FWD-AMR-RefLabCap 1st 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

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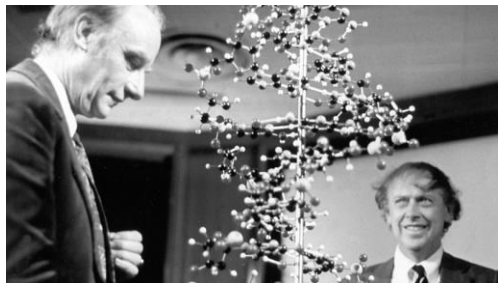
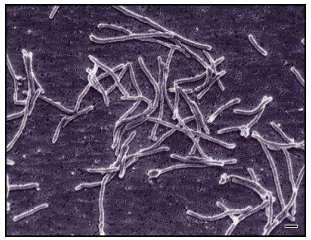
History of PCR



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***

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Histroy of PCR

Cold Spring Harbor Symposia on Quantitative Biology

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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

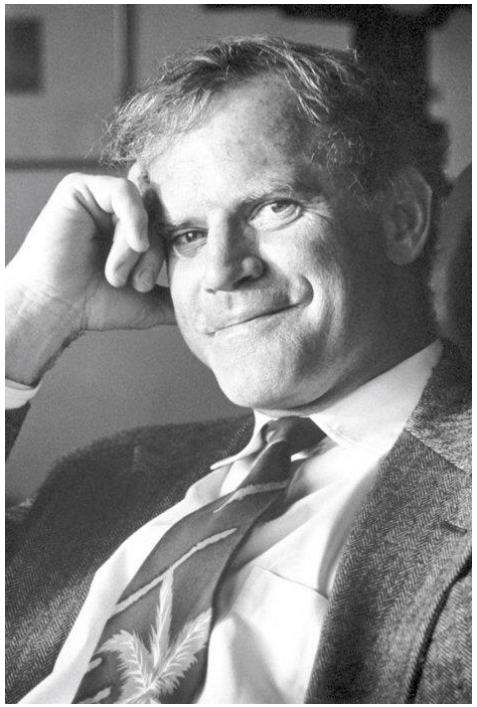
+ See all authors and affiliations

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

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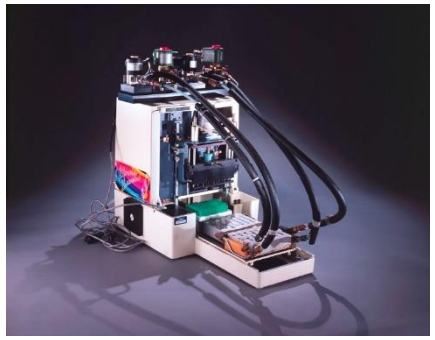
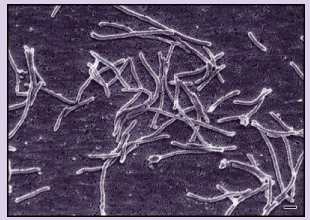
Histroy of PCR

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

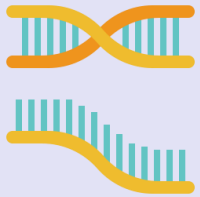


Amplification of DNA in a test tube using a thermocycler

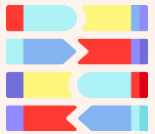


DNA Polymerization

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

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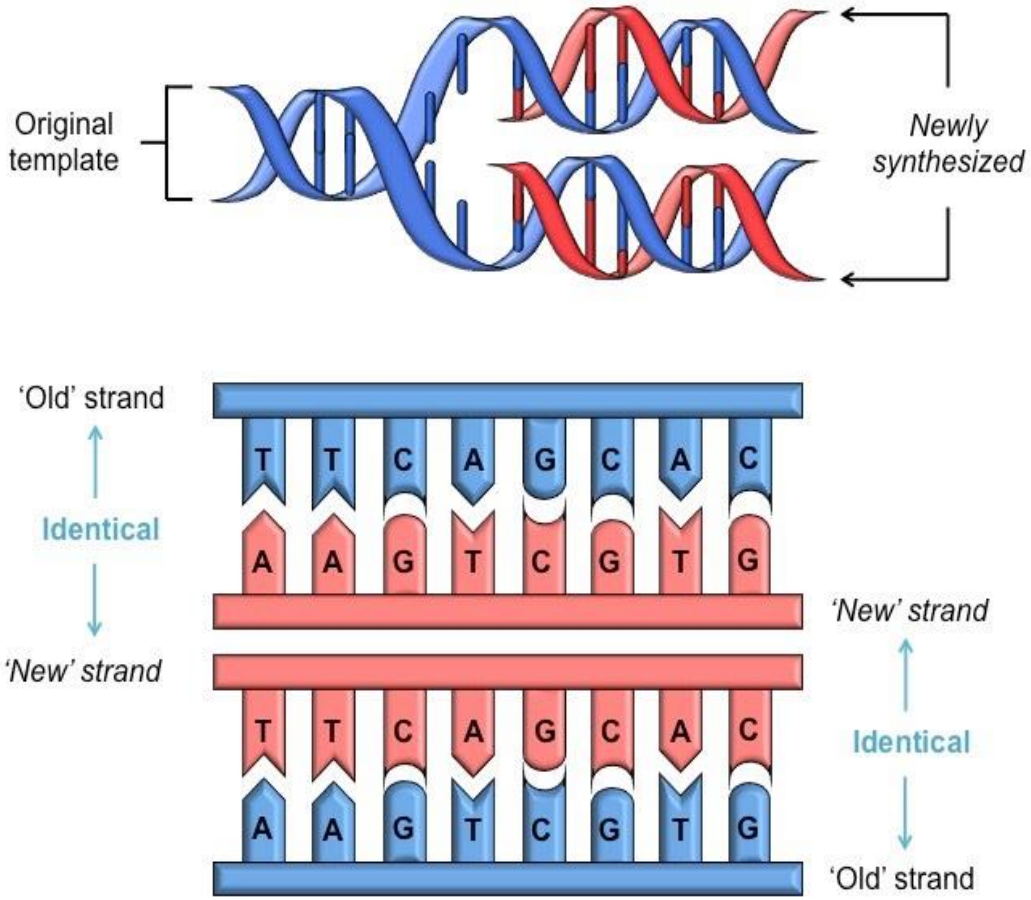
DNA Replication

- 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)

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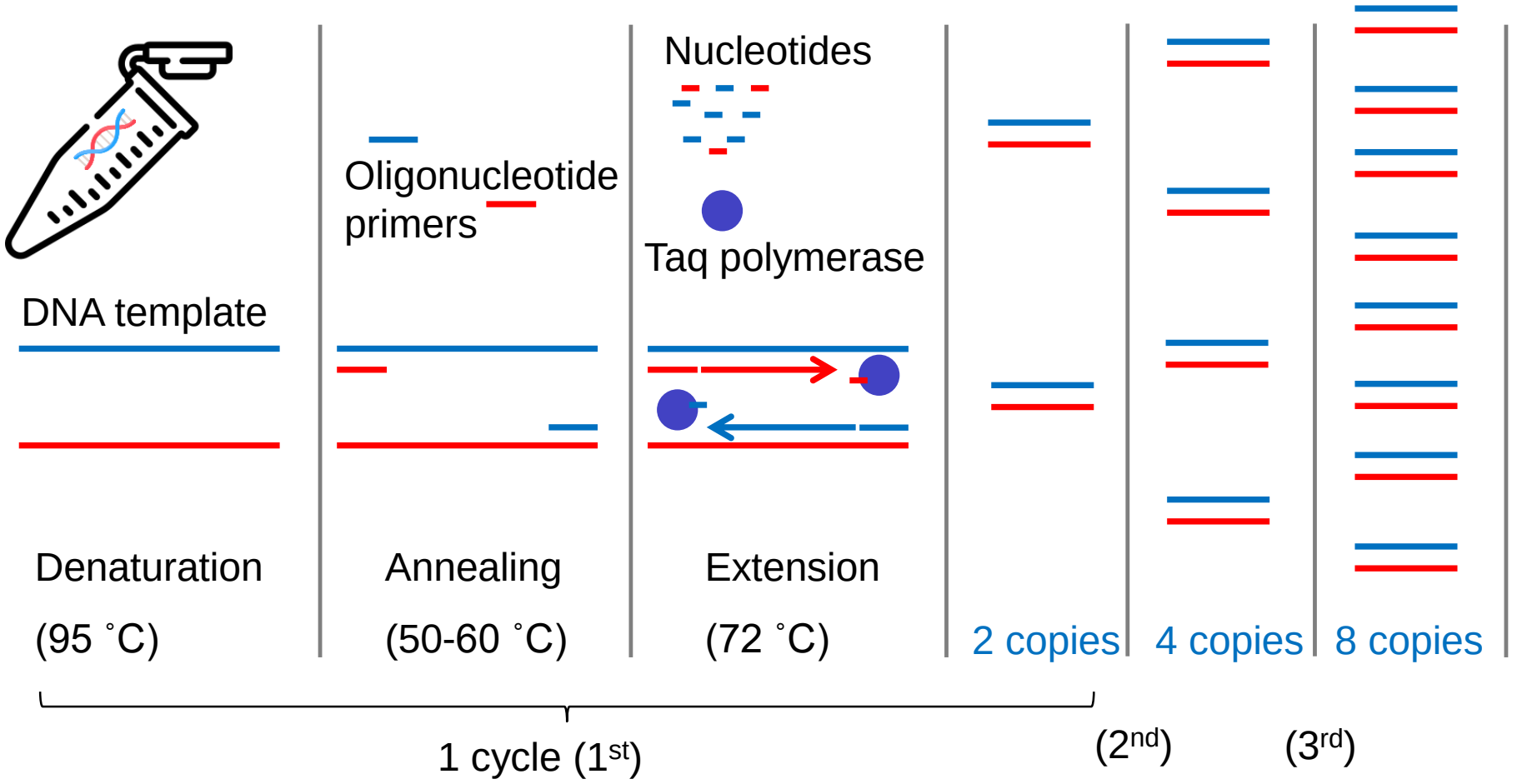
PCR - DNA “amplify” technique



No. of copies = 2^n

(n = cycles)

Example

No. of copies = 2^3 = 8 copies

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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

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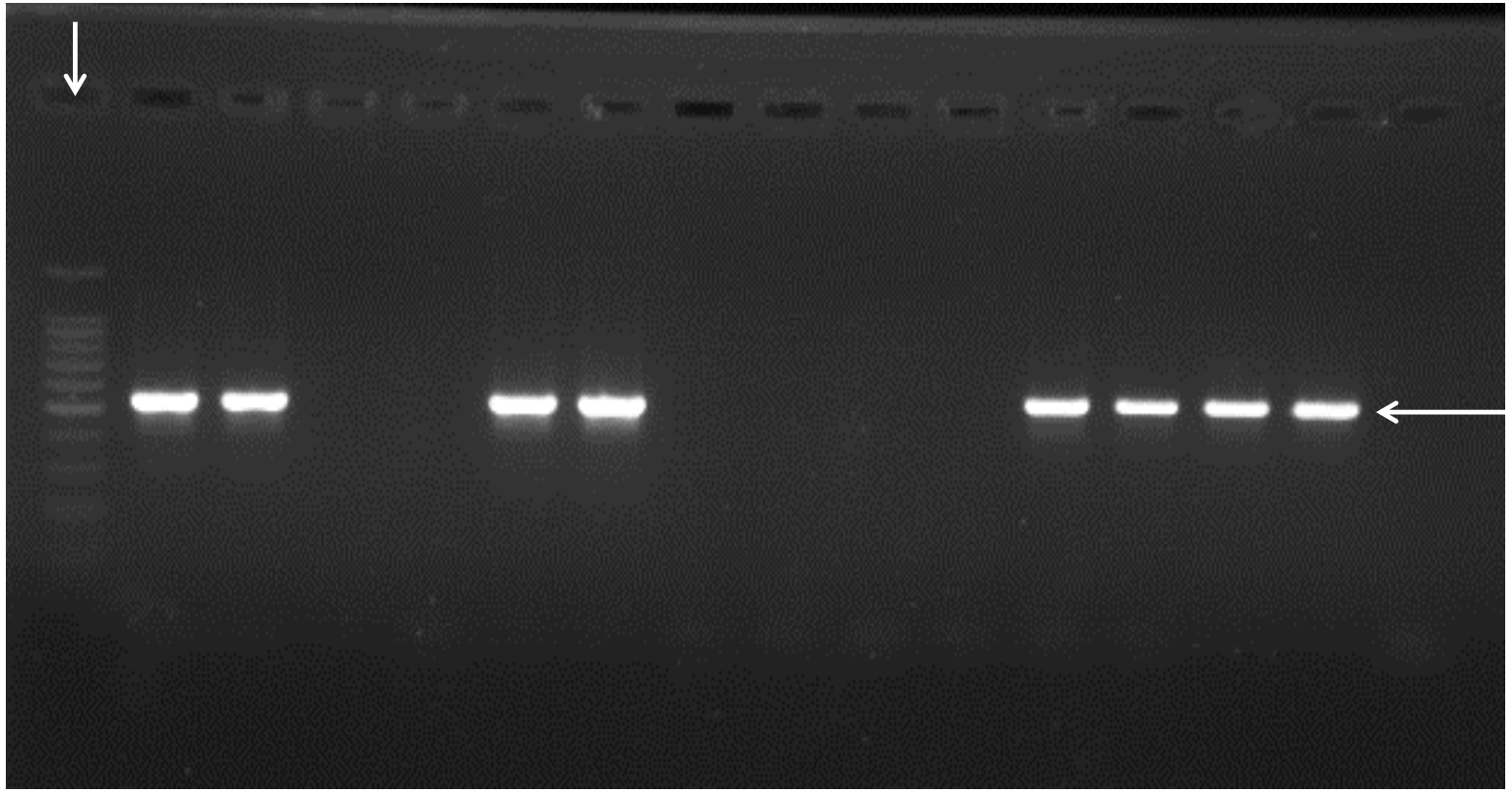
Visualization of PCR

100 base pair DNA ladder marker



Positive

PCR product

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Types of PCR

Standard or conventional PCR

DNA template

Oligonucleotide primers

Nucleotide (A, T, C, G)

Taq polymerase

others eg. Mg2+, 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

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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

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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

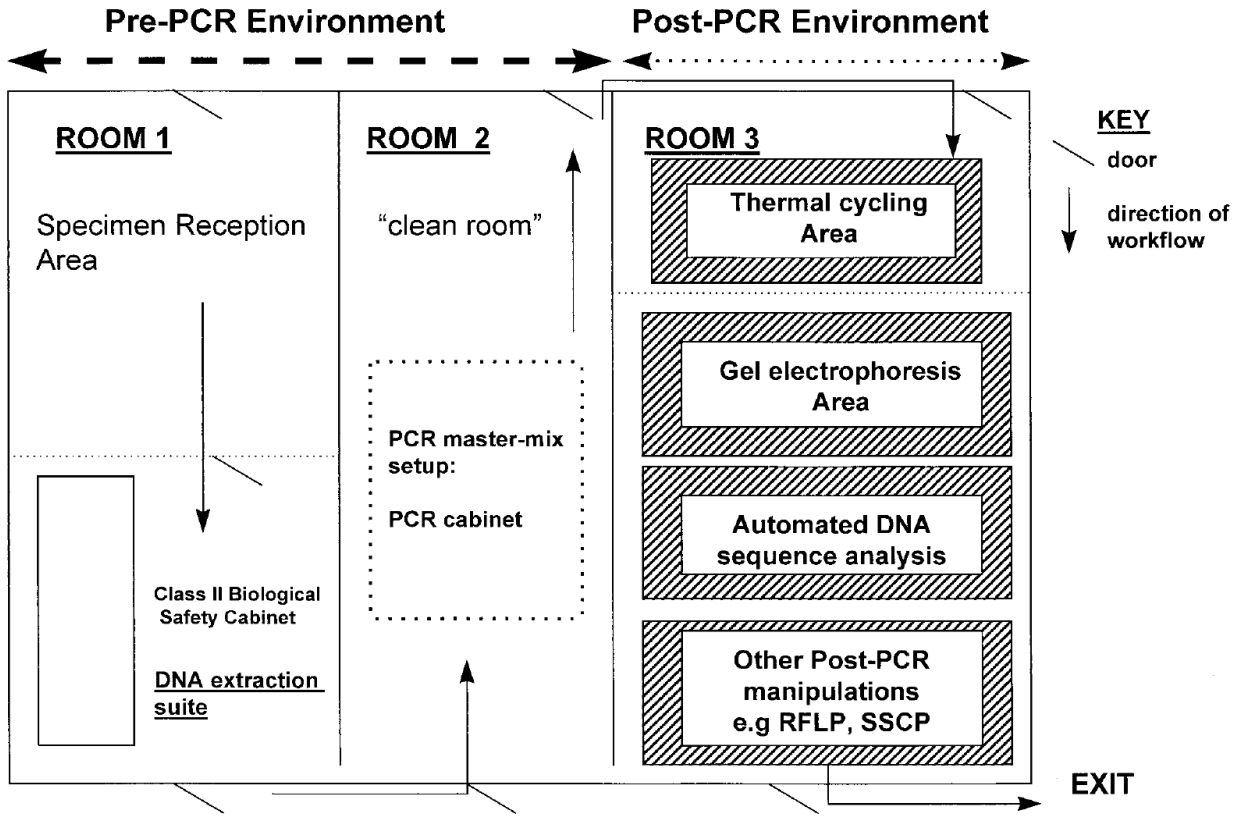

Figure. Proposed layout of PCR laboratory space

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

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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

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Make a plan for PCR

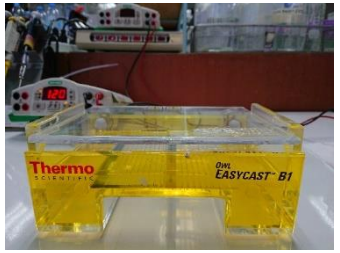
Materials

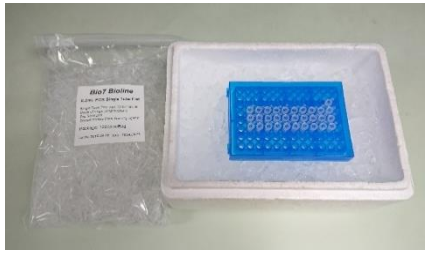
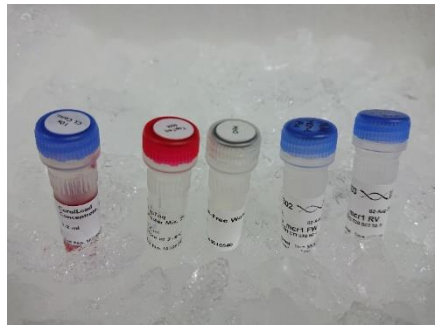
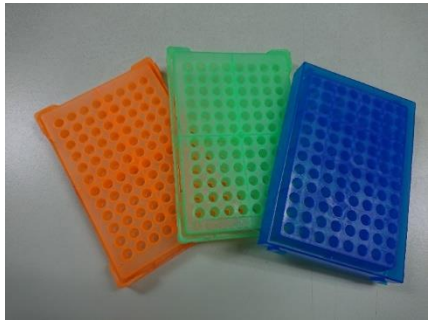
- Molecular marker (Ladder 100bp)
- Electrophoresis buffer (TAE or TBE)
- Tips (filter) for pipettes 1 µL to 1000 µL
- Agarose
- PCR components
- TE buffer
- Tris HCl buffer
- Crushed ice
- Mineral oil (if necessary)
- etc

Equipment

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







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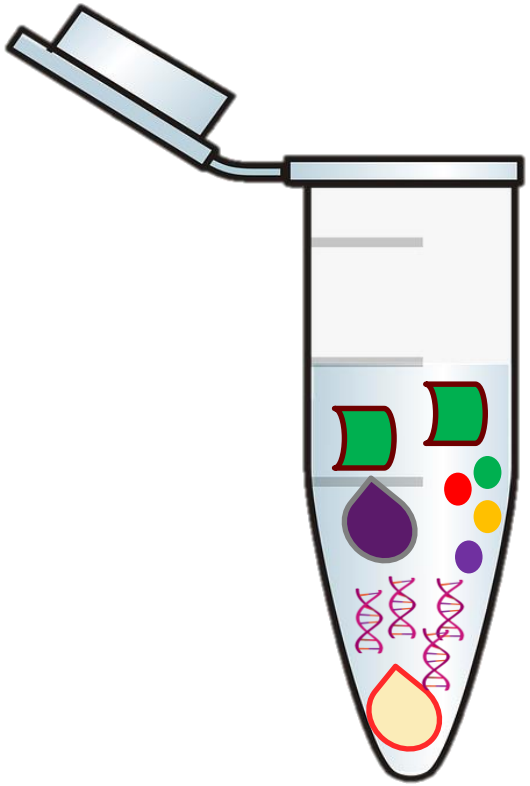
Components of a PCR reaction



- Template DNA
- Thermostable DNA polymerase
- Primers
- dNTPs
- Buffer and salts (KCl, $MgCl_2$)
- PCR grade water
- Optional: DMSO, Formamide

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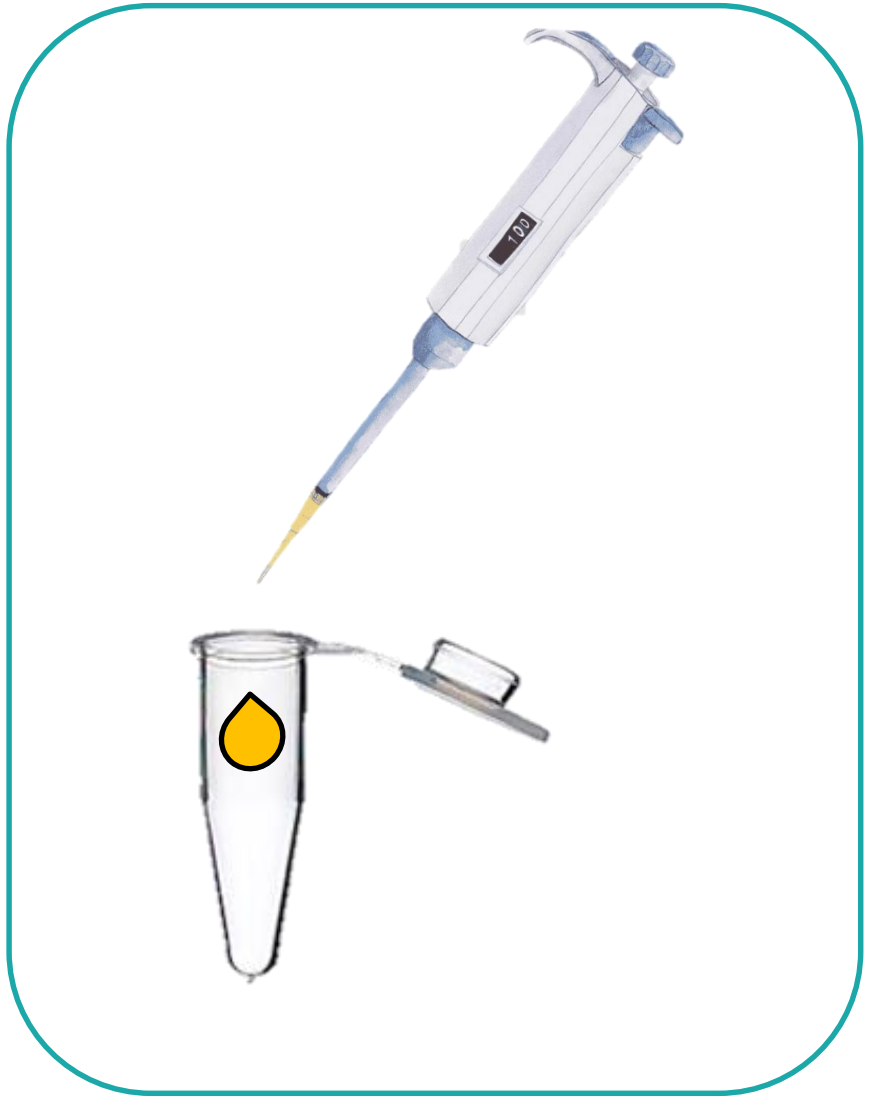
Components of a PCR reaction

Template DNA

- ✓ **A starting amount in a 50 µL 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
  - ↑ Amounts, ↑Non-specific amplification
  - ↓ Amounts, ↓ PCR product yields



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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

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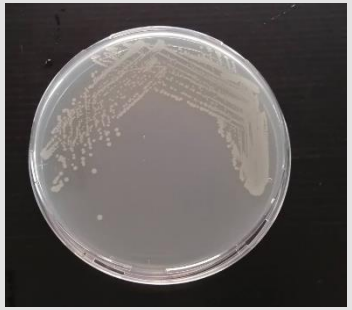
Components of a PCR reaction

Preparation of DNA template

Boiling methods

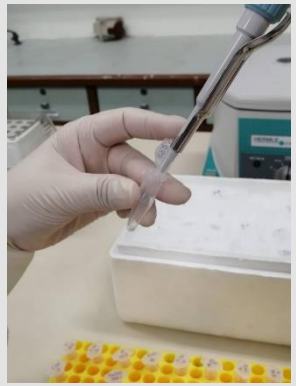


✓ Suspend a loopful of culture in 100 µl of sterile DW.

✓ Boiled 10 min at 100ºC

✓ Place on ice

✓ Store at -20ºC



✓ Transfer supernatant to a new tube

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

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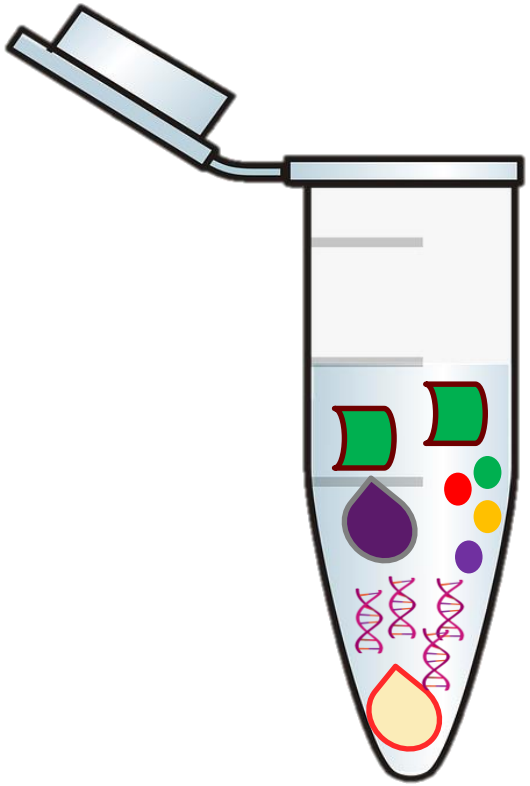
Components of a PCR reaction



- Template DNA
- Thermostable DNA polymerase
- Primers
- dNTPs
- Buffer and salts (KCl, $MgCl_2$)
- PCR grade water
- Optional: DMSO, Formamide

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Components of a PCR reaction

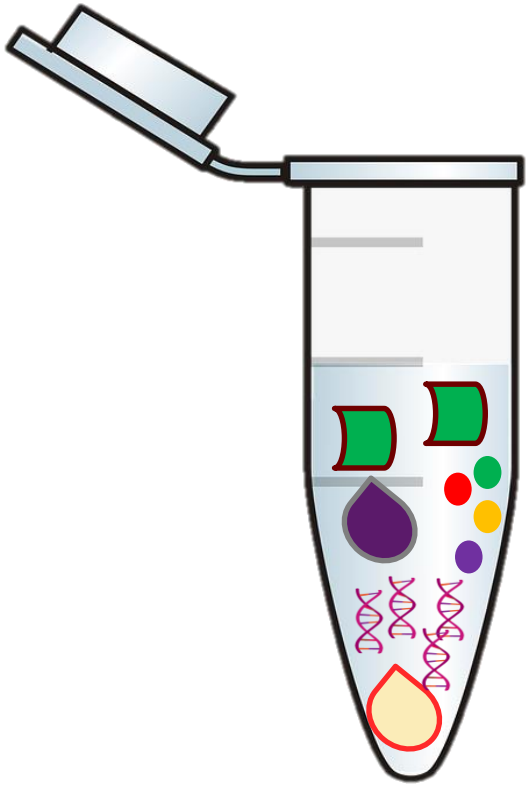
Thermostable DNA polymerase

Taq Polymerases

- a thermostable enzyme from hyperthermophilic *Thermus aquaticus*.
- Most active around **70-72°C**.
- incorporates ≈ 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 µL reaction.
- "Hot start"
  - contain a thermolabile inhibitor that is denatured during denaturation and active polymerase is released.
  - improve the specificity of the PCR amplification

https://ftw.usatoday.com/2019/08/yellowstone-tourist-walks-up-to-old-faith

Components of a PCR reaction



- Template DNA
- Thermostable DNA polymerase
- Primers
- dNTPs
- Buffer and salts (KCl, $MgCl_2$)
- PCR grade water
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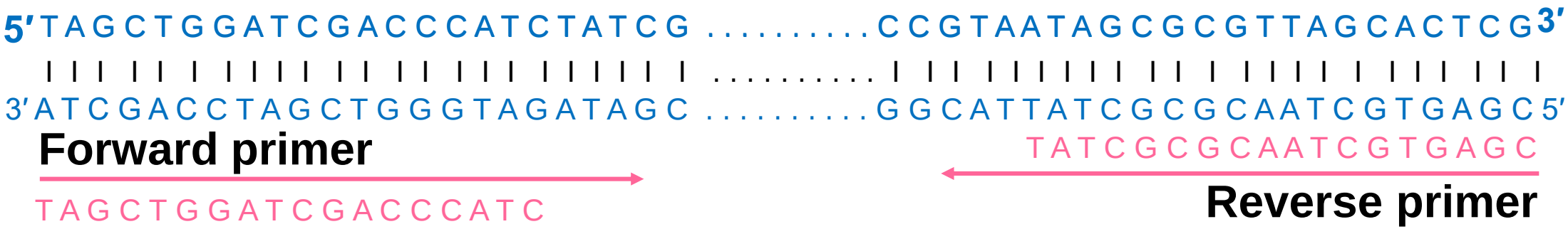
Components of a PCR reaction

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 (≤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

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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 (Tm)

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Melting temperature (Tm)

What is Tm?

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

What is annealing temperature (Ta)?

- Temperature that primers bind to template DNA
- Annealing temperature is about 5ºC 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

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Melting temperature (Tm)

Tm calculation

Tm = 2AT + 4GC

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

Annealing temperature (Ta) = Tm - 5

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

T_m = 2AT + 4GC
= (2 x 7) + (4 x 11)
= 58

T_a = Tm – 5
= 58 – 5 = 53
= 53

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

N = primer length
K = concentration of cations (nM)

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

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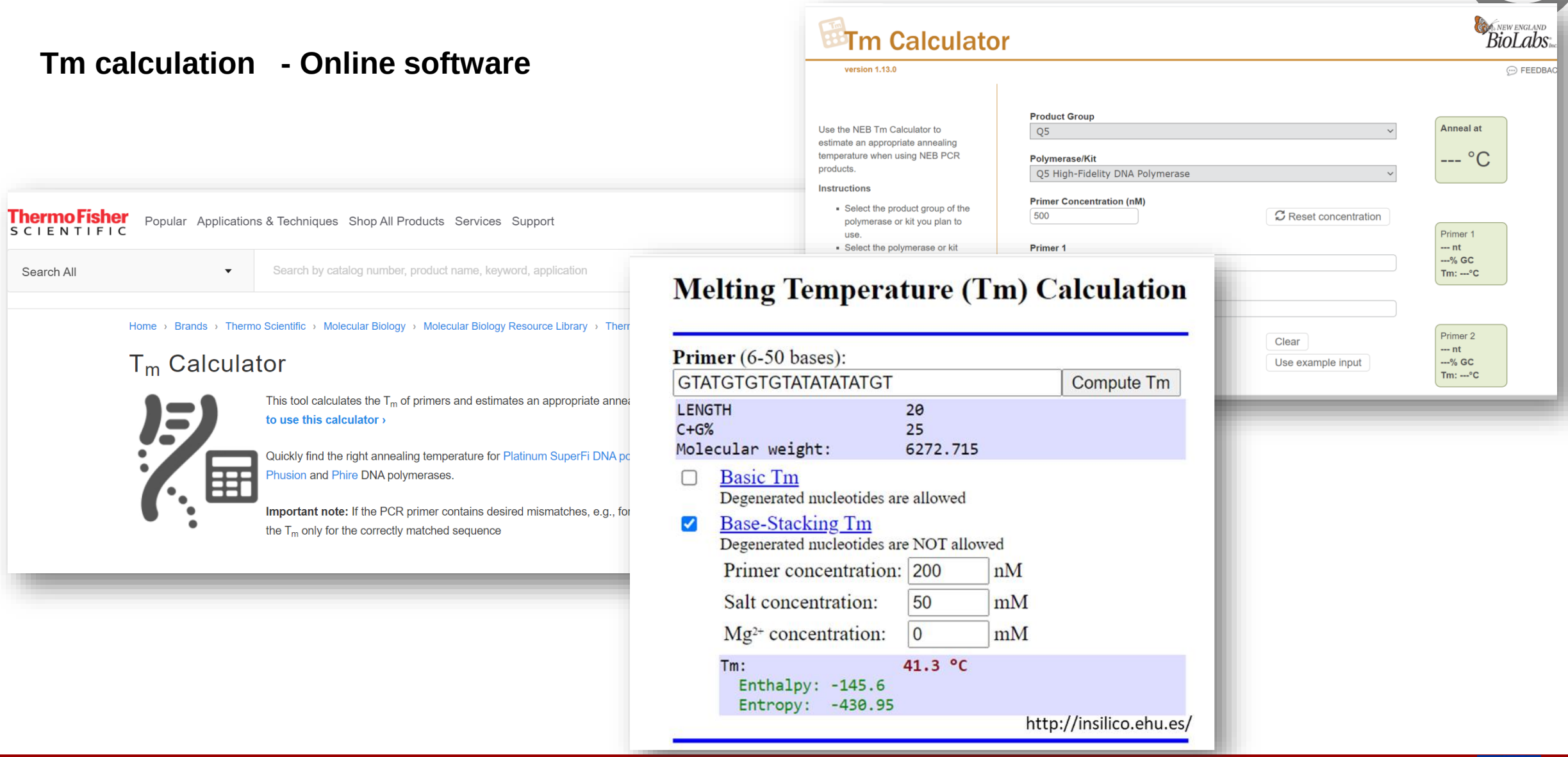
Melting temperature (Tm)

Tm calculation - Online software



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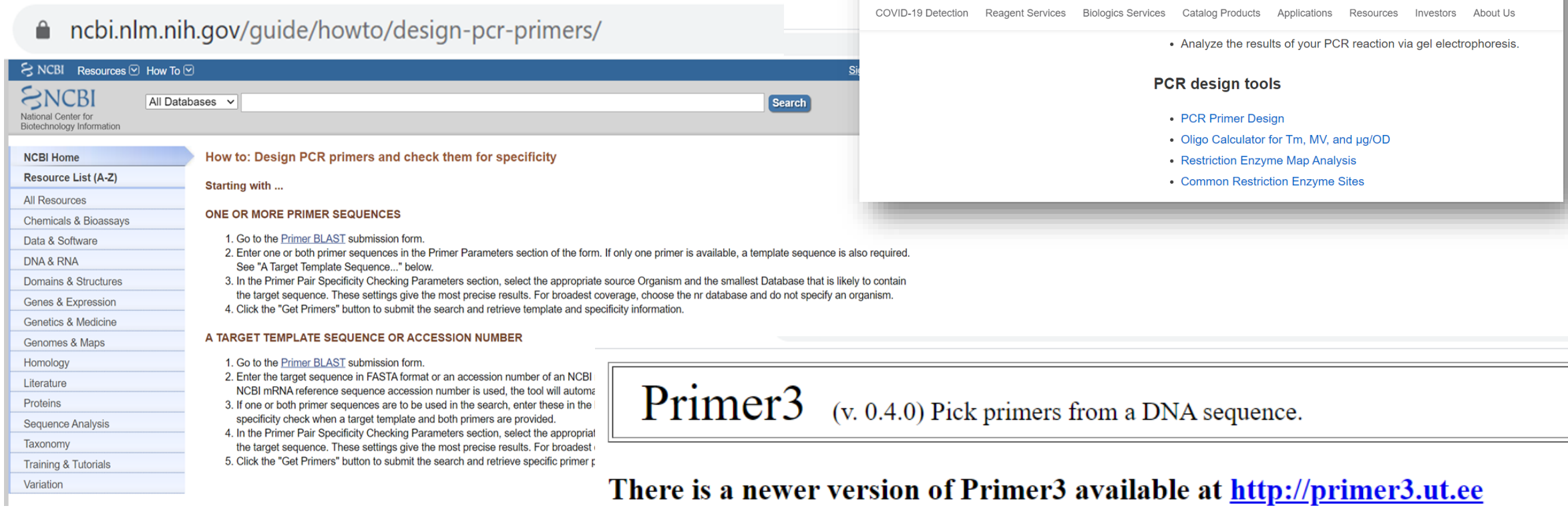
Internet software for primer design



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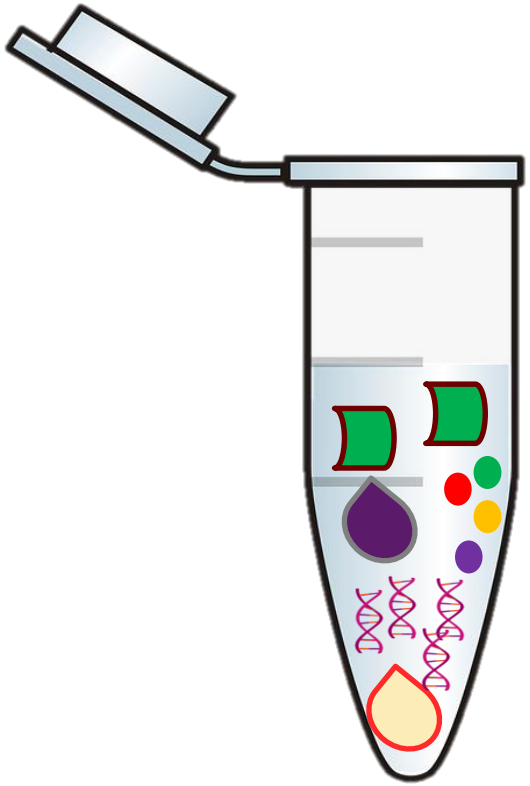
Components of a PCR reaction



- Template DNA
- Thermostable DNA polymerase
- Primers
- dNTPs
- Buffer and salts (KCl, $MgCl_2$)
- PCR grade water
- Optional: DMSO, Formamide

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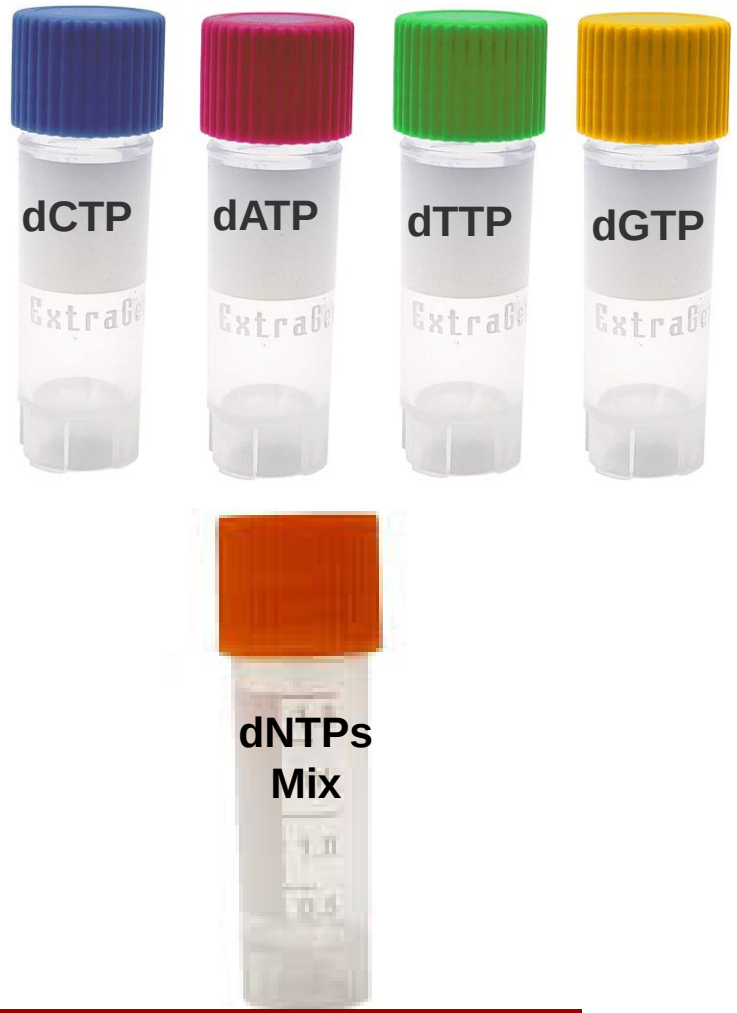
Components of a PCR reaction

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 µM.

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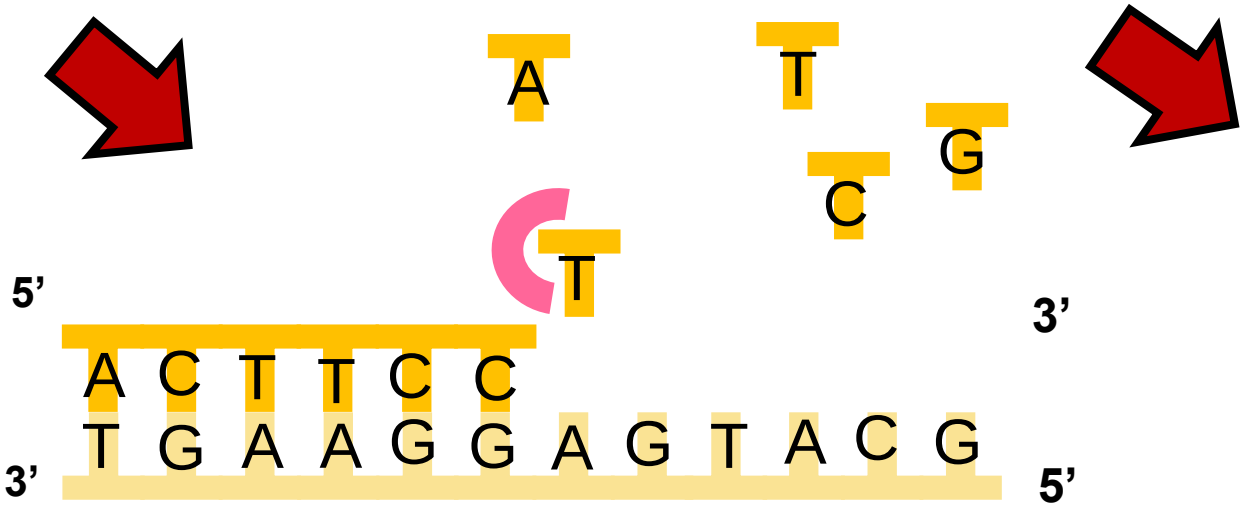
Components of a PCR reaction

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

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Components of a PCR reaction

PCR grade water

- purified
- double-distilled
- deionized
- autoclaved.

Must free of all DNA, RNase and DNase contamination.

https://www.fishersci.se/shop/products/hyclone-water-injection-wfi-quality-water-water-2/p-7076316

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Setting up traditional PCR reaction

Keep and thaw all reagents on ice.

Assemble reaction mix into 50 µL volume in a thin walled 0.2 mL PCR tubes.

Add reagents in following order:

Water → buffer → dNTPs → $MgCl_2$, → template DNA → primers → 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.

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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

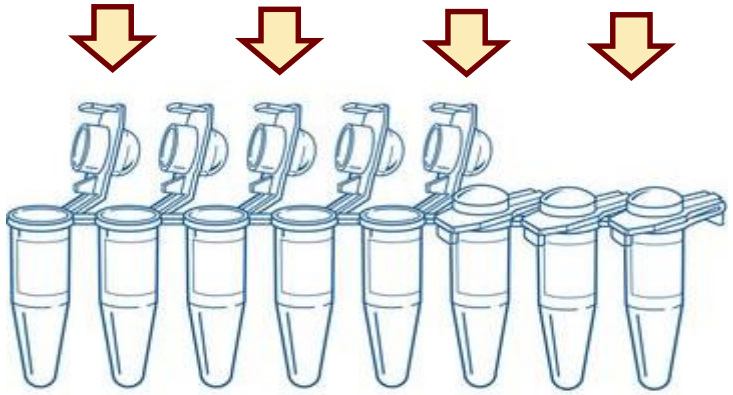
DNA polymerase

dNTPs

reaction buffer

water

Aliquot into PCR tubes



Add DNA as the last step

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To prevent lots of confusion...

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

Service contract for the provision of EU networking and support for public health reference laboratory functions for antimicrobial resistance in *Salmonella* species and *Campylobacter* species in human samples

SC 2019 74 09

Multiplex PCR for differentiation of *C. coli* and *C. jejuni*.

May 2022

STATENS SERUM INSTITUT

FWD AMR· RefLabCap

Health and Digital Executive Agency

Third EU Health Programme

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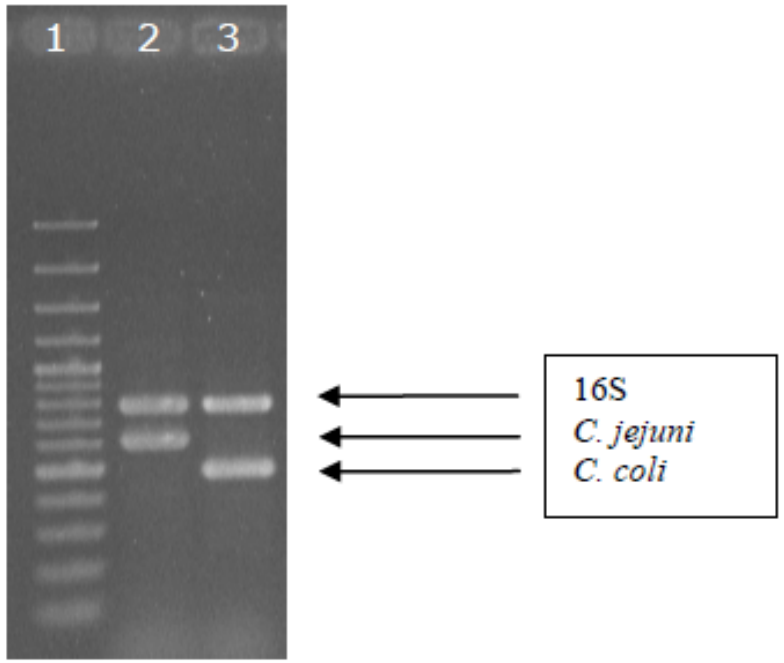
To prevent lots of confusion...

...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

Funded by the European Union

Acknowledgement

Curtesy to:
Dr. Taradon Luangtongkum D.V.M., Ph.D.
Assist. Prof. Pattrarat Chanchaithong DVM PhD
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Thank you for your attention

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