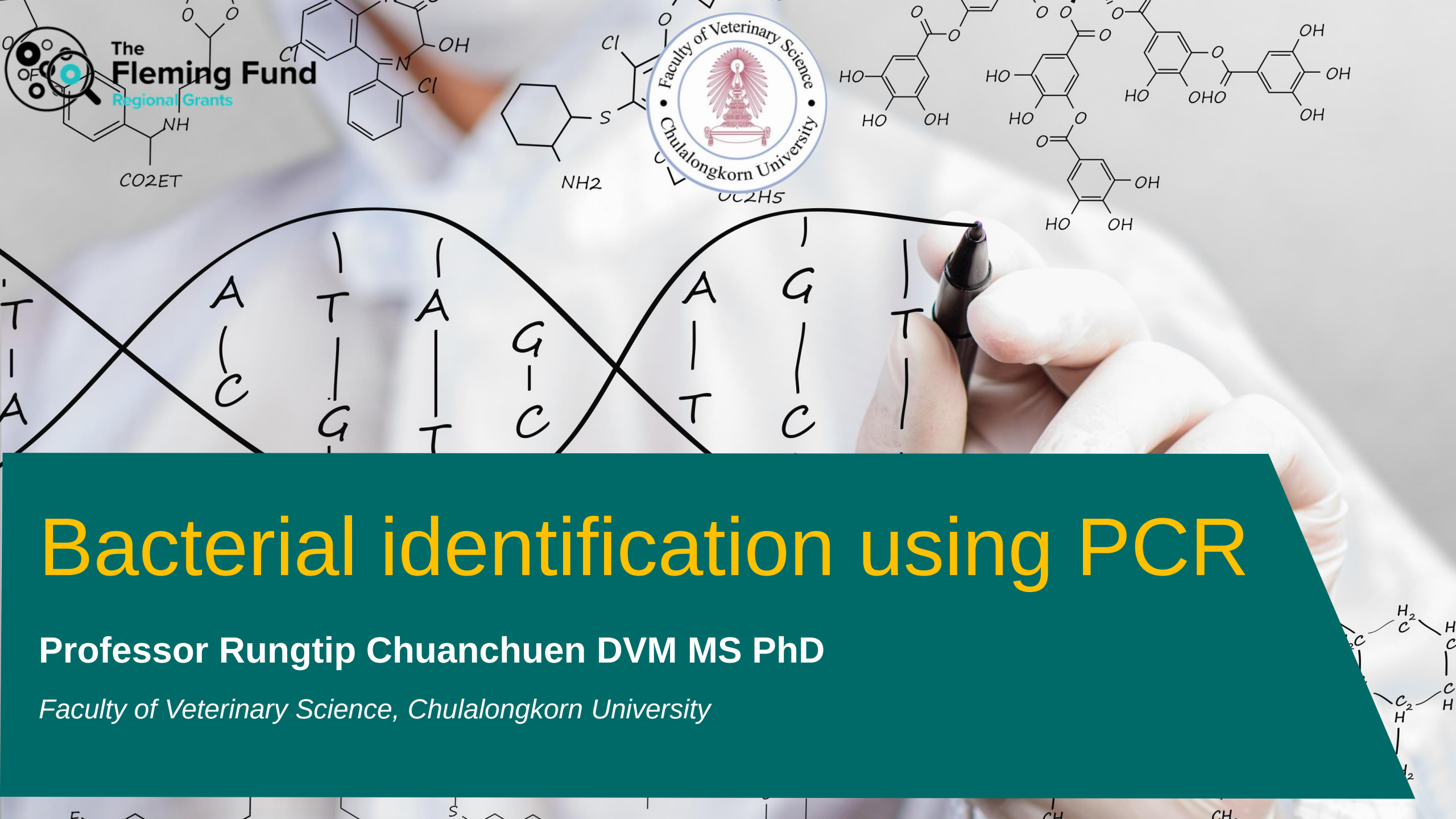


Bacterial identification using PCR

Professor Rungtip Chuanchuen DVM MS PhD

Faculty of Veterinary Science, Chulalongkorn University

Polymerase chain reaction - PCR

- Conventional PCR assays are suited to detect the presence or absence of the target genes.
- NOT their function or expression.

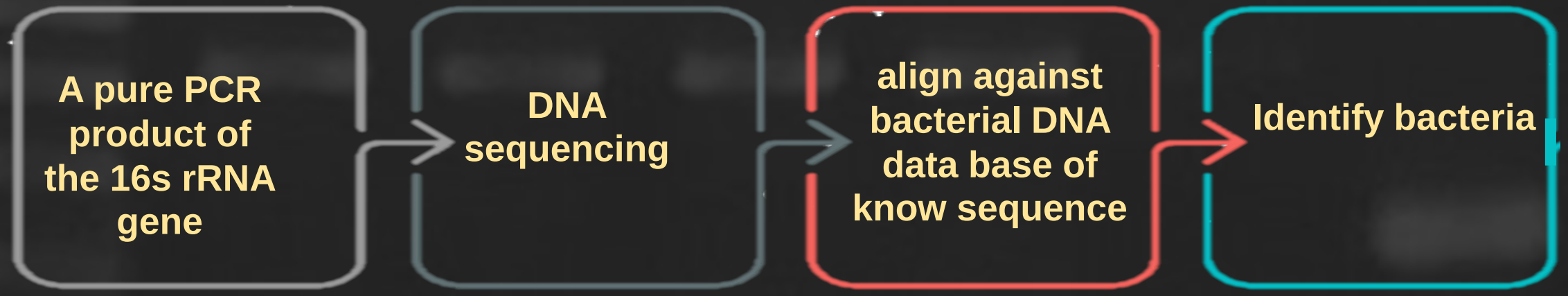
  - Rapid detection
  - Confirm species identity because biochemical and phenotypic tests do not always produce correct species differentiation.
  - Detect and identify either live or dead bacteria.

- Various PCR based bacterial identification
  - DNA sequencing based
  - PCR amplicon pattern of highly conserved genes

A universal method for bacterial identification

PCR amplification of 16s rRNA gene

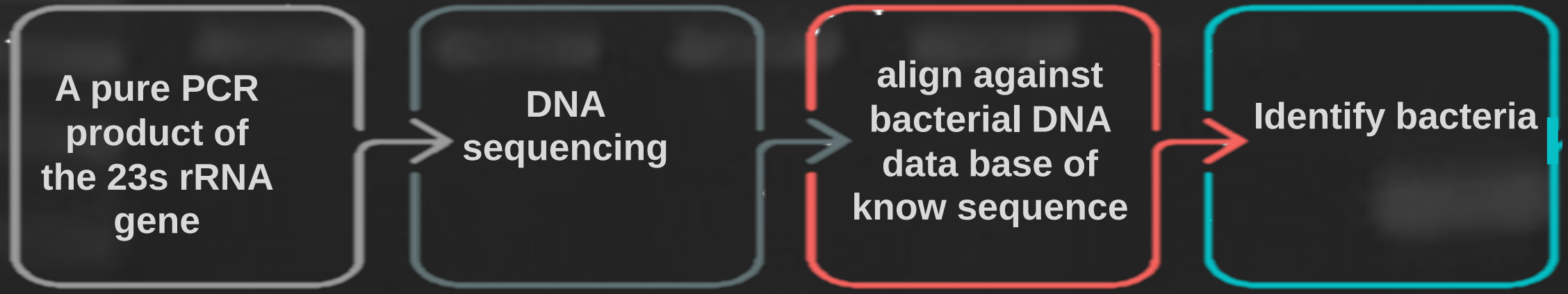
- widely used as a means to identify an unknown
- 16S rRNA
  - present in all bacterial species
  - highly conserved sequences
  - genus- or species-specific.



PCR amplification of 23s rRNA gene

23s rRNA gene:

- universal distribution
- highly conserved sequences
- genus - or species-specific.
- a greater length and sequence variations
- possibly better phylogenetic resolution
- the lack of established broad-range bacterial PCR amplification and sequencing primers.



Other PCR based-bacterial identification

- Amplified 16S Ribosomal DNA Restriction Analysis (16S-ARDRA)
- real-time PCR *Salmonella* screening method (*invA* gene)
- Amplification of *sodA* (superoxide dismutase) gene and *ddl* (D-alanine-D-alanine ligase) gene for *E. faecium* & *E. faecalis* identification

- etc

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Basic for PCR

Flow of conventional PCR

1

Make a plan

2

Prepare PCR components

3

Set PCR reaction

4

Run thermocycler

5

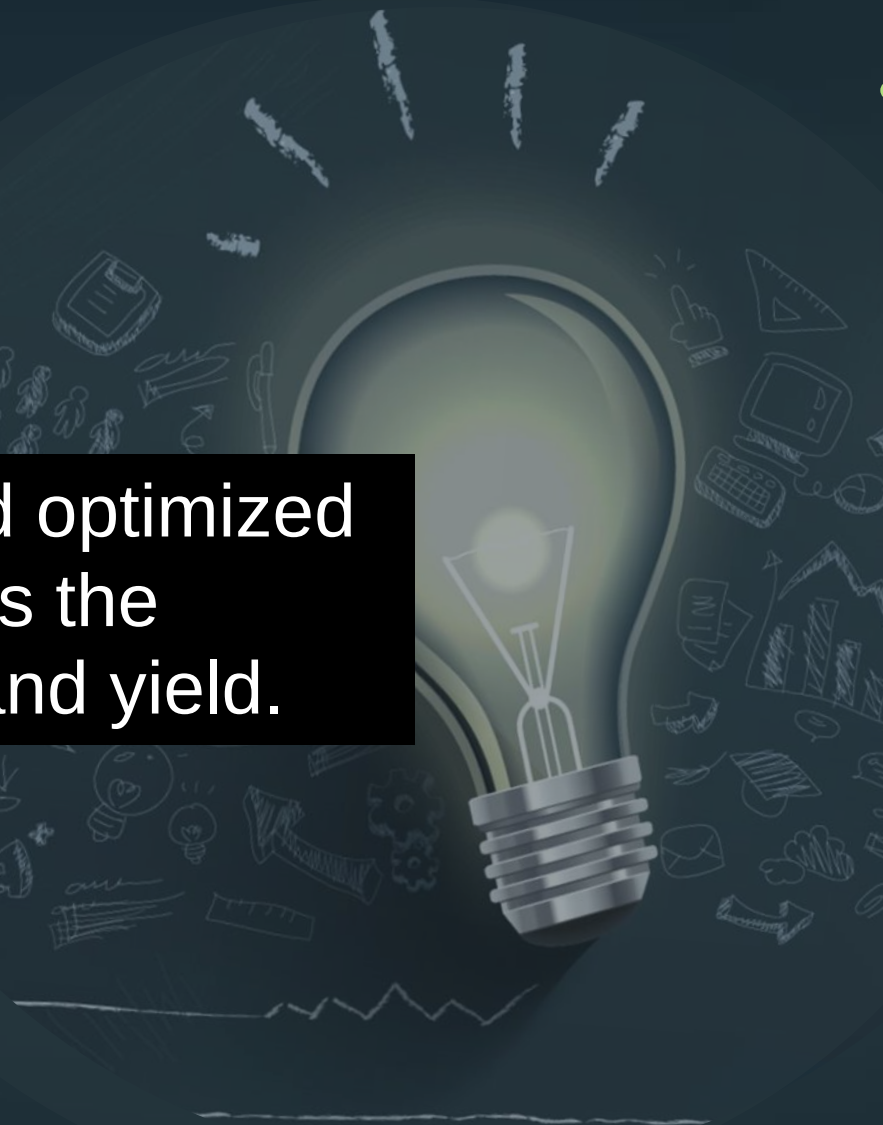
Run electrophoresis

6

PCR product visualization

Make a plan for PCR

1



A well-designed and optimized PCR assay provides the highest specificity and yield.

- What is or are the purpose (s) of your PCR?
- What are the DNA sequence of your target gene and surroundings?
- What is or are your target gene (s)?
- What materials are needed?

etc

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Make a plan for PCR

1

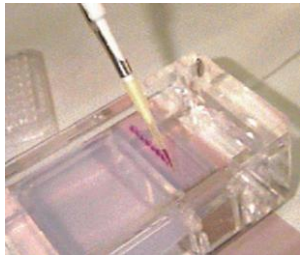
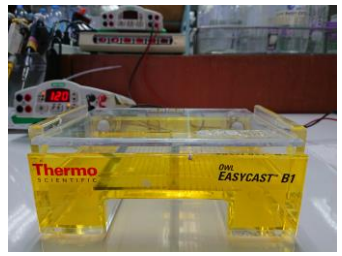
Materials

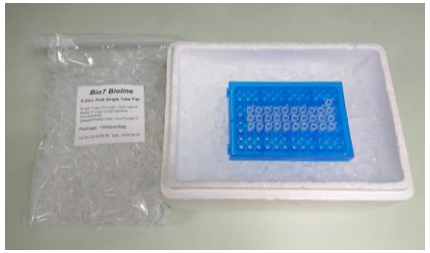
- Molecular marker (Ladder 100bp)
- Electrophoresis buffer (TAE or TBE)
- Tips (filter) for pipettes 1 µL to 1000 µL
- Agarose
- PCR components
- TE buffer
- Tris HCl buffer
- Crushed ice
- Mineral oil (if necessary)
- etc

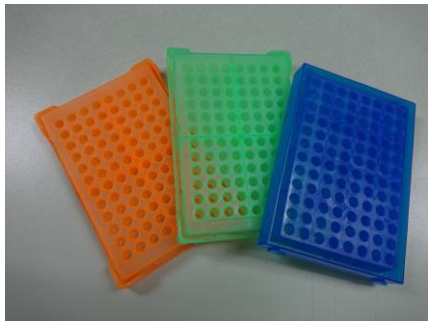
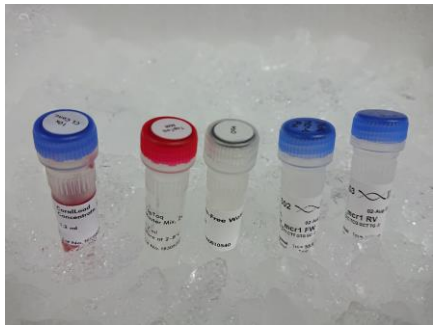
Equipment

- 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





etc

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PCR components

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

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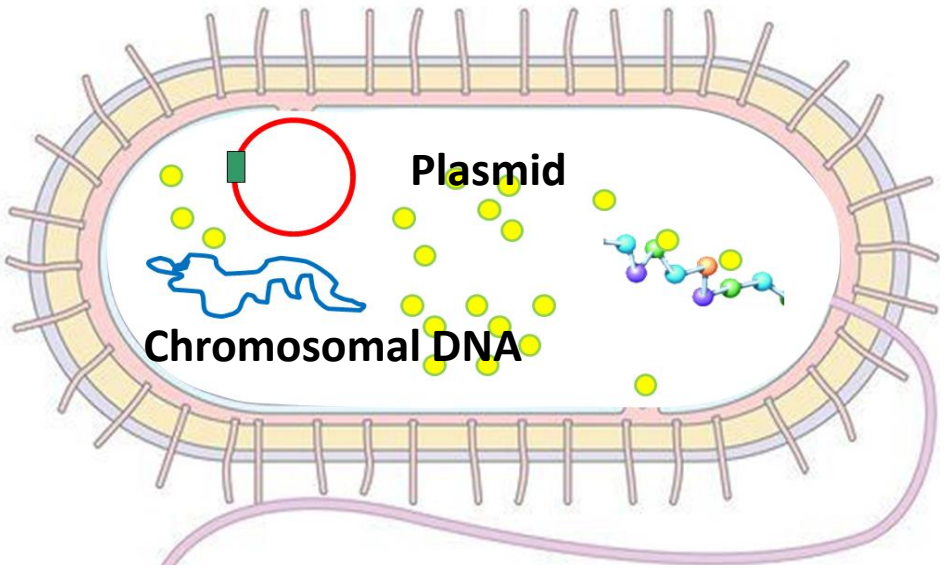
Template DNA

- ✓ Any DNA sources
  - Genomic DNA (gDNA)
  - Plasmid DNA
  - DNA fragments
  - Complementary DNA (cDNA)



- ✓ **Template DNA properties:**
  - highly purified
  - free from chemical contaminants and other DNA contaminants.
  - with 50% to 55% GC content

- ✓ **A starting amount in a 50 µL PCR**,
  - 0.1–1 ng of plasmid DNA
  - 5–50 ng of gDNA

- ✓ Optimization of DNA amounts
  - ↑ Amounts, ↑ Nonspecificity
  - ↓ Amounts, ↓ PCR product yields

Template DNA

DNA preparation

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

Conventional methods

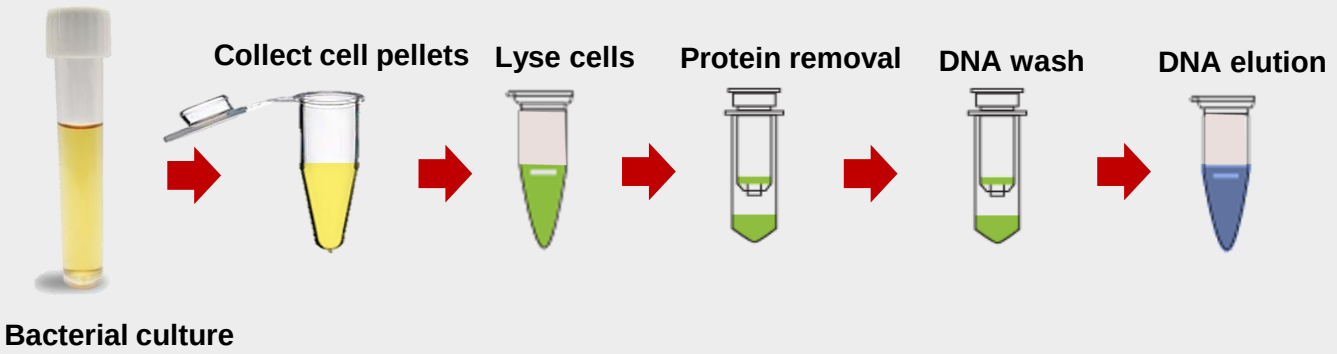
- Alkaline lysis
- Phenol chloroform extraction

Commercial kits

- Pure DNA if used properly
- Phenol chloroform extraction

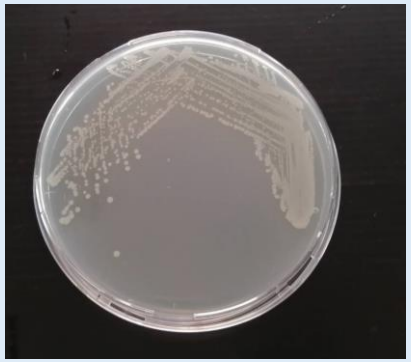
Boiling methods

- Whole cell DNA
- Simple, reproducible, rapid and economical
- Contain protein, enzyme etc



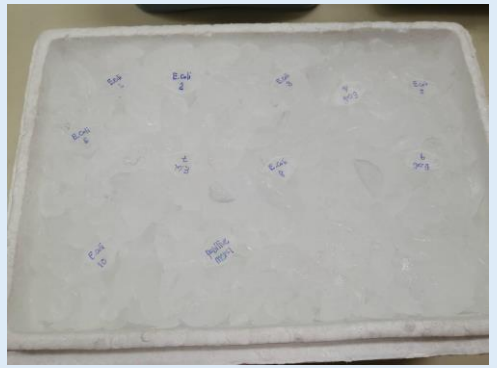
Template DNA

Preparation of DNA template by whole cell boiling method



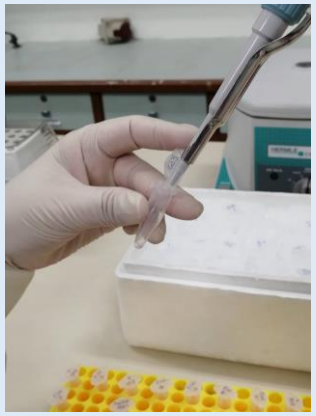
✓ Suspend a loopful of culture in 100 µl of sterile DW.

✓ Boiled 10 min at 100ºC



✓ Place on ice

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



✓ Transfer supernatant to a new tube

✓ Store at -20ºC

Template DNA

Assessing the quality of DNA by spectrometry:

- **Absorption peak for DNA**
  = at ~260 nm. (A_{260})
- **Absorption peak for protein**
  = at ~280 nm. (A_{280})

- DNA sample purity
  = A_{260}/A_{280} ratio
  = ~1.8 for dsDNA
- A ration of < 1.7
  = protein contamination.

Assessing the quality of DNA DNA by spectrometry:

- One A260 unit is the amount of nucleic acid contained in 1 mL and producing an OD of 1.
- Conversion factors:
  - 1 A260 unit dsDNA = 50 µg
  - 1 A260 unit ss DNA = 33 µg
  - 1 A260 unit ssRNA = 40 µg
- **Example,**
  If A260 of a DNA sample is = 2.5

  ∴ dsDNA amount = 2.5 x 50
  = 125 µg

PCR components

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

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.

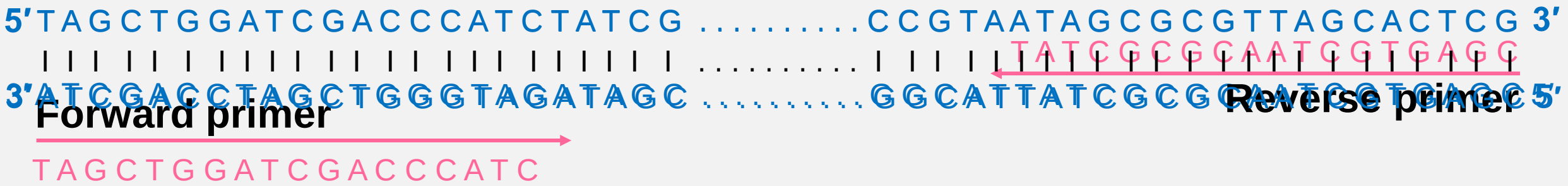
PCR components

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

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



Oligonucleotide primers

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^{o}C)
- No self-complementary sequence between primers
- No inverted repeat sequence (Max. 3 bp)

How to get primers

- Design primers (Manual design & Computer-assisted design)
- Send for custom oligo synthesis
- Prepare and use

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 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 (Tm)

Tm calculation

1. 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

2. Tm = 81.5 + 16.6(logK^+) + 0.41[%(G+C)] - (675/n)

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

- Good for 14-70 bp primers
- Cation concentration of ≤ 4M

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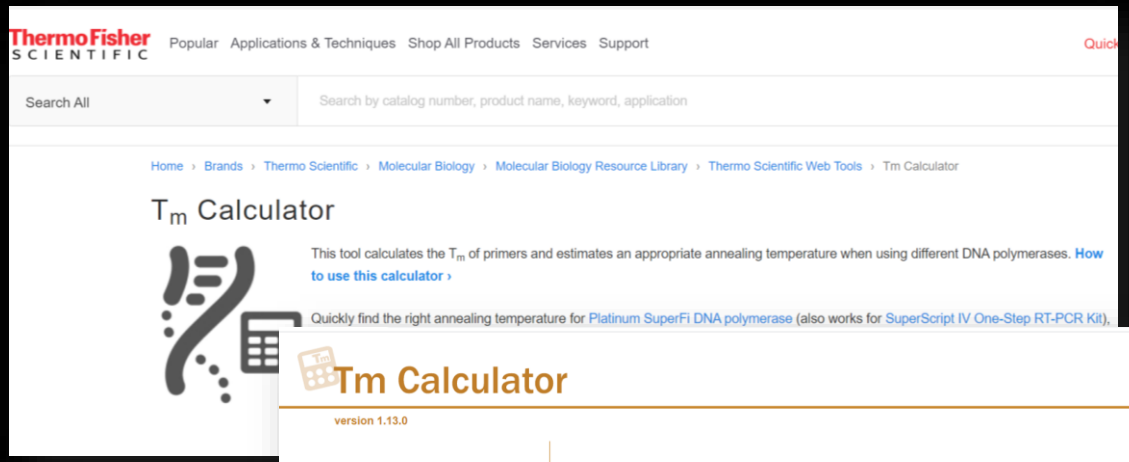
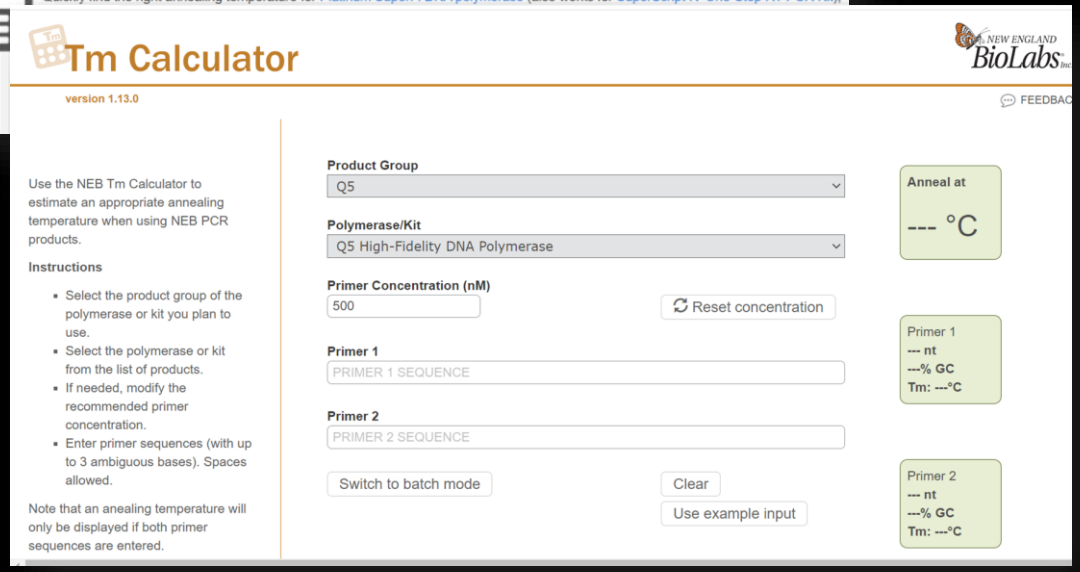
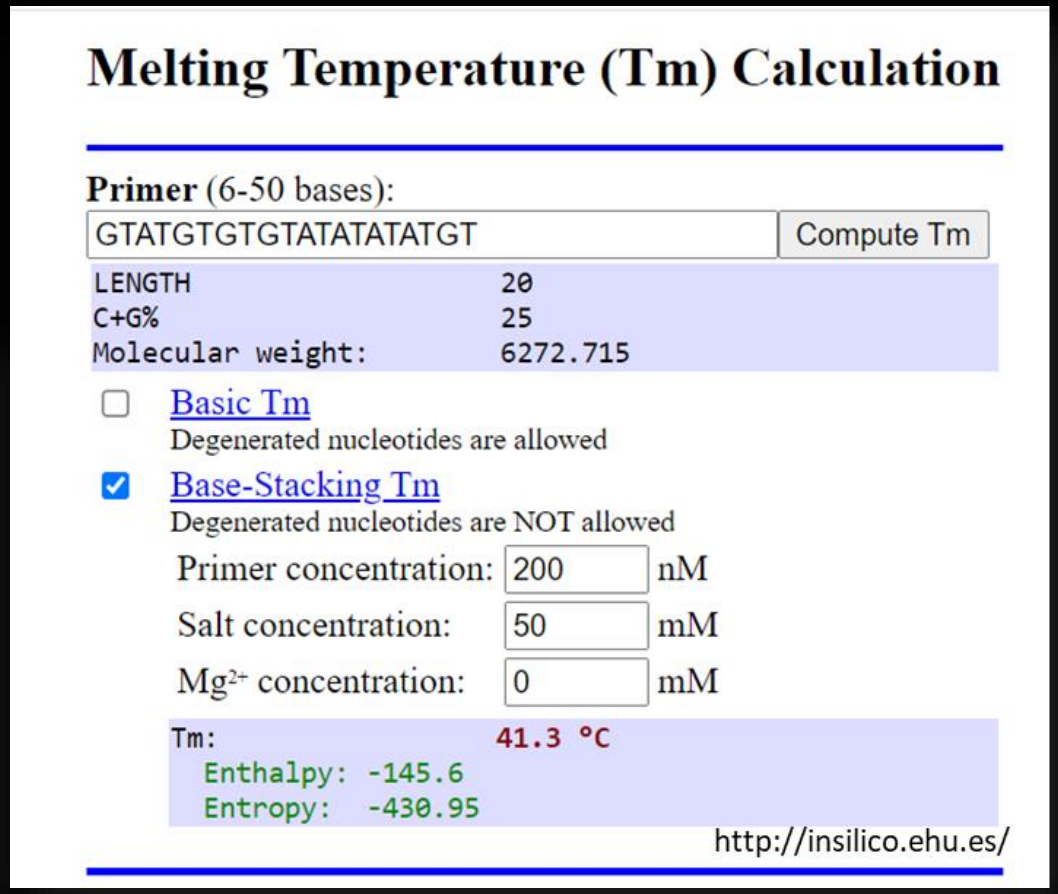
Melting temperature (Tm)

Tm calculation

3. Online software







Primer design

2

Manual design

100

430

```
5’-TAGCTGGATCGACCCATCTATCG..........CCGTAATAGCGCGTTAGCACTCG-3’
   |||||||||||||||||||||||||||||||||||||||||||||||||||||||
3’-ATCGACCTAGCTGGGTAGATAGC..........GGCATTATCGCGCAATCGTGAGC-5’
```

Forward primer

Reverse primer

```
   TAGCTGGATCGACCCATC
3’-ATCGACCTAGCTGGGTAGATAGC..........GGCATTATCGCGCAATCGTGAGC-5’
```

PCR amplicon size = 330 bp

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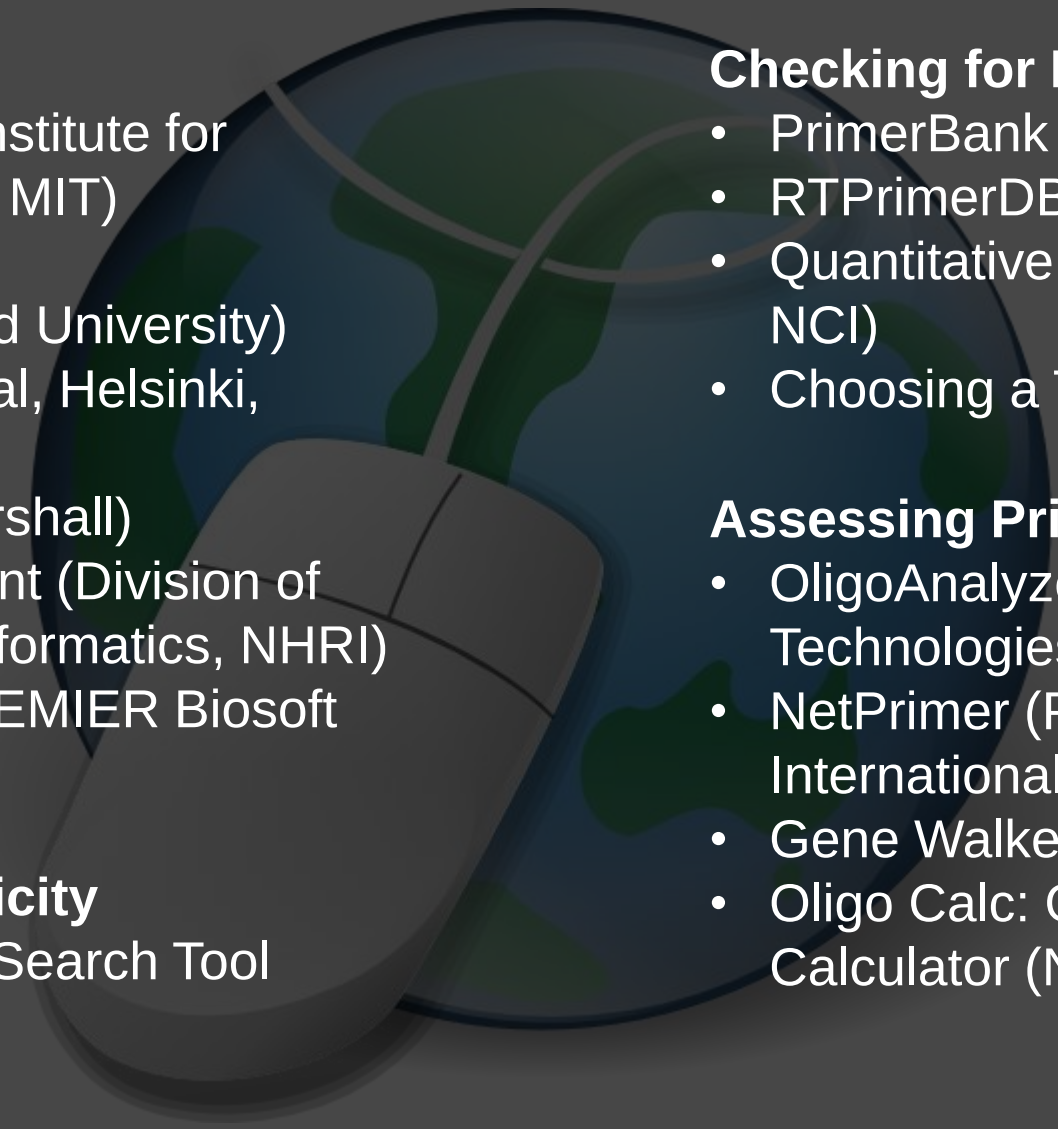
Internet software for primer design

2



Designing Primers

- NCBI
- Primer3 (Whitehead Institute for Biomedical Research, MIT)
- GenScript
- GeneFisher2 (Bielefeld University)
- FastPCR (PrimerDigital, Helsinki, Finland)
- PerlPrimer (Owen Marshall)
- Primer Design Assistant (Division of Biostatistics and Bioinformatics, NHRI)
- Beacon Designer (PREMIER Biosoft International)*

Checking Primer Specificity

- Basic Local Alignment Search Tool (BLAST; NCBI)

Checking for Existing PCR Primers

- PrimerBank (Massachusetts General Hospital)
- RTPrimerDB
- Quantitative PCR Primer Database (QPPD; NCI)
- Choosing a Target Sequence

Assessing Primer Properties

- OligoAnalyzer 3.1 (Integrated DNA Technologies)
- NetPrimer (PREMIER Biosoft International)
- Gene Walker (CyberGene AB)
- Oligo Calc: Oligonucleotide Properties Calculator (Northwestern University)

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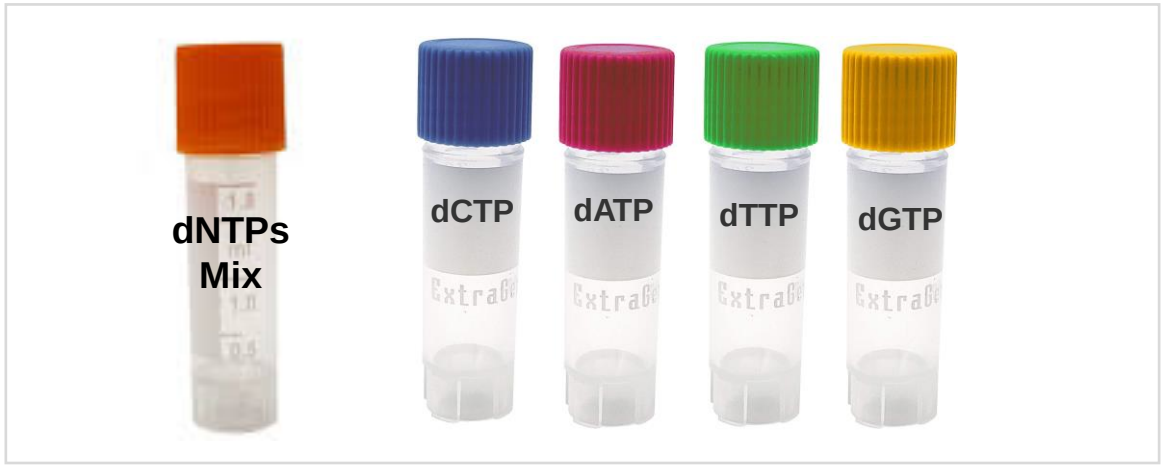
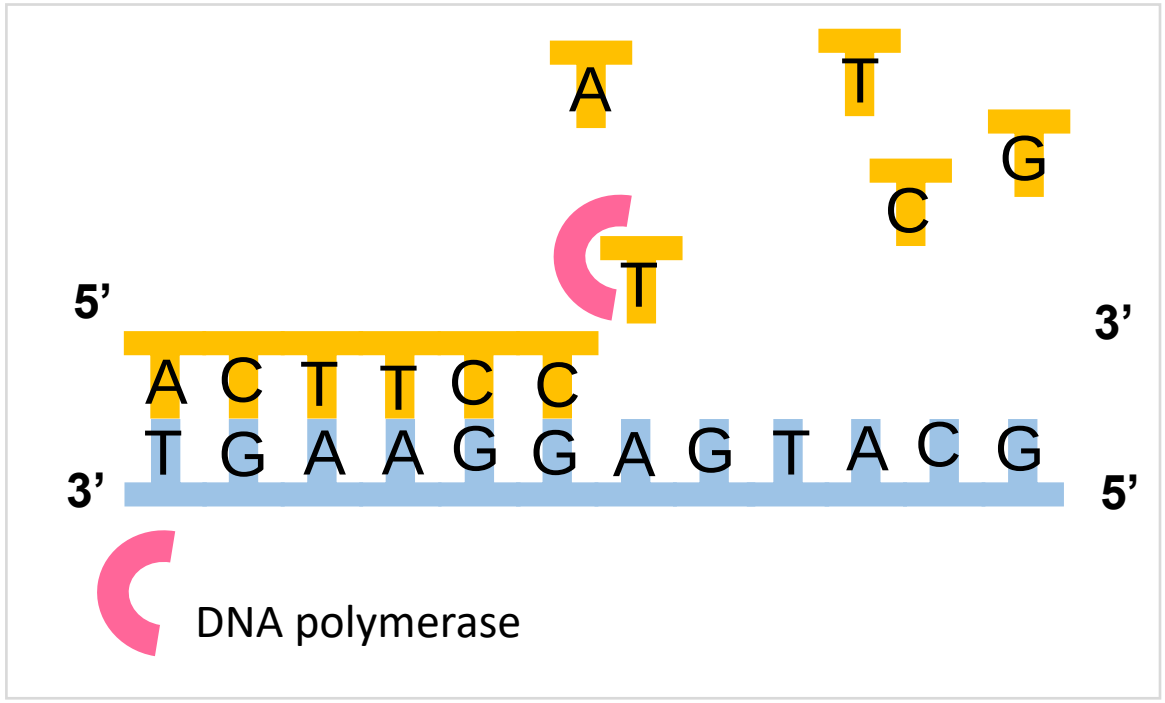
PCR components

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

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.

PCR components

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

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PCR grade water

- Must free of all DNA, RNase and DNase contamination.
- purified
- double-distilled
- deionized
- autoclaved.

https://www.fishersci.se/shop/products/hyclone-water-injection-wfi-quality-water-9/10484075

https://www.fishersci.co.uk/shop/products/ultrapure-dnase-rnase-free-distilled-water-2/p-7076316

Other PCR components

Dimethyl sulfoxide (DMSO)

- an organosulfur compound with a high polarity and high dielectric constant
- can greatly reduce the activity of *Taq* polymerase.
- benefit for longer and high GC rich DNA that are hard to amplify with basic PCR components and normal PCR protocol
- test a variety of DMSO concentrations between 1-10% w/v to find the best balance.

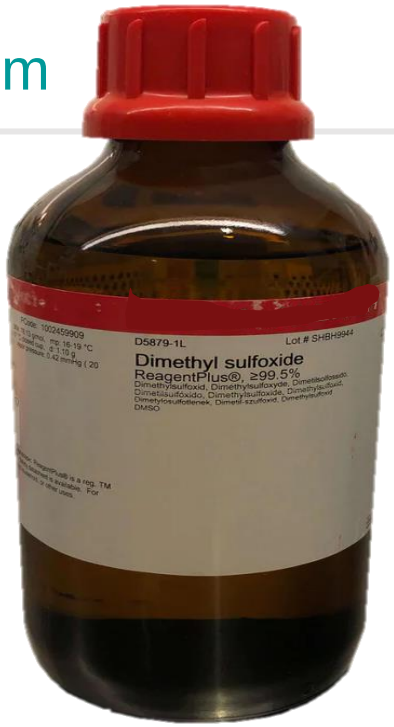
directly binds to C of the GC rich region

↓

changes the conformation of C
makes GC rich DNA more heat-labile

↓

reduces Tm



PCR reaction

A traditional PCR reaction (50 µl)

Component	Concentration range	Stock concentration	A PCR standard reaction
Sterile DW	Add to 50 µl		Add to 50 µl
PCR buffer	10x	10x	1x
dNTPs	200-250 µM	10mM	200 µM
MgCl2	0.1-5 mM	25mM	1.5 mM
Forward primer	0.1-0.5 µM	20 pmol/µl (20 µM)	20 pmol
Reverse primer	0.1-0.5 µM	20 pmol/µl (20 µM)	20 pmol
Taq polymerase	0.5 – 2.5 units	5 unit/µl	0.5 units
DNA template	1 pg to 1 µg	25 ng/µl	10 ng
DMSO	1 to 10% w/v		

Note: These PCR conditions are suitable for products ranging up to 3 kb in length. PCRs that result in longer products may require optimization of the dNTP and primer concentrations and the use of special DNA polymerase kits.

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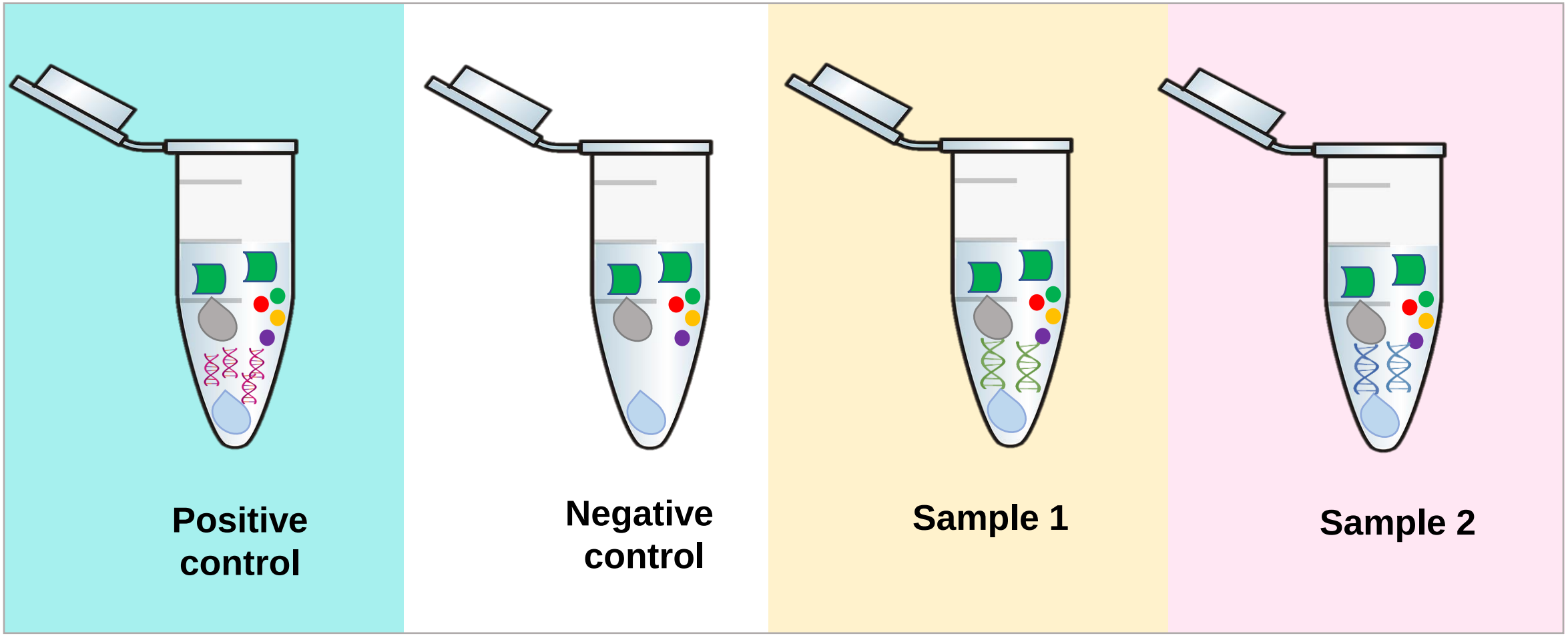
Setting up traditional PCR reaction

3



Setting up traditional PCR reaction

3

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.

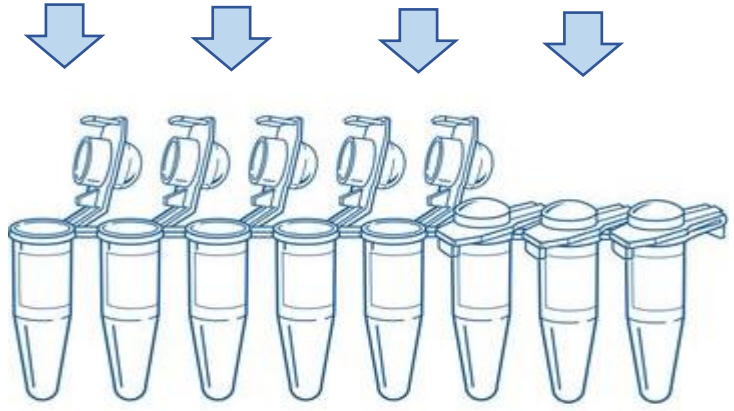
PCR cocktail

- 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

PCR cocktail

Example of a PCR cocktail for 10 DNA samples (11 50 µl-reactions)

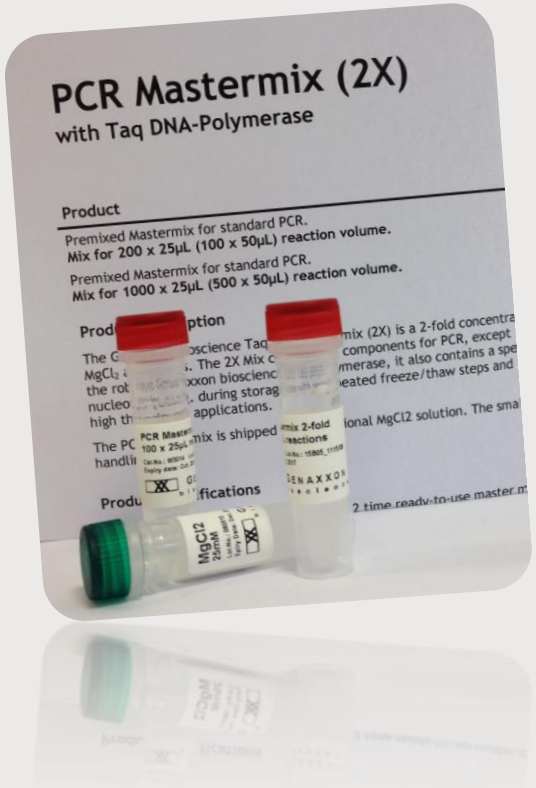
Component	Stock conc.	Volume in 1 rxn	No of rxn	Volume in cocktail (11rxn)	Final conc. in 1 rxn (50 ul)
Sterile DW		36.5	11	401.5	
PCR buffer	10x	5 µl	11	55	1x
dNTPs	10mM	1 µl	11	11	200 µM
MgCl2	25mM	3 µl	11	33	1.5 mM
Forward primer	20 pmol/µl (20 µM)	1 µl	11	11	20 pmol
Reverse primer	20 pmol/µl (20 µM)	1 µl	11	11	20 pmol
Taq polymerase	5 unit/µl	0.5 µl	11	5.5	2.5 units
DNA template	25 ng/µl	2 µl	11	22	50 ng
Total		**50 µl**		**550**	

48 µl

+

2 µl - DNA template

PCR Master Mix



1. MASTERMIX

- Sterile Water
- 10X PCR Buffer
- $MgCl_2$
- dNTP's
- DNA Polymerase

2. Primers (Forward + Reverse)

3. DNA Template

WHY?

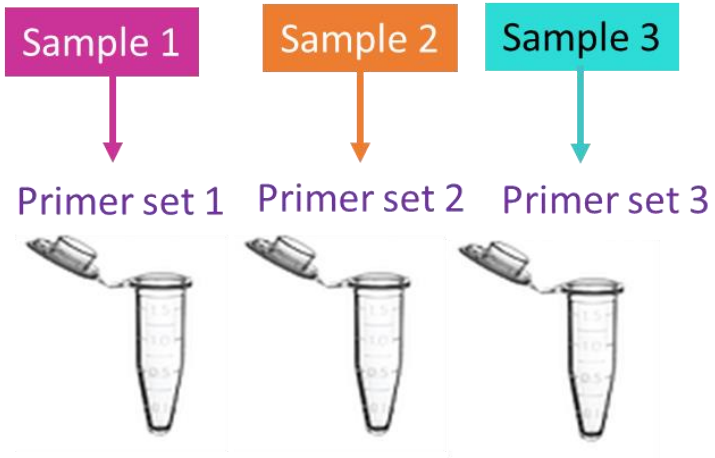
- reduce pipetting error
- Reduce risk of contamination
- convenient
- saves time
- prevent possible errors in mixing
- Improve consistency

Multiplex PCR

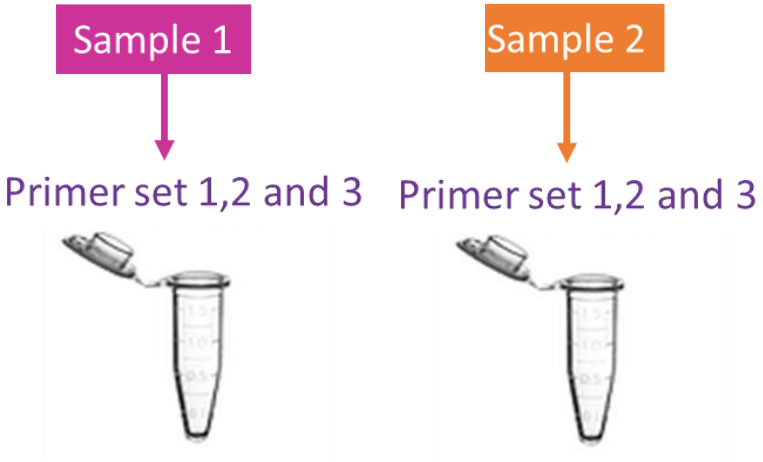
Simplex PCR amplifies a single gene.

Component	A PCR standard reaction
Sterile DW	Add to 50 µl
PCR buffer	1x
dNTPs	200 µM
MgCl2	1.5 mM
Forward primer	20 pmol
Reverse primer	20 pmol
Taq polymerase	0.5 units
DNA template	10 ng
DMSO	

Simplex PCR



Multiplex PCR



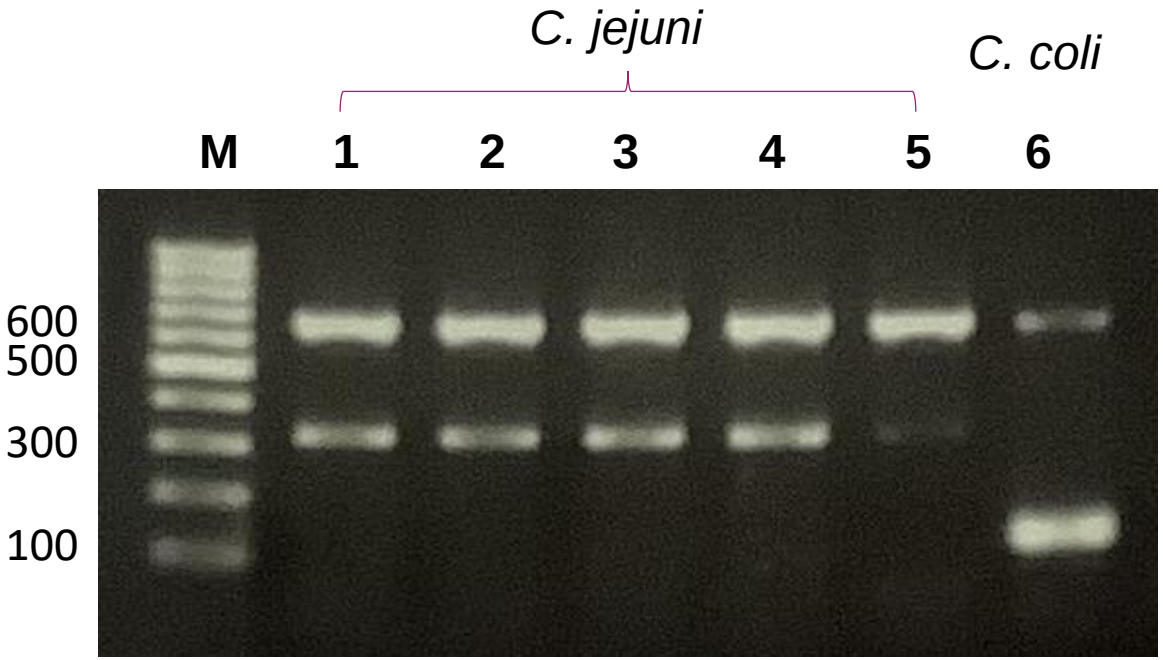
Multiplex PCR amplifies multiple target DNA simultaneously

- several target DNA fragments are amplified simultaneously with different primers.
- The products must be of different sizes and can be visualized by gel electrophoresis.
- Often designed to detect different genes, all relating to the same resistance phenotype
  - detection of ESBL genes
  - detect genus and species of bacteria

Example of multiplex PCR

Detection of *Campylobacter* spp.

	Reagents	Volume used (µl)	Final conc.
	10X PCR buffer	10	1X
	25 mM $MgCl_2$	3	1.5 mM
	10 mM dNTPs	1	200 µM
	DNA template	5	100-500 nM
1.	CJF (10 µM)	2.5	0.5 µM
	CJR (10 µM)	2.5	0.5 µM
2	CCF (20 µM)	2.5	1.0 µM
	CCR (20 µM)	2.5	1.0 µM
3	CLF (20 µM)	2.5	1.0 µM
	CLR (20 µM)	2.5	1.0 µM
4	23SF(10 µM)	1	0.2 µM
	23SR (10 µM)	1	0.2 µM
	Tag polymerase	1	5 units
	dH_2O	13	
	Total	50	



Campylobacter	650 bp
C. jejuni	323 bp
C. coli	126 bp

Wang et al 2002

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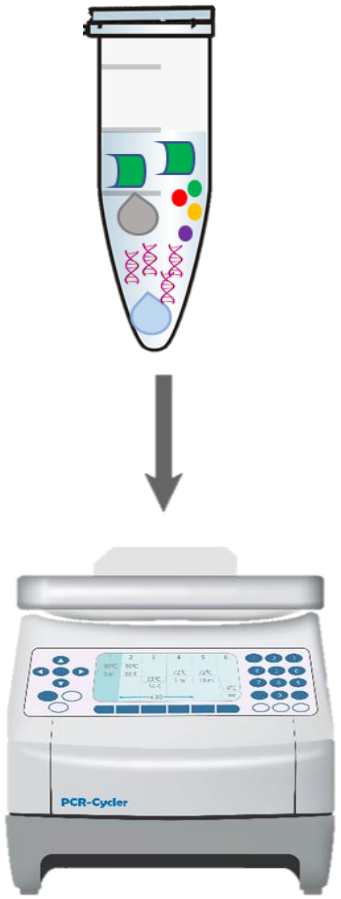
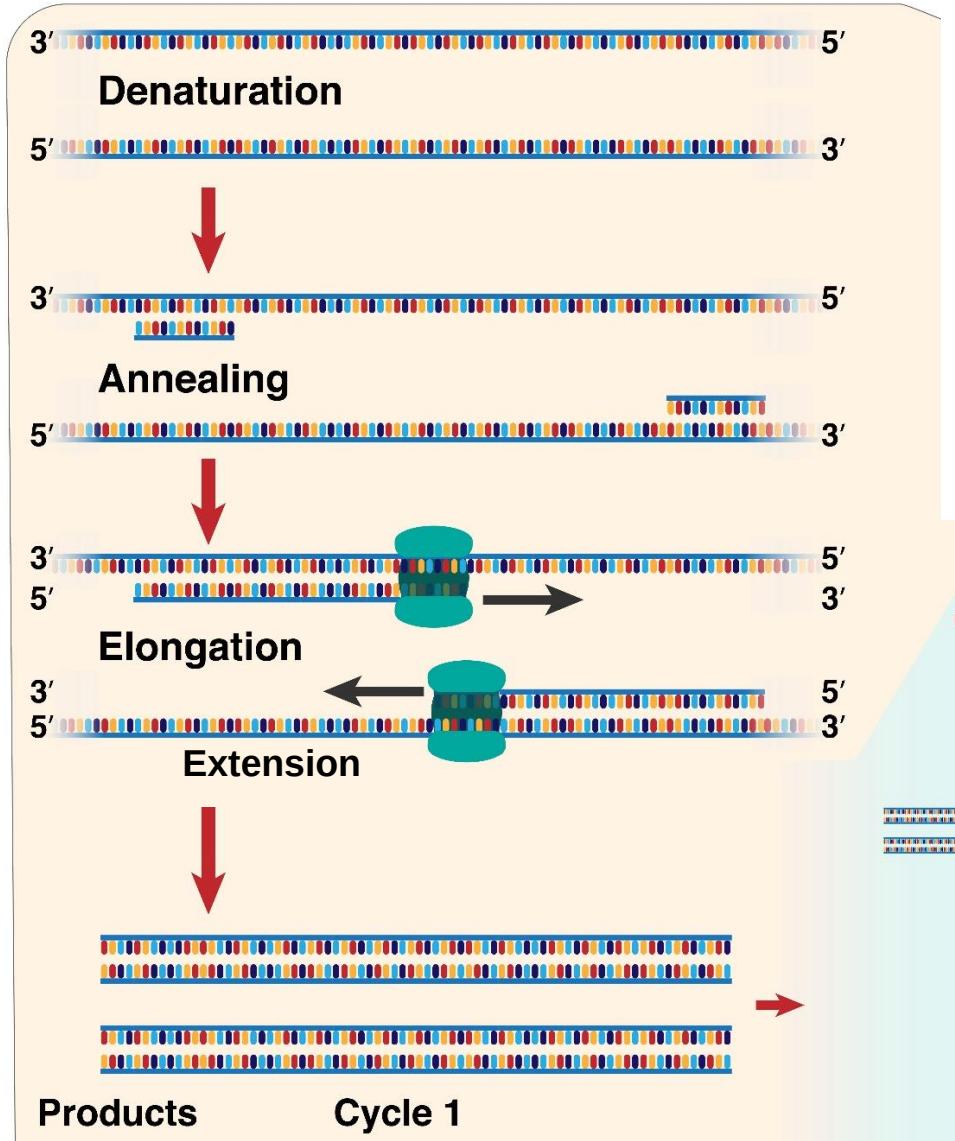
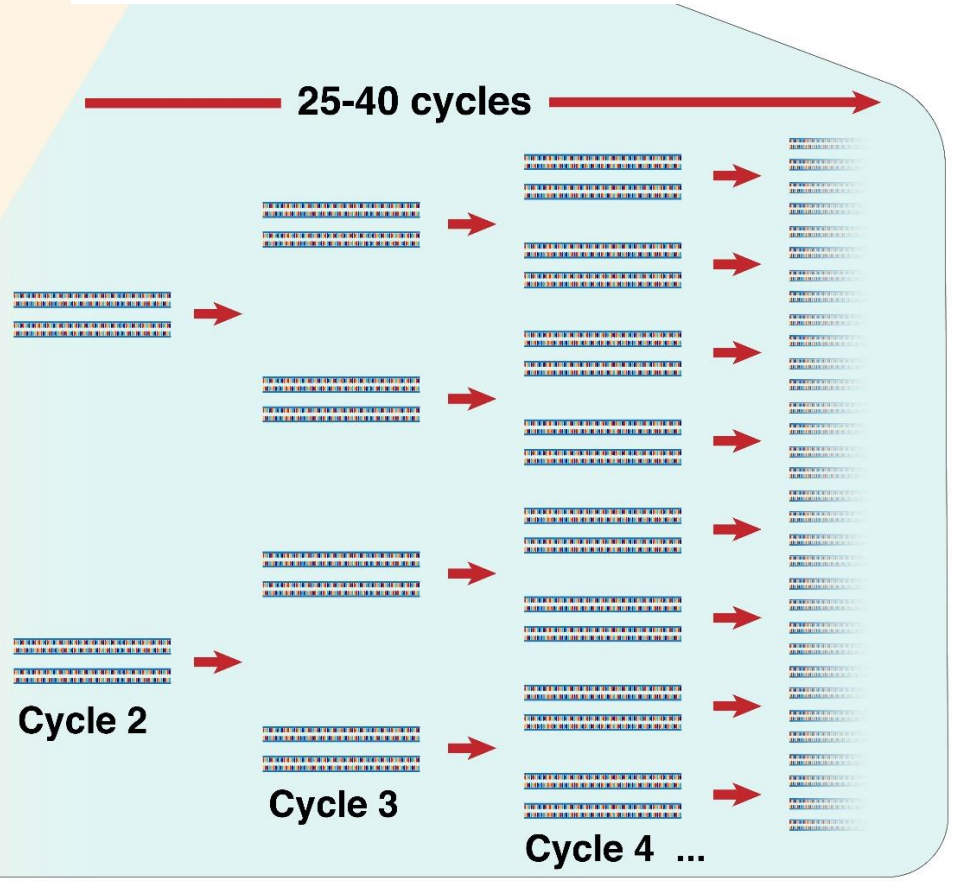
4

PCR cycles







DNA copies in n cycle $= 2^n$

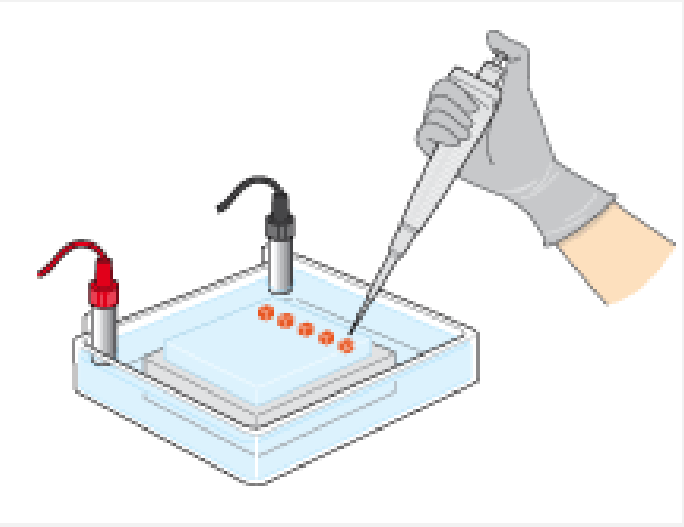
Typical PCR Conditions

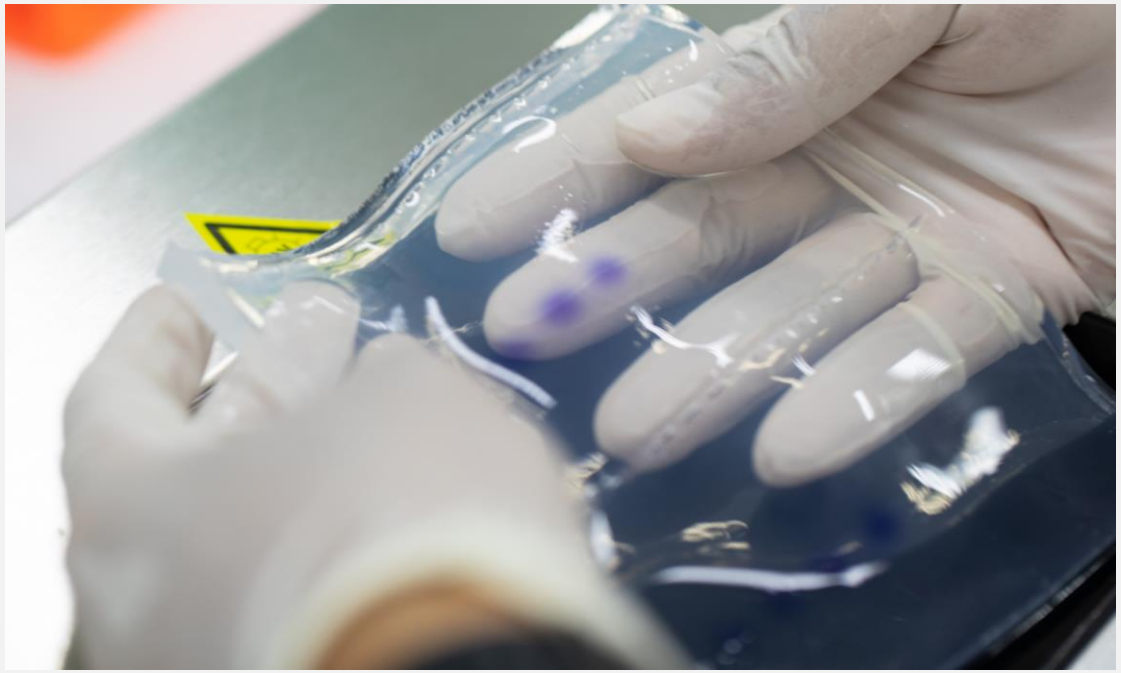
1. Predenaturation	94-98 °C	1-5 min
2. Denaturation 3. Annealing 4. Extension	94-98 °C Ta 72°C	0.5-2 min 0.5–2 min (~1,000bp/min)
Go to step 2, repeat 25-35 cycles		
5. Final extension	72°C	3-10 min
6. Storage	4-8 °C	α

Analysing amplified DNA

Gel Electrophoresis

- A method that separates macromolecules-either nucleic acids or proteins-on the basis of size, electric charge, and other physical properties.
- **Gel is made from "Agarose"** that is a linear polysaccharide and made up of the basic repeat units of agarobiose





Agarose gel

- Decide what percentage of agarose to use
- Agarose concentration should be appropriate for molecular size of interest.
- If there are a wide range of sizes to be separated on a gel, it is recommended to start with a 1.20% agarose gel concentration.

Agarose % (w/v)	Resolution
0.50%	1,000 – 30,000 bp
0.70%	800 – 12,000 bp
1.00%	500 – 10,000 bp
1.20%	400 – 7,000 bp
1.50%	200 – 3,000 bp
2.00%	50 – 2,000 bp

https://toptipbio.com/percentage-agarose-gel/

Buffer solution

➡ able to conduct electricity & working at 1X

Tris acetate EDTA (TAE)

10x Stock

- 48.5 g tris
- 11.4 mL glacial acetic acid
- 20 mL 0.5M EDTA (pH 8.0)
- Adjust volume to 1 L.

Tris borate EDTA (TBE)

10X Stock

- 108 g tris base
- 55 g boric acid
- 900 ml double-distilled H_2O
- 40 ml 0.5 M EDTA solution (pH 8.0)
- Adjust volume to 1 L.

NOTE

- TBE gives a better conductive medium than TAE and is less prone to overheating.
- use TBE for long runs
- Use TBE for <2kb DNA fragments
- Use TAE for isolating the DNA for downstream enzymatic steps.

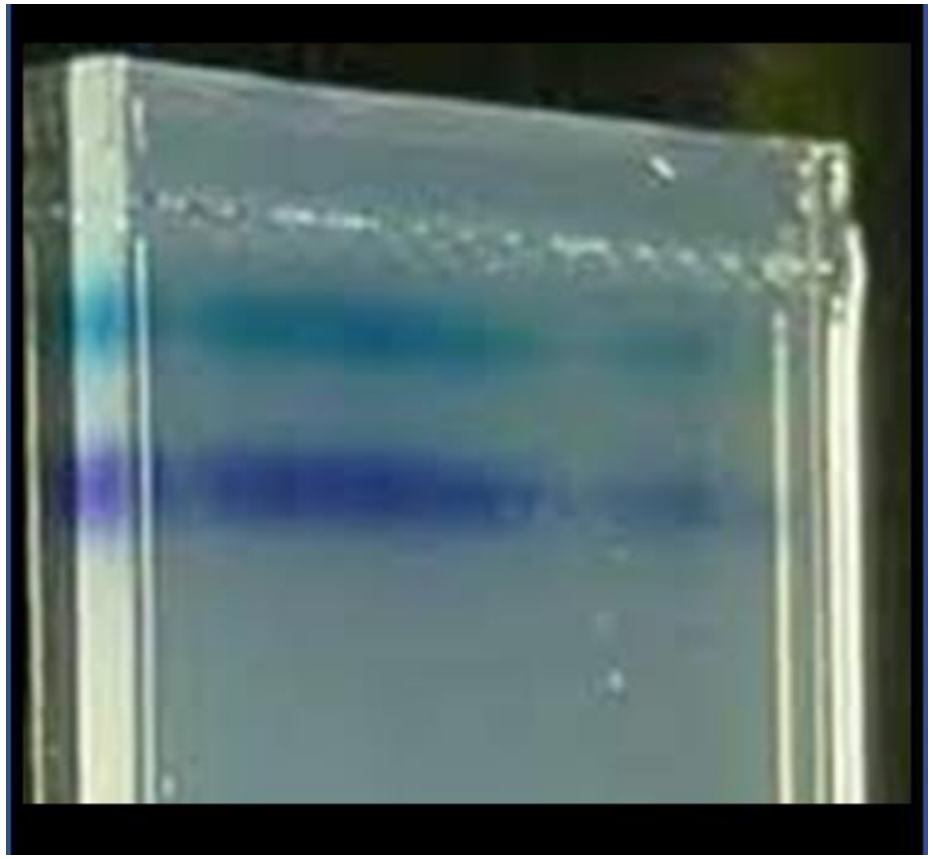
Gel loading dye

- Used to prepare DNA markers and DNA samples for **loading** on agarose **gels.**
- Used as **a tracking dye** during electrophoresis allowing the user to monitor the progress of molecules moving through the **gel**

- **Common recipe**
  - Bromophenol blue
  - Ficoll 400
  - water
  - Optional Xylene cyanol, Tris and EDTA, sucrose, glycerol)

Xylene cyanol

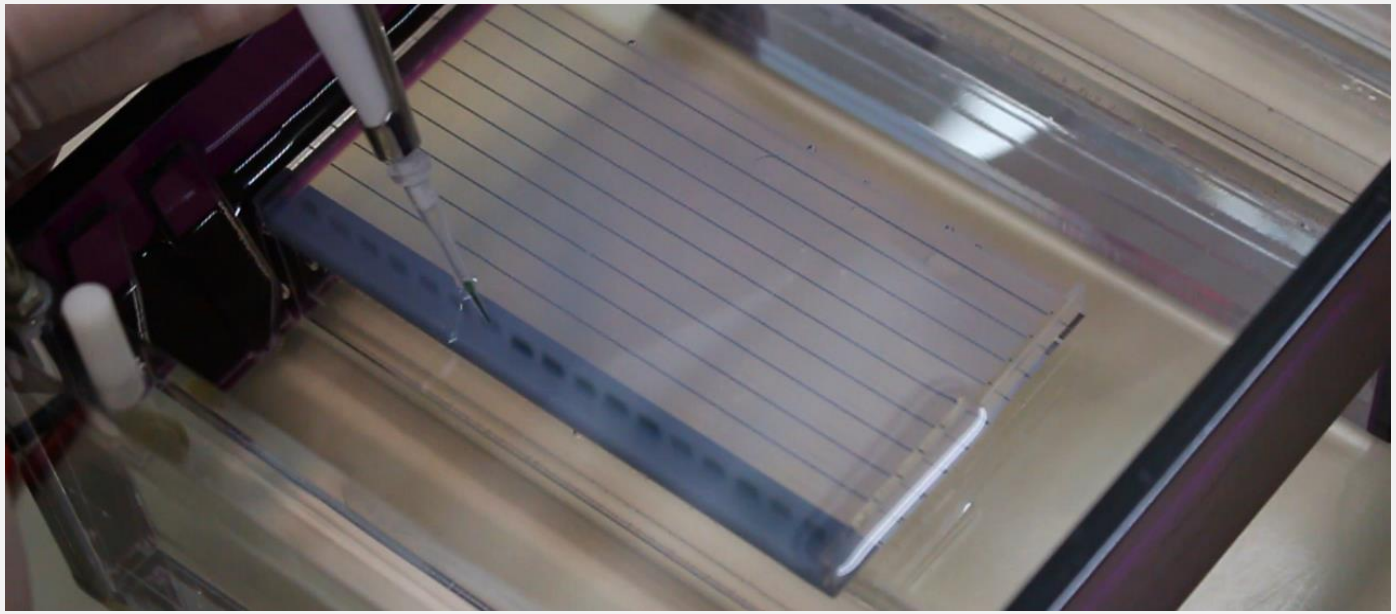
Bromophenol blue

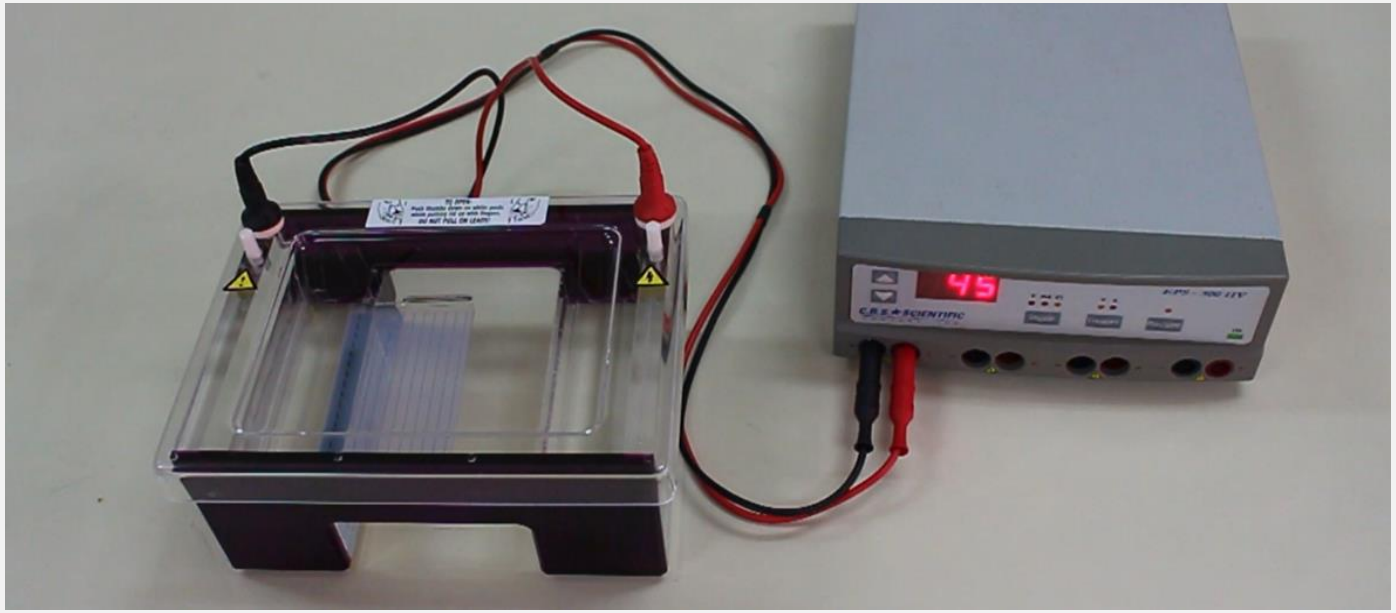


https://toptipbio.com/percentage-agarose-gel/

DNA loading & running gel electrophoresis

- Mix DNA with loading dye
- Load to a gel.
- Apply electricity (Run DNA on gel)
- Staining
- Visualize DNA
- PCR products are visible as different “bands”.





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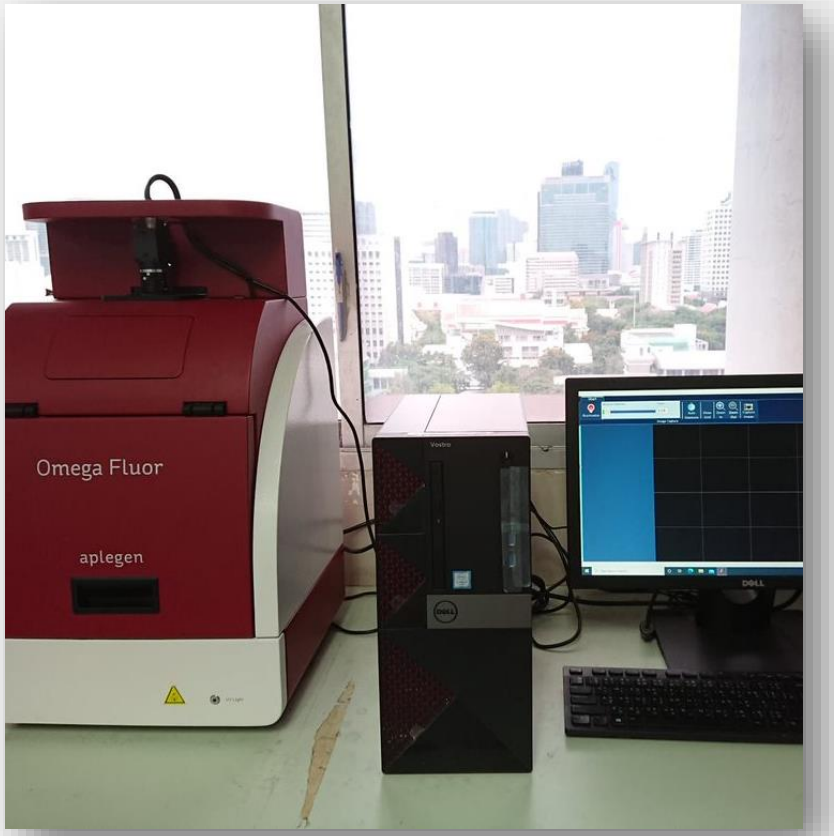
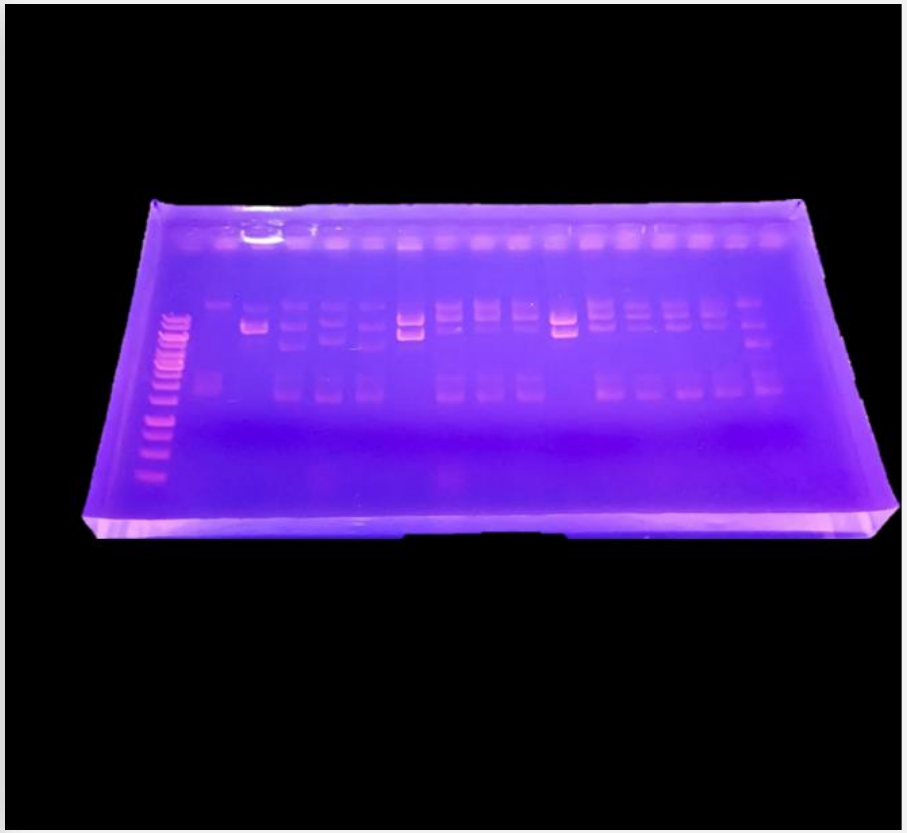
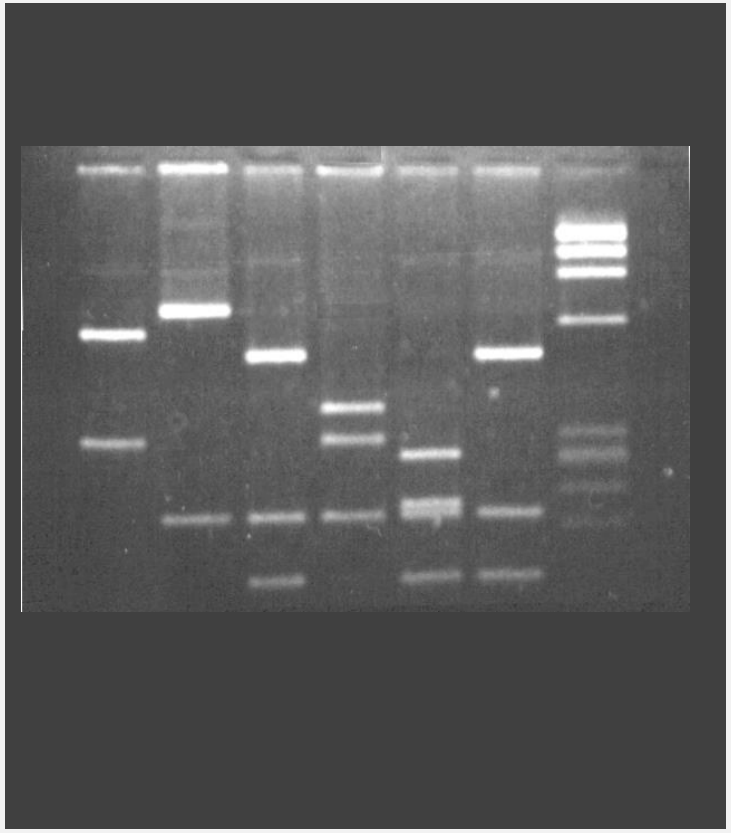
Visualizing DNA



UV transilluminator





DNA gel

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Thank you

This programme is being funded by the UK Department of Health and Social Care.
The views expressed do not necessarily reflect the UK Government's official policies.