

# FWD-AMR-RefLabCap 1<sup>st</sup> Training Course

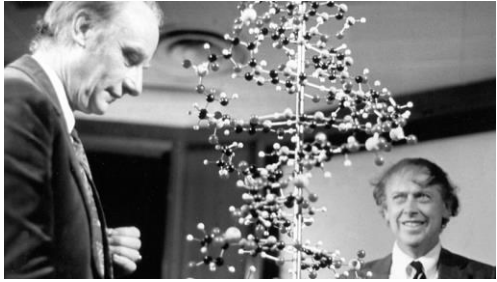
## Introduction to a PCR assay for speciation of *Campylobacter*

Tuesday, 19 May 2022  
12:15 -12:45 CET at DTU Food



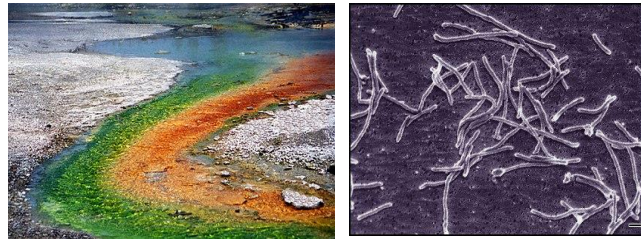
**HaDEA Service Contract 20197409**  
Provision of EU networking and support  
for public health reference laboratory  
functions for antimicrobial resistance in  
*Salmonella* species and *Campylobacter*  
species in human samples

# History of PCR

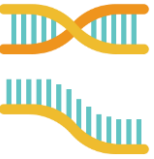


**1953 - Watson and Crick**  
 • Discovery of DNA

**1969 - Brock and Freeze**  
 • First isolation of *Thermus aquaticus*, a thermophilic bacterium



**1985 - Susanne Stoffel & David Gelfand**  
 • Isolation of DNA polymerase from *T. aquaticus*



**1985 - Randy Saikai**  
 • DNA strand separation by **heat (95°C)**  
 • primers binding at 50-60 °C

Pre-PCR experiments

**1957 - Korenberg**  
 • Discovery of **DNA polymerase**



**1971 - Khorana**  
 • Discovery of **genetic code**  
 • DNA repairing and synthesis by **primers**

ARTICLES

Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia

RK Saiki, S Scharf, F Faloona, KB Mullis, GT Horn, HA Erlich, N Arnheim  
 + See all authors and affiliations

Science 20 Dec 1985:  
 Vol. 230, Issue 4732, pp. 1350-1354  
 DOI: 10.1126/science.2999980

**Kjell**  
 Two-primer system  
 First **DNA replication in vitro**





Cold Spring Harbor Symposia  
on Quantitative Biology

HOME | ABOUT | ARCHIVE | PURCHASE | ADVERTISE | ALERTS | CONTACT | HELP

## Specific Enzymatic Amplification of DNA In Vitro: The Polymerase Chain Reaction

K. Mullis, F. Faloona, S. Scharf, R. Saiki, G. Horn, and H. Erlich

### 1986 - Kary Mullis

#### Amplification of DNA

- primers
- nucleotides: building block (A, T, C, G)
- DNA polymerase

#### ARTICLES

### Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase

RK Saiki, DH Gelfand, S Stoffel, SJ Scharf, R Higuchi, GT Horn, KB Mullis, HA Erlich

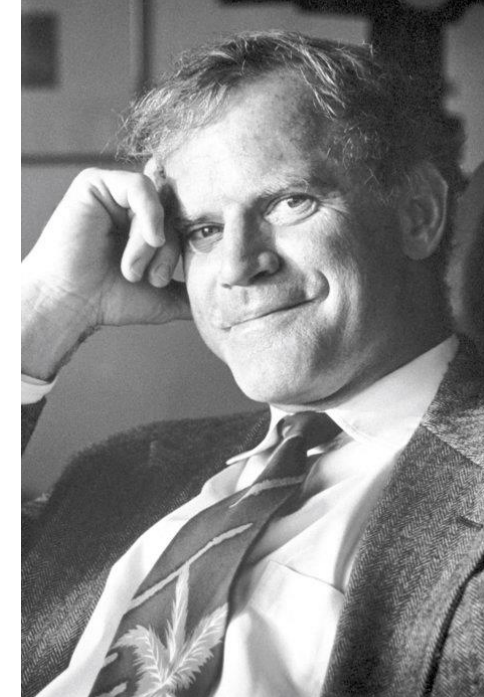
+ See all authors and affiliations

Science 29 Jan 1988:  
Vol. 239, Issue 4839, pp. 487-491  
DOI: 10.1126/science.239.4839.487

### 1988

Use **Taq polymerase** in PCR

*Taq* polymerase –  
molecule of the year in 1990



(www.nobelprize.org)

### Katy B. Mullis (1944-2019)

- Inventor of PCR
- Nobel Prize in Chemistry in 1993

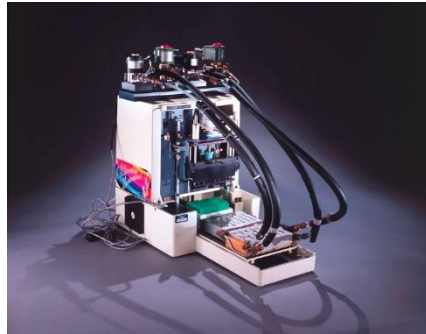


# Histroy of PCR

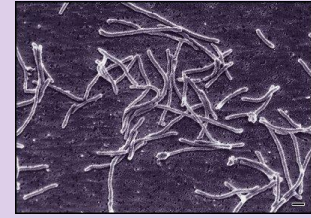


## DNA

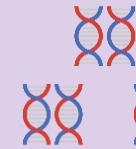
- Isolation of DNA from organisms
- DNA extraction
- DNA template



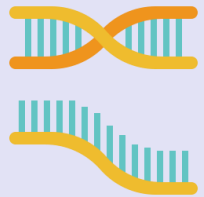
## Amplification of DNA in a test tube using a thermocycler



## DNA Polymerization



## DNA replication



## DNA denaturation

- Separation of DNA strands
- Heating (95 °C)
- Denaturation



## Primers

- Oligonucleotides
- DNA repairing
- Binding to template at 50-60°C- Annealing

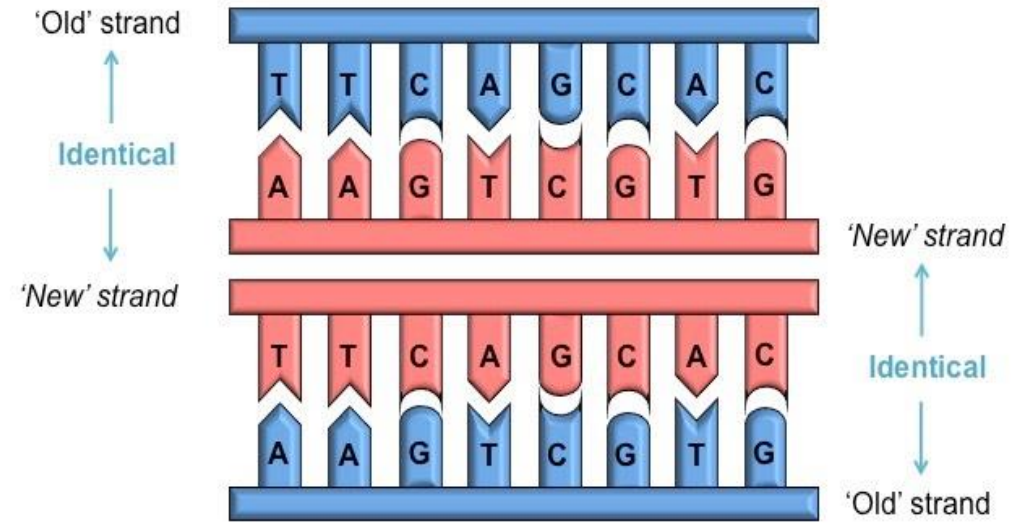
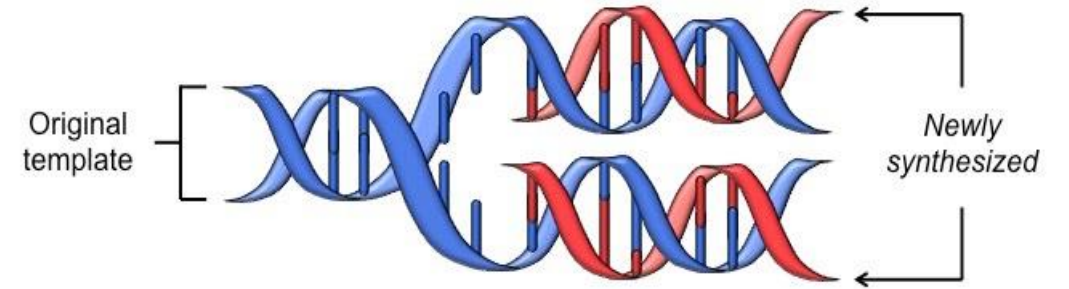
## DNA Polymerization

- *Taq* polymerase
  - Optimal temp. at 72 °C
- Nucleotide (building blocks)
  - A, T, C, G
- Extension or elongation



# DNA Replication

- A process copying genetic information for transmission to the next generation
- A semi-conservative process
  - Original template strand
  - Newly synthesized strand
- Two identical DNA molecules (complementary base pairing)



(<https://ib.bioninja.com.au>)

# PCR - DNA “amplify” technique



DNA template

Denaturation  
(95 °C)

Oligonucleotide primers

Annealing  
(50-60 °C)

Nucleotides

Taq polymerase

Extension  
(72 °C)

2 copies

4 copies

8 copies

1 cycle (1<sup>st</sup>)

(2<sup>nd</sup>)

(3<sup>rd</sup>)

No. of copies =  $2^n$   
(n = cycles)

Example

No. of copies =  $2^3 = 8$  copies





# Steps of PCR



PCR thermocycler

- **Initial denaturation**

95 °C for 5-10 min

- **Denaturation**

95 °C for 30 sec

- **Annealing**

$T_a$  °C for 30 sec

( $T_a$  = annealing temperature)

- **Extension**

72 °C for  $t$  sec

( $t$  = extension time)

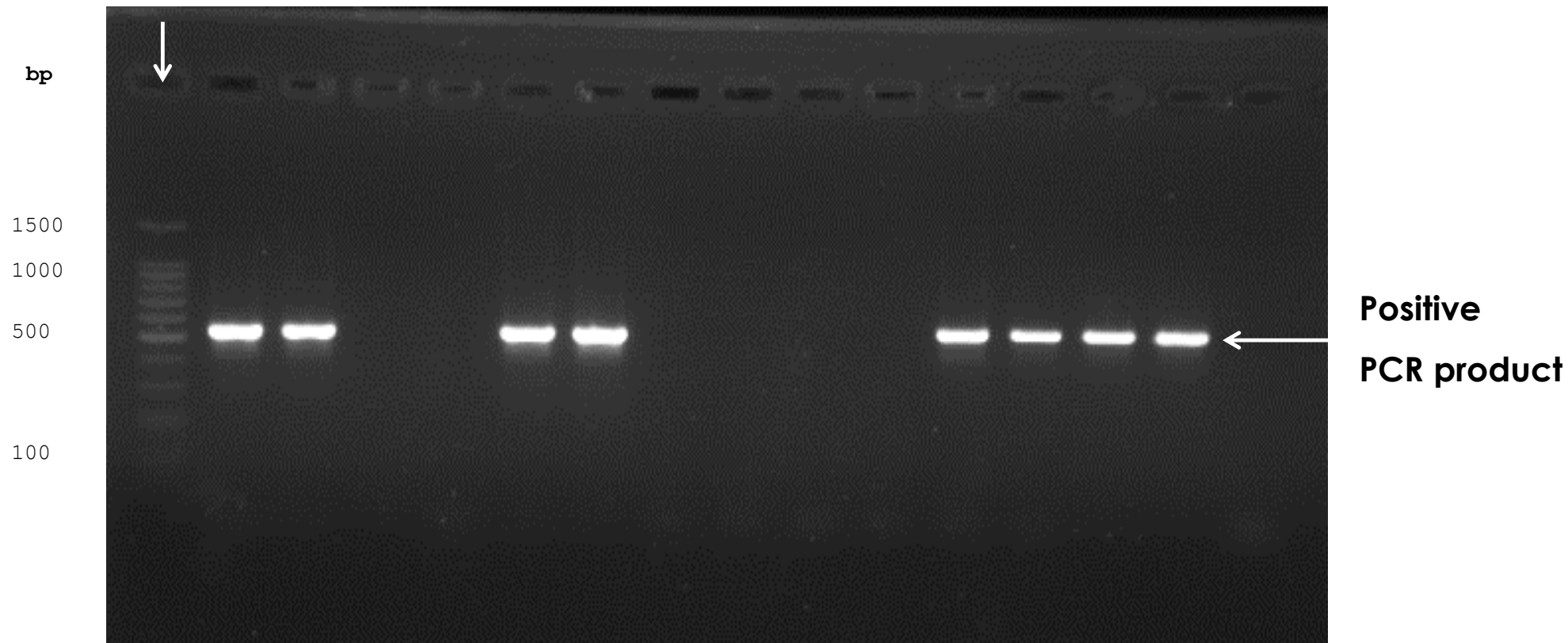
30 cycles

- **Final extension**

72 °C for 5-10 min

# Visualization of PCR

100 base pair DNA ladder marker





# Types of PCR

## Standard or conventional PCR



DNA template



Oligonucleotide primers



Nucleotide (A, T, C, G)



*Taq* polymerase

others eg. Mg<sup>2+</sup>, buffer

## Multiplex PCR

- Amplify more than one specific DNA segments



DNA template



Nucleotide (A, T, C, G)



Primers

(>1 pairs)



*Taq* polymerase

## Real-time PCR

- Real-time report of amplified fragment



+ Reporter

eg. fluorescent probes etc.



DNA template



Nucleotide (A, T, C, G)



Oligonucleotide primers



*Taq* polymerase



# Advantages of PCR

## Why PCR ?

“lets you pick the piece of DNA you’re interested in and have as much of it as you want”

(Mullis, 1990)

- A very sensitive technique
- Rapid amplification
- Specificity by primers

makes billion of copies of  
a specific DNA fragment  
or gene



## PCR product

- Detection by visualization
  - Diagnosis
    - Infectious diseases
    - Direct from sample
    - Culture-independent
  - Gene sequencing
  - Other molecular techniques

# Limitations of PCR

## A very sensitive technique

- Sensitive to contaminated DNA also
- False positive result

## Requirement

- Skills
- Laboratory capacities
- Prior DNA sequence data
  - Primer designation

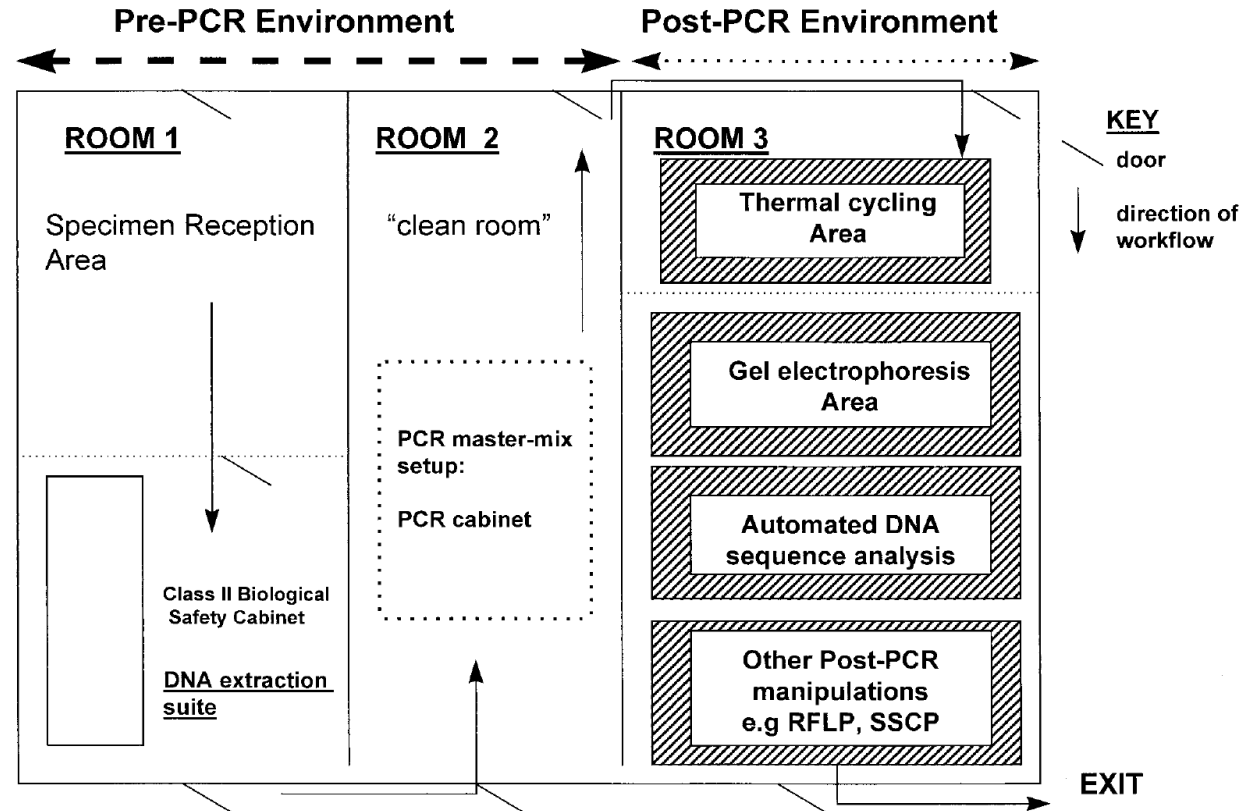


Figure. Proposed layout of PCR laboratory space

Millar et al. (2002) J Clin Microbiol. 40(5): 1575-1580.

# How to perform PCR



1

Make a plan



2

Prepare PCR components



3

Set PCR reaction



4

Run thermocycler



5

Run electrophoresis



6

PCR product visualization



# Make a plan for PCR

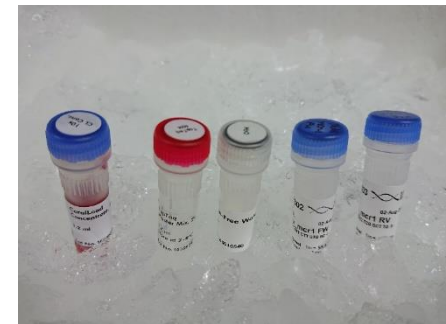
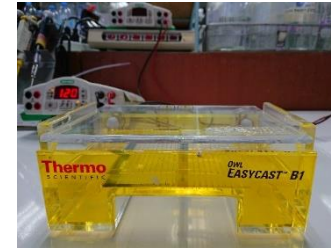
## Materials

- Molecular marker (Ladder 100bp)
- Electrophoresis buffer (TAE or TBE)
- Tips (filter) for pipettes 1  $\mu$ L to 1000  $\mu$ L
- Agarose
- PCR components
- TE buffer
- Tris HCl buffer
- Crushed ice
- Mineral oil (if necessary)
- etc

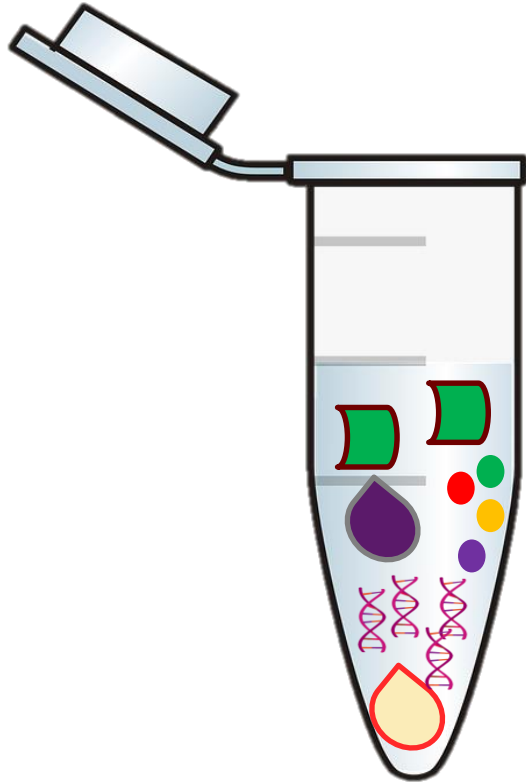


## Equipment

- PCR thermocycler
- Pipettes for 1  $\mu$ L to 1000  $\mu$ L
- Eppendorf tubes
- PCR tubes
- Electrophoresis unit
- Microwave
- Autoclave
- Eppendorf centrifuge (PCR tubes)
- Photo camera
- UV-transilluminator
- Water bath



# Components of a PCR reaction



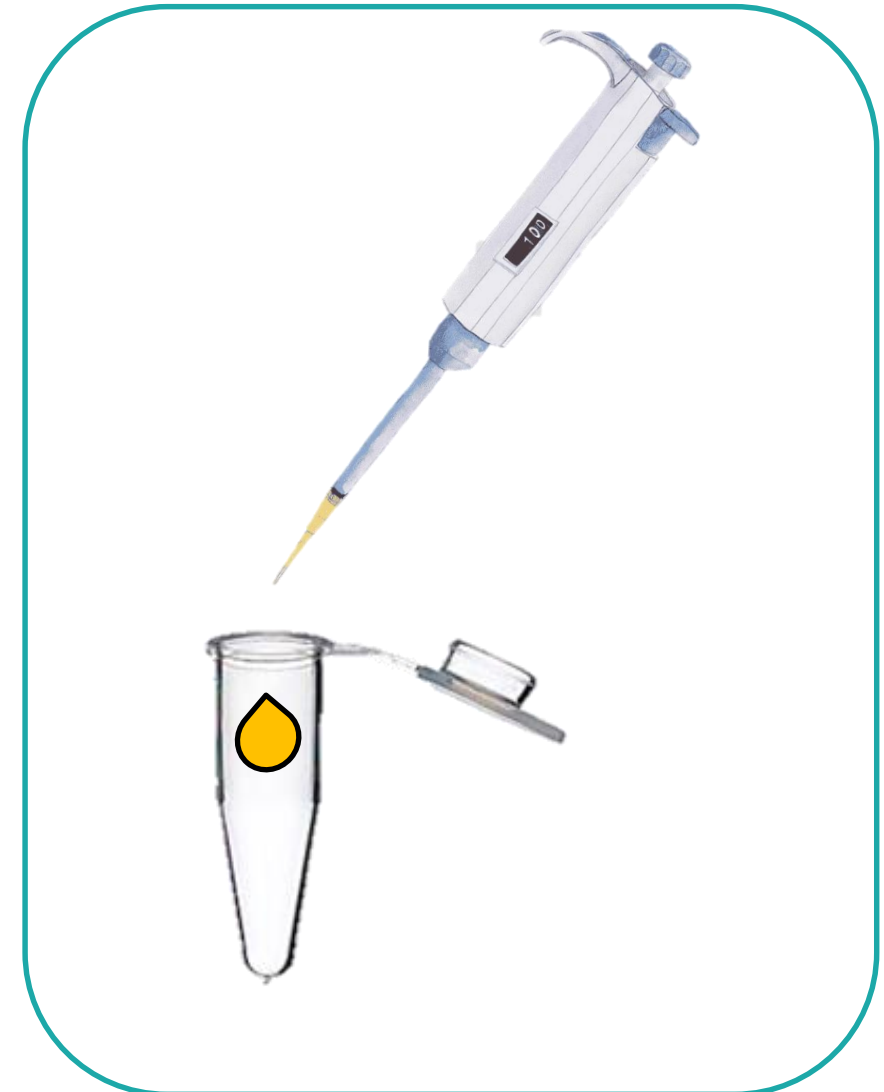
- Template DNA
- Thermostable DNA polymerase
- Primers
- dNTPs
- Buffer and salts (KCl, MgCl<sub>2</sub>)
- PCR grade water
- Optional: DMSO, Formamide



# Components of a PCR reaction

## Template DNA

- ✓ **A starting amount in a 50  $\mu$ L PCR,**
  - 0.1–1 ng of plasmid DNA
  - 5–50 ng of gDNA
- ✓ Optimal template amounts vary based on the type of DNA polymerase used.
- ✓ Optimization of DNA amounts
  - $\uparrow$  Amounts,  $\uparrow$  Non-specific amplification
  - $\downarrow$  Amounts,  $\downarrow$  PCR product yields



# Components of a PCR reaction

## Preparation of DNA template

### DNA extraction

- Purifying DNA by using physical and/or chemical methods from a sample

#### Conventional methods

- Alkaline lysis
- Phenol chloroform extraction

#### Commercial kits

- Pure DNA if used properly
- Phenol chloroform extraction

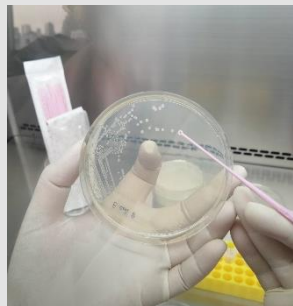
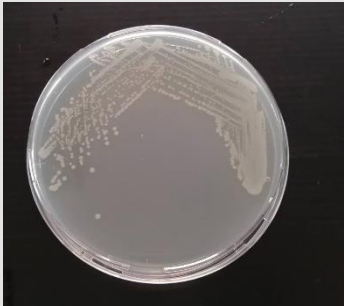
#### Boiling methods

- Use heat to lyse bacterial cell.
- Whole cell DNA
- Simple, reproducible, rapid and economical
- Contain protein, enzyme etc

# Components of a PCR reaction

## Preparation of DNA template

### Boiling methods



✓ Suspend a loopful of culture in 100 µl of sterile DW.

✓ Boiled 10 min at 100°C

✓ Place on ice

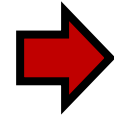
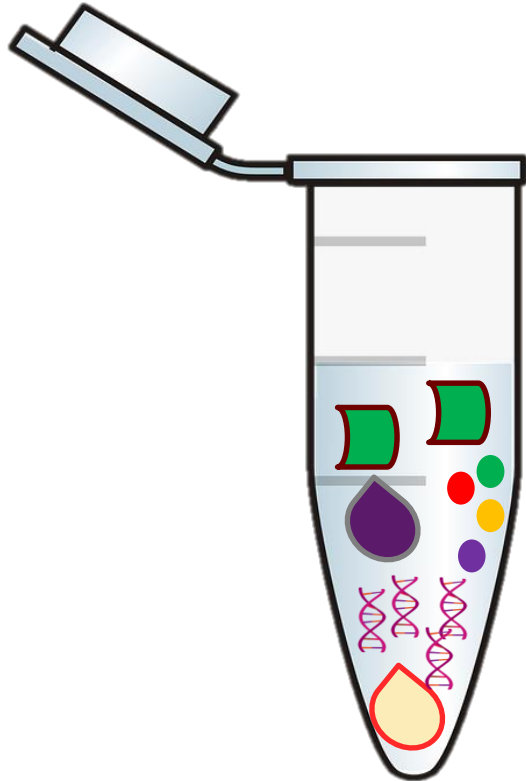


✓ Store at -20°C

✓ Transfer supernatant to a new tube

✓ Centrifuged 5 min at 12,000-13,000xg

# Components of a PCR reaction



- Template DNA
- Thermostable DNA polymerase
- Primers
- dNTPs
- Buffer and salts (KCl, MgCl<sub>2</sub>)
- PCR grade water
- Optional: DMSO, Formamide

## Thermostable DNA polymerase

### Taq Polymerases

a thermostable enzyme from hyperthermophilic *Thermus aquaticus*.

Most active around **70-72°C**.

incorporates  $\approx 1,000$  bp per a min

makes an error in approx. every 125,000 nucleotides

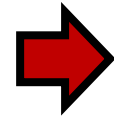
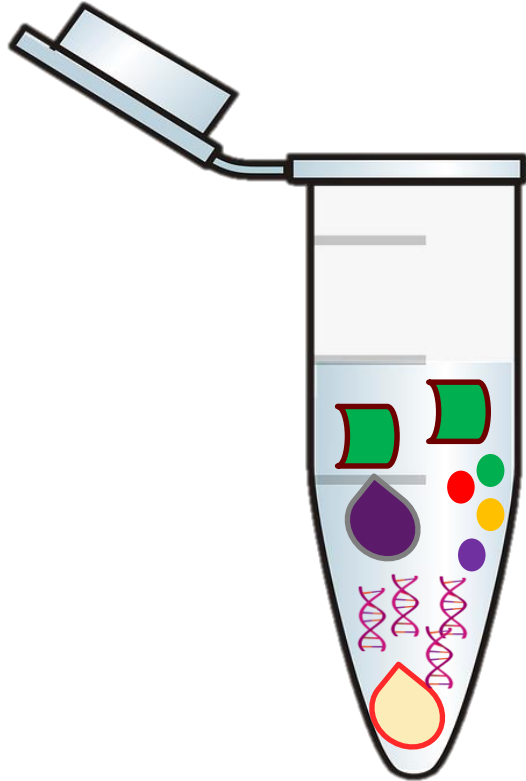
0.5–2 units of DNA polymerase are sufficient in a typical 50  $\mu$ L reaction.

“Hot start“

- contain a thermolabile inhibitor that is denatured during denaturation and active polymerase is released.
- improve the specificity of the PCR amplification



# Components of a PCR reaction



- Template DNA
- Thermostable DNA polymerase
- Primers
- dNTPs
- Buffer and salts (KCl, MgCl<sub>2</sub>)
- PCR grade water
- Optional: DMSO, Formamide

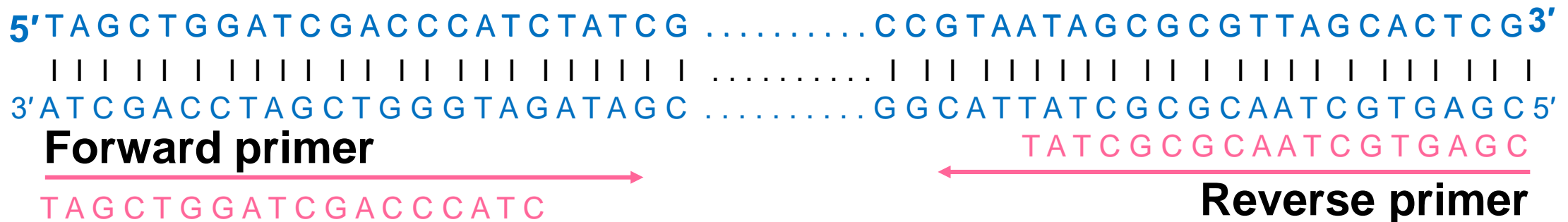


# Components of a PCR reaction

## Oligonucleotide primers

- A piece of single-stranded DNA that matches the sequences at the ends of or within the target DNA in such a way that the 3' end of it is available to serve as the starting point for the new DNA.

## Template (target) DNA



# Components of a PCR reaction

## Primer criteria :

Optimal length = 18-25 bases long (up to 30)

Best to have G or C ( $\leq 3$  base) at least 5 bases from 3' end

Should contain 40-60% G + C content

Primers should have approximately equal melting temperatures (within 5°C)

No self-complementary sequence between primers

Avoid runs!! No inverted repeat sequence (Max. 3 bp)

Avoid repeats for TATATATA (max. 4 bp)

## How to get primers

- Design primers
- Send for custom oligo synthesis

- Prepare and use



# Components of a PCR reaction

## Primer design



Manual design



Computer-assisted design

## What we need to know:

- Sequence of target DNA
- Expected PCR amplicon size
- Melting temperature ( $T_m$ )

# Melting temperature ( $T_m$ )

## What is $T_m$ ?

The temperature at which one-half of the DNA duplex will dissociate to become single stranded.

## What is annealing temperature ( $T_a$ )?

- Temperature that primers bind to template DNA
- Annealing temperature is about 5°C below the  $T_m$  of the primers.
- Optimal annealing temperatures give the highest product yield of the correct amplicon.

- **If  $T_a$  is too low**  
= nonspecific PCR amplification
- **If  $T_a$  is too high**  
= reduced yield of PCR amplicons

# Melting temperature (T<sub>m</sub>)



## T<sub>m</sub> calculation

$$T_m = 2AT + 4GC$$

- Good for 15-20 bp primers
- High ionic strength solvent

$$\text{Annealing temperature (T}_a\text{)} = T_m - 5$$

5' - G T G C T G G A T C G A C C C T A C -3'

$$\begin{aligned} T_m &= 2AT + 4GC \\ &= (2 \times 7) + (4 \times 11) \\ &= 58 \end{aligned}$$

$$\begin{aligned} T_a &= T_m - 5 \\ &= 58 - 5 = 53 \\ &= 53 \end{aligned}$$

$$T_m = 81.5 + 16.6(\log K^+) + 0.41[\%(\text{G+C})] - (675/n)$$

N = primer length

K = concentration of cations (nM)

- Good for 14-70 bp primers
- Cation concentration of  $\leq 4\text{M}$



# Melting temperature (T<sub>m</sub>)

## T<sub>m</sub> calculation - Online software

**ThermoFisher SCIENTIFIC** Popular Applications & Techniques Shop All Products Services Support

Search All Search by catalog number, product name, keyword, application

Home > Brands > Thermo Scientific > Molecular Biology > Molecular Biology Resource Library > Thermo Scientific

### T<sub>m</sub> Calculator

This tool calculates the T<sub>m</sub> of primers and estimates an appropriate annealing temperature. [to use this calculator >](#)

Quickly find the right annealing temperature for [Platinum SuperFi DNA polymerase](#) and [Phire DNA polymerase](#).

**Important note:** If the PCR primer contains desired mismatches, e.g., for the T<sub>m</sub> only for the correctly matched sequence

### T<sub>m</sub> Calculator

version 1.13.0

Use the NEB T<sub>m</sub> Calculator to estimate an appropriate annealing temperature when using NEB PCR products.

**Product Group:** Q5

**Polymerase/Kit:** Q5 High-Fidelity DNA Polymerase

**Primer Concentration (nM):** 500 Reset concentration

**Instructions:**

- Select the product group of the polymerase or kit you plan to use.
- Select the polymerase or kit

**Anneal at:** --- °C

**Primer 1:** --- nt, ---% GC, T<sub>m</sub>: ---°C

**Primer 2:** --- nt, ---% GC, T<sub>m</sub>: ---°C

Clear Use example input

### Melting Temperature (T<sub>m</sub>) Calculation

**Primer (6-50 bases):** GTATGTGTATATATATGT Compute T<sub>m</sub>

LENGTH	20
C+G%	25
Molecular weight:	6272.715

[Basic T<sub>m</sub>](#)  
Degenerated nucleotides are allowed

[Base-Stacking T<sub>m</sub>](#)  
Degenerated nucleotides are NOT allowed

Primer concentration:  nM

Salt concentration:  mM

Mg<sup>2+</sup> concentration:  mM

**T<sub>m</sub>:** 41.3 °C

Enthalpy: -145.6  
Entropy: -430.95

<http://insilico.ehu.es/>





# Internet software for primer design

ncbi.nlm.nih.gov/guide/howto/design-pcr-primers/

NCBI Resources How To

NCBI National Center for Biotechnology Information

All Databases Search

- NCBI Home
- Resource List (A-Z)
- All Resources
- Chemicals & Bioassays
- Data & Software
- DNA & RNA
- Domains & Structures
- Genes & Expression
- Genetics & Medicine
- Genomes & Maps
- Homology
- Literature
- Proteins
- Sequence Analysis
- Taxonomy
- Training & Tutorials
- Variation

**How to: Design PCR primers and check them for specificity**

Starting with ...

**ONE OR MORE PRIMER SEQUENCES**

1. Go to the [Primer BLAST](#) submission form.
2. Enter one or both primer sequences in the Primer Parameters section of the form. If only one primer is available, a template sequence is also required. See "A Target Template Sequence..." below.
3. In the Primer Pair Specificity Checking Parameters section, select the appropriate source Organism and the smallest Database that is likely to contain the target sequence. These settings give the most precise results. For broadest coverage, choose the nr database and do not specify an organism.
4. Click the "Get Primers" button to submit the search and retrieve template and specificity information.

**A TARGET TEMPLATE SEQUENCE OR ACCESSION NUMBER**

1. Go to the [Primer BLAST](#) submission form.
2. Enter the target sequence in FASTA format or an accession number of an NCBI mRNA reference sequence accession number is used, the tool will automatically generate a target template sequence.
3. If one or both primer sequences are to be used in the search, enter these in the Primer Parameters section. The tool will automatically perform a specificity check when a target template and both primers are provided.
4. In the Primer Pair Specificity Checking Parameters section, select the appropriate source Organism and the smallest Database that is likely to contain the target sequence. These settings give the most precise results. For broadest coverage, choose the nr database and do not specify an organism.
5. Click the "Get Primers" button to submit the search and retrieve specific primer information.

GenScript® Make Research Easy

Gene Synthesis

COVID-19 Detection Reagent Services Biologics Services Catalog Products Applications Resources Investors About Us

- Analyze the results of your PCR reaction via gel electrophoresis.

**PCR design tools**

- PCR Primer Design
- Oligo Calculator for Tm, MV, and µg/OD
- Restriction Enzyme Map Analysis
- Common Restriction Enzyme Sites

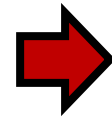
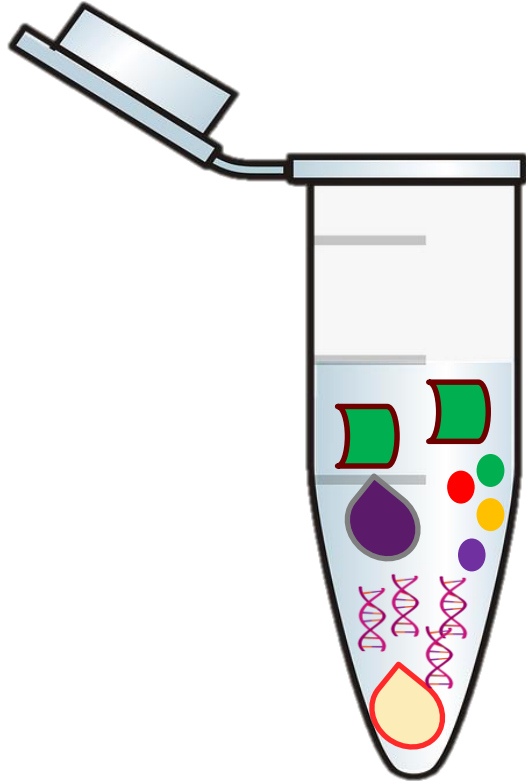
**Primer3** (v. 0.4.0) Pick primers from a DNA sequence.

There is a newer version of Primer3 available at <http://primer3.ut.ee>

Paste source sequence below (5'->3', string of ACGTNacgtn -- other letters treated as N -- numbers LINEs, etc.) or use a [Mispriming Library \(repeat library\)](#):



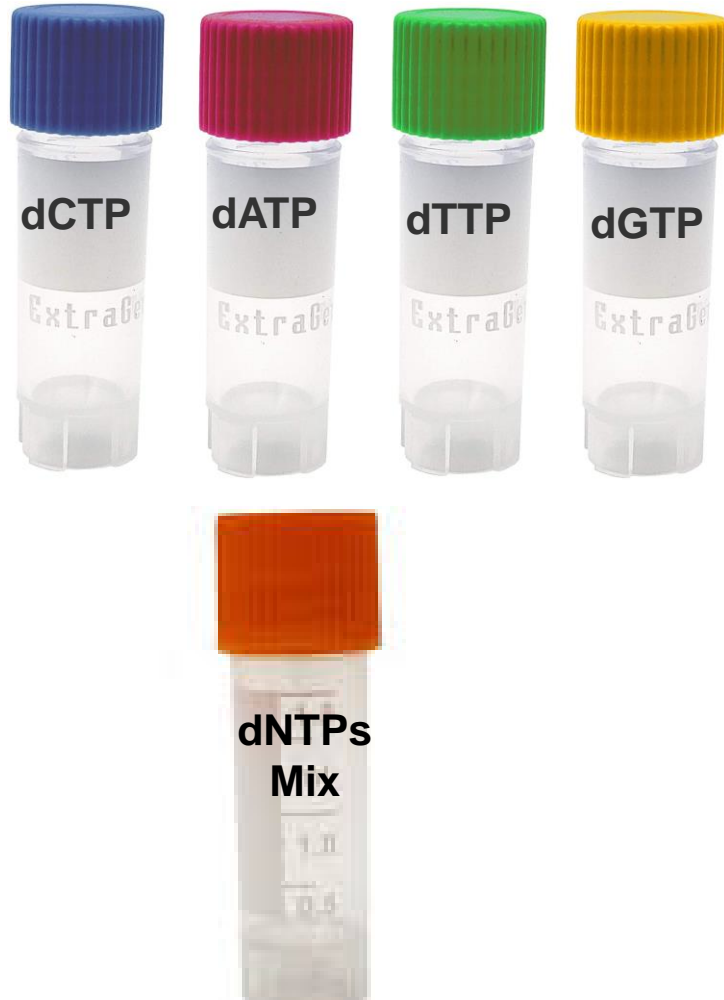
# Components of a PCR reaction



- Template DNA
- Thermostable DNA polymerase
- Primers
- dNTPs
- Buffer and salts (KCl, MgCl<sub>2</sub>)
- PCR grade water
- Optional: DMSO, Formamide

# Components of a PCR reaction

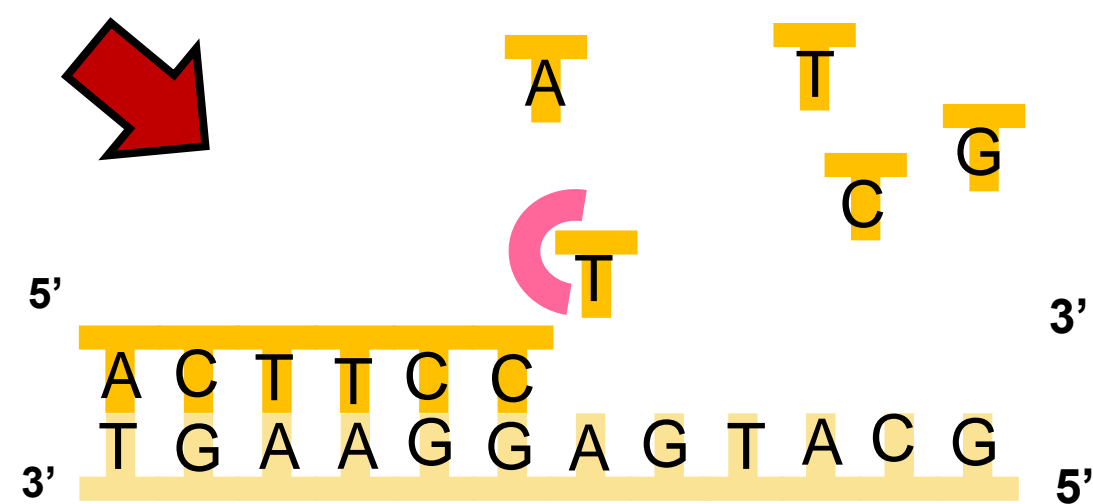
## Deoxynucleotide Triphosphates (dNTPs)



- Four nucleotides are typically added to the PCR reaction in equimolar amounts for optimal base incorporation.
- Incorporate into a new DNA strand by DNA polymerase
- For efficient incorporation by DNA polymerase, free dNTPs should be present in the reaction at a concentration of **no less than 0.010–0.05 mM**.
- In most PCR applications, the recommended final concentration of each dNTP is generally 200  $\mu$ M.

# Components of a PCR reaction

## Deoxynucleotide Triphosphates (dNTPs)



- In the extension step, DNA polymerase adds free dNTPs from the reaction mixture to the template DNA in the 5'-to-3' direction = the elongation of DNA strand.
- $Mg^{2+}$  binds to dNTPs and reduces dNTPs availability for incorporation. Therefore, higher dNTPs concentrations may be needed.
- When using non-proofreading DNA polymerases, fidelity can be improved by lowering dNTP concentrations (0.01–0.05 mM) and proportionally reducing  $Mg^{2+}$ .

DNA polymerase

# Components of a PCR reaction

## PCR grade water

purified

double-distilled

deionized

autoclaved.

**Must free of all DNA, RNase and DNase contamination.**



# Setting up traditional PCR reaction



Keep and thaw all reagents on ice.

Assemble reaction mix into 50  $\mu$ L volume in a thin walled 0.2 mL PCR tubes.

**Add reagents in following order:**

Water  $\rightarrow$  buffer  $\rightarrow$  dNTPs  $\rightarrow$  MgCl<sub>2</sub>  $\rightarrow$  template DNA  $\rightarrow$  primers  $\rightarrow$  Taq polymerase.

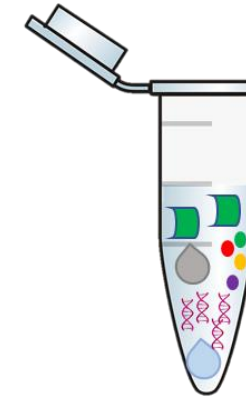
Gently mix by tapping tube. Briefly centrifuge to settle tube contents.

Prepare negative control reaction without template DNA.

Prepare positive control reaction with template of known size and appropriate primers.

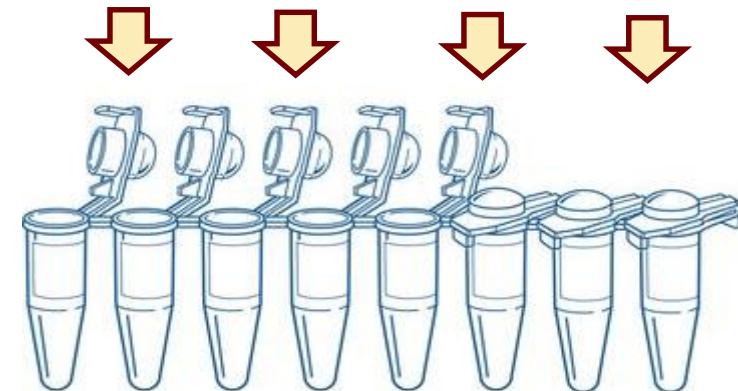
# Setting up a PCR cocktail

- PCR cocktail is good for multiple PCR experiments.
- assemble a mixture of reagents common to all reactions (i.e., Master Mix).
- The amount of each reagent added to the cocktail is equivalent to the total number plus one whole reaction.



**PCR cocktail**  
 DNA polymerase  
 dNTPs  
 reaction buffer  
 water

**Aliquot into PCR tubes**



**Add DNA as the last step**



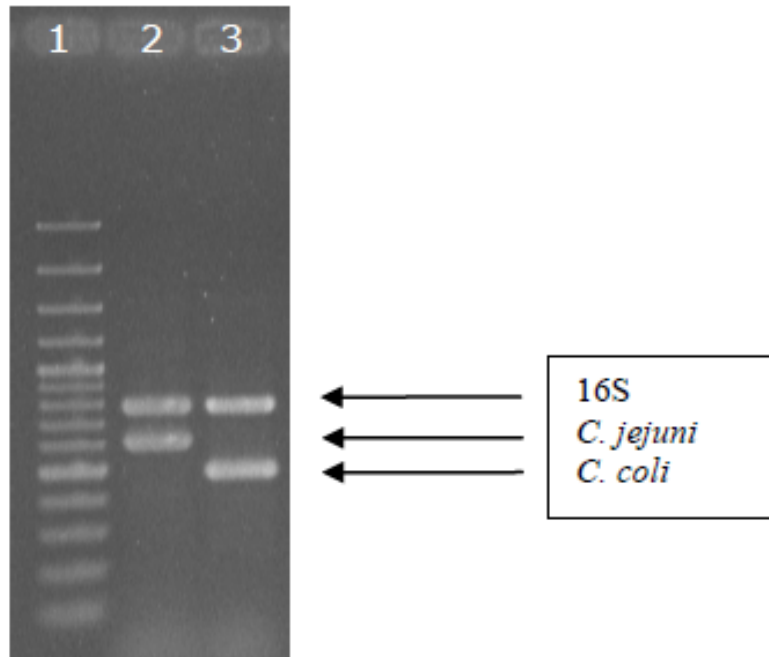
# To prevent lots of confusion...

...use PCR to confirm *C. jejuni* and *C. coli*



# To prevent lots of confusion...

...use PCR to confirm *C. jejuni* and *C. coli*



- The PCR protocol target the identification of *Campylobacter jejuni* and *Campylobacter coli* based on the amplification of the two genes:
  - *mapA* in *C. jejuni*
  - *ceuE* in *C. coli*

**Figure 1.** Multiplex PCR for detection of *C. jejuni* and *C. coli*

Lane 1: 100 bp ladder

Lane 2: *Campylobacter jejuni* ATCC 33560 (CCUG 11284)

Lane 3: *Campylobacter coli* ATCC 33559 (CCUG 11283)

M. Denis, C. Soumet, K. Rivoal, G. Ermel, D. Blivet, G. Salvat and P. Colin. Development of a m-PCR assay for simultaneous identification of *Campylobacter jejuni* and *C. coli*. Letters in Applied Microbiology 1999, 29, 406–410

# Acknowledgement

Curtesy to:

Dr. Taradon Luangtongkum D.V.M., Ph.D.

Assist. Prof. Pattrarat Chanchaithong DVM PhD

Professor Rungtip Chuanchuen DVM MS PhD

Faculty of Veterinary Science, Chulalongkorn University

Bangkok, Thailand

for sharing some slides

# Thank you for your attention

Prof. Rene S. Hendriksen, PhD

Head of Research Group Global Capacity Building

WHO Collaborating Centre for Antimicrobial Resistance in Food borne Pathogens and Genomics

European Union Reference Laboratory for Antimicrobial Resistance

FAO Reference Laboratory for Antimicrobial Resistance

National Food Institute, Technical University of Denmark

[rshe@food.dtu.dk](mailto:rshe@food.dtu.dk)

