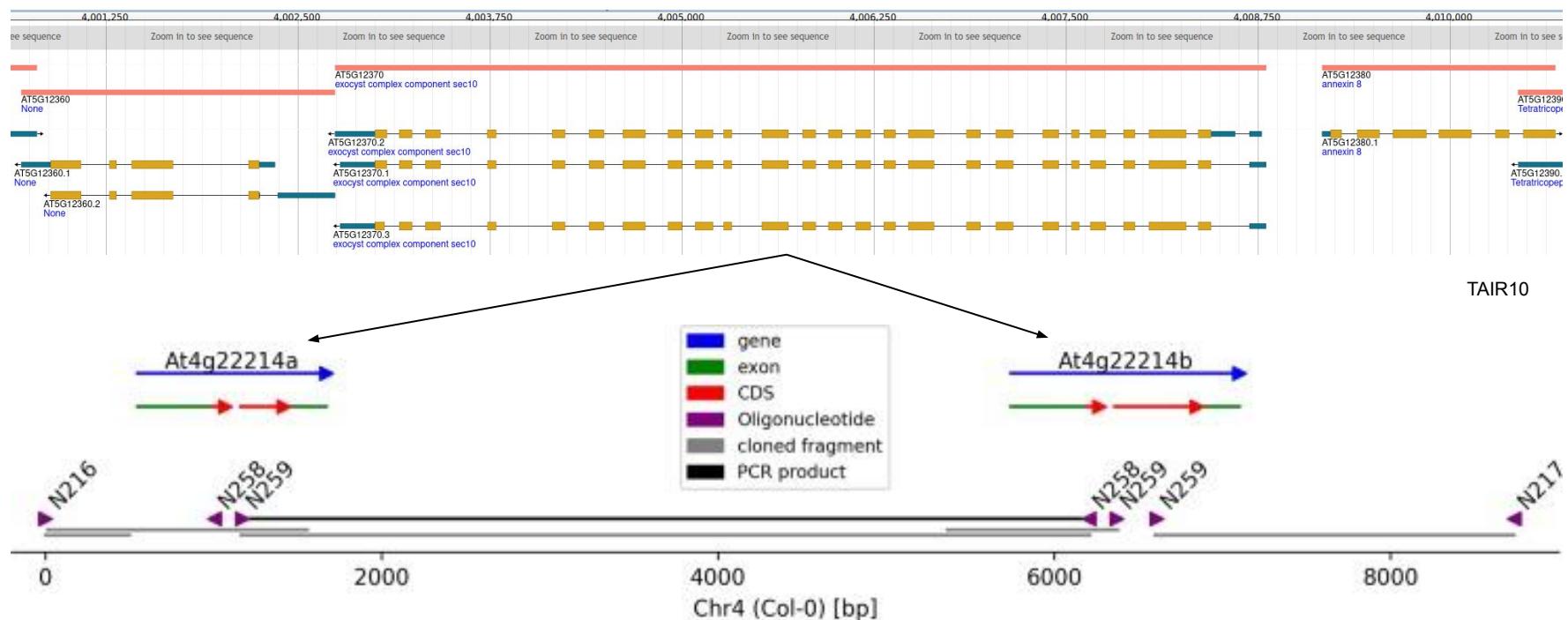
Droplet digital PCR (ddPCR)

- 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)
- Copy number can be quantified



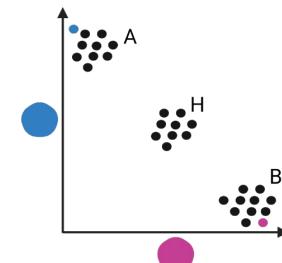
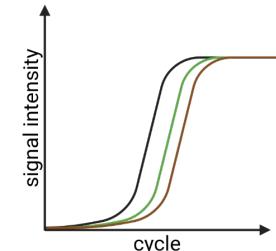
10.1371/journal.pone.0216233

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. Which different types of DNA polymerases are suitable for a PCR?
6. Which types of PCR can be applied to quantify DNA concentrations?

