MUNI
MED

Genetics in Dentistry – Practice

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Practical Part

Practical Part

1. PCR
2. ELPHO
3. Evaluation of ELPHO

Practical implementation of PCR

volume in microtube: 25 µL each sample

Composition (1 sample):

MASTER MIX

- 2 Primers 10 pmols (1.25 µL)
- $MgCl_2$ 25mM (4 µL)
- dNTP mix (0.5 µL)
- Taq polymerase 1U (1 µL)
- Buffer DYNEX (2.5 µL)
- PCR H_2O (12.5 µL)

- Template DNA 50ng (2µL)

1 drop of mineral oil

1. 95°C…..5 minutes
2. 95°C…..1 minute
3. 60°C…..1 minute
4. 72°C…..1 minute

35 ×

5. 72°C…..7 minutes
6. 10°C…..10 minutes

Practical implementation of PCR

1) Prepare Master Mix for all samples you have by multiplying prescription by the number of samples (plus 1 extra sample).

2) Divide Master Mix to each microtubes by 23 µL.

3) Add one sample (2 µL) to each microtube.

Practical implementation of PCR

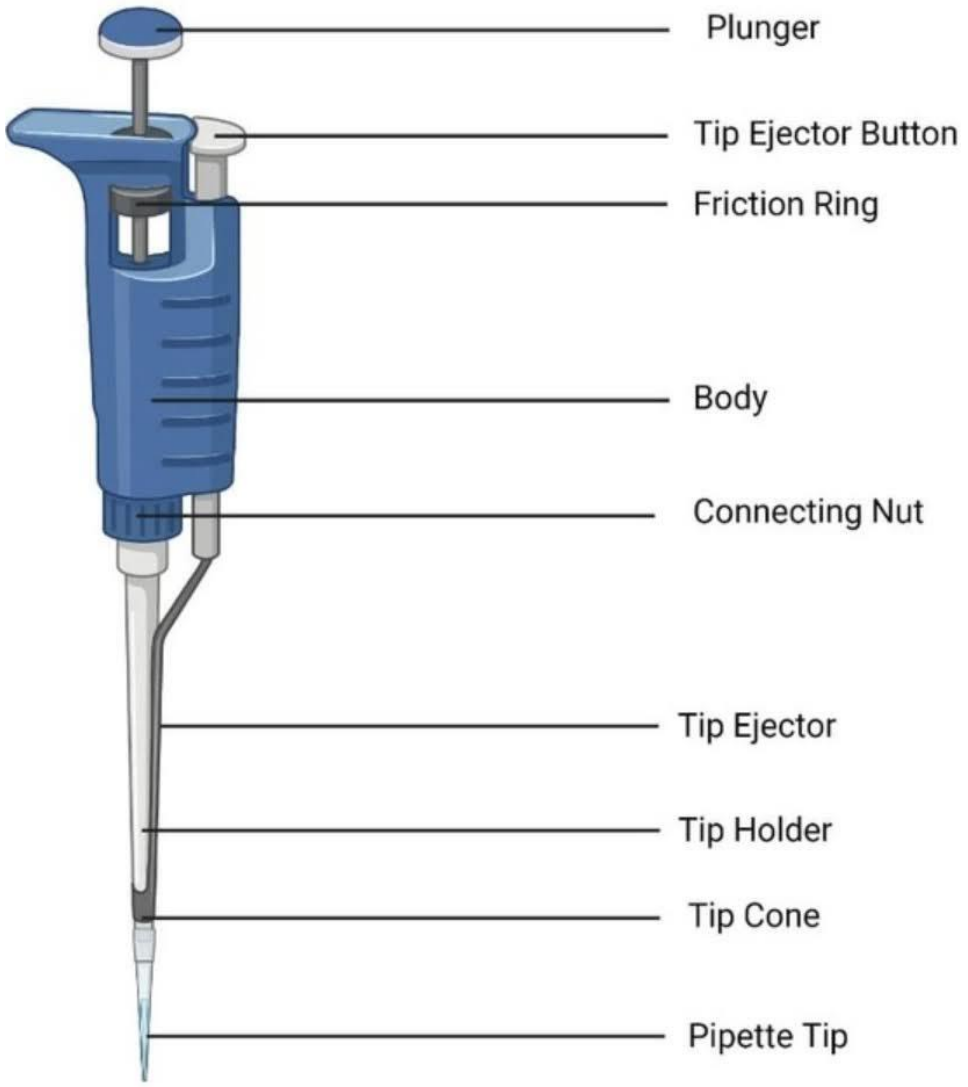
1 drop of mineral oil

1. 95°C…..5 minutes
2. 95°C…..1 minute
3. 60°C…..1 minute
4. 72°C…..1 minute

35 ×

5. 72°C…..7 minutes
6. 10°C…..10 minutes

How to Use a Micropipette



- Always hold the pipette **vertically** (tip pointing down).
- Hold the pipette in a palm hanging from an index finger and operated by a thumb.
- Choose optimal volume range!!! Never set the pipette outside of the range in either direction!!!
- Before pipetting, use a corresponding tip (according to the volume range of the pipette).
- Always use a new sterile tip.
- While pipetting the same solution multiple times, use the same tip during the whole time.
- Tip ejector button on the side of the handle is for ejecting a used tip.

How to Use a Micropipette

Procedure:

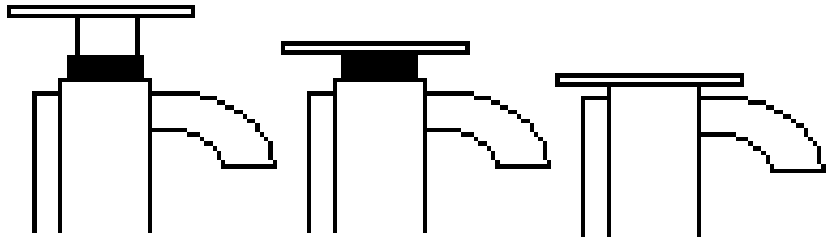
- Set desired volume on the pipette. Horizontal line on display is a decimal point.
- Put a tip on a pipette (seal thoroughly) – not by hand!
- Pick up the pipette so that the finger rest of the handle is on the index finger and thumb can operate the two-position plunger.

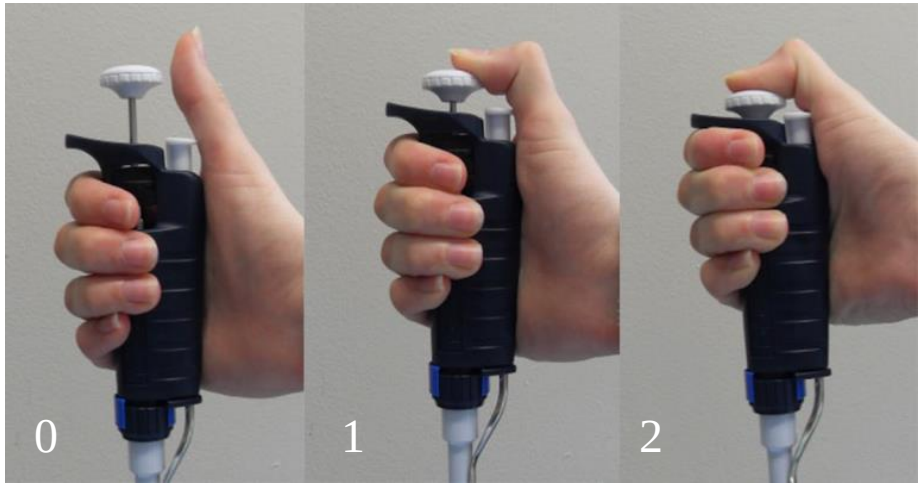
- Aspirating: Press the plunger into the position „1“ (pipette tip is in the air), immerse the tip into the liquid, slowly release the plunger.

- Dispensing: Immerse the pipette into the solution into which you want to dispense the aspirated liquid. Dispense by pressing the plunger into the position „1“, finalize by pressing into the position „2“ and take the tip out of the solution (still in position „2“).

- Release the plunger, throw away the tip.

Plunger position:





Methods in Molecular Biology

Biological Materials

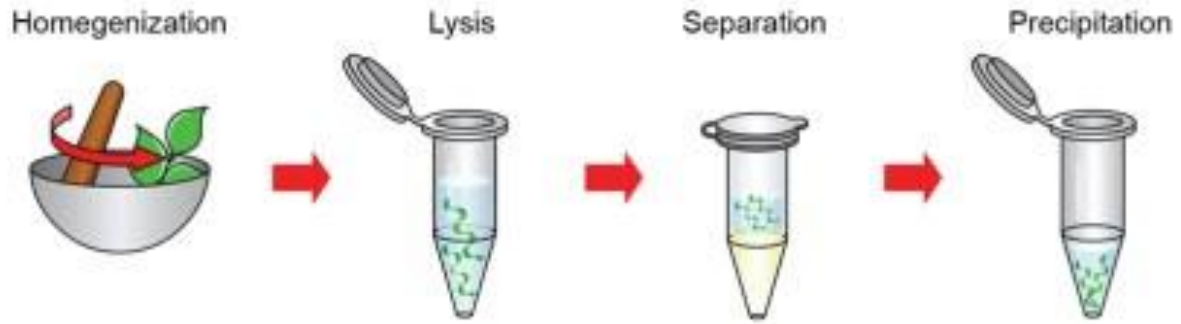
- Biological material is everything that **was** or **is** a **part** or a **product** of a living organism
  - dried herbal tea mixture
  - apple core
  - oak plank
  - cat droppings/fur
  - tube with SARS-CoV-2 virus, smallpox virus
  - bodily fluids – urine, blood, plasma, serum, spit, ejaculate, phlegm
  - tissues, cells

Methods in Molecular Biology

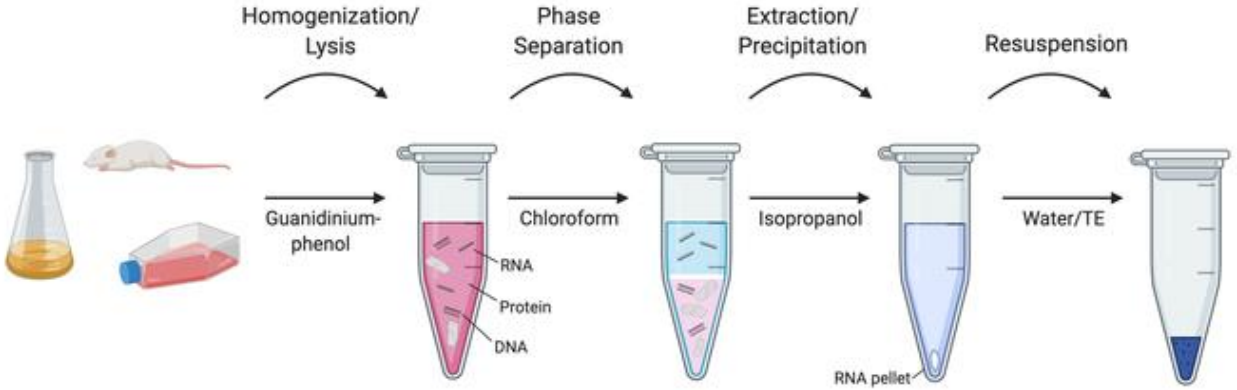
- Determination of nucleic acids
  - PCR, RFLP-PCR – detection by ELPHO
  - Real time PCR
  - Sequencing
- Determination of proteins
  - ELISA
  - Western blot
  - other methods based on antigen-antibody interactions
- Other molecular biology methods

Nucleic Acid Isolation

- In native state from native material – in sufficient quantity and required purity.
- NAs **need to** be **devoid** of all **substances** that would **after lysis** become a part of the crude substrate and that **would impair** the specific **effects** of enzymes used for further analyses.



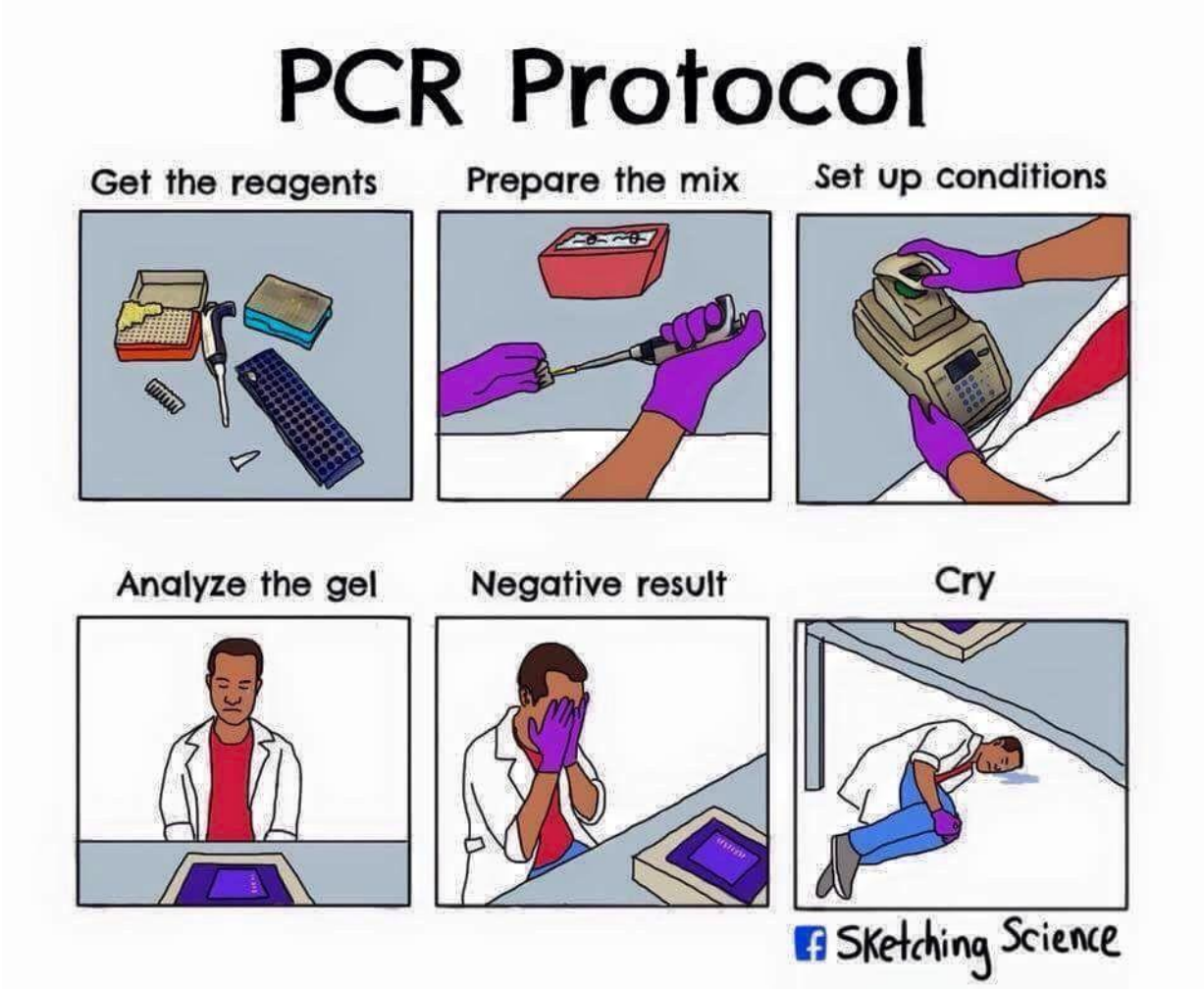
- Isolation of genomic DNA
- Isolation of RNA – focus on protection against degradation!



PCR – Polymerase Chain Reaction

- **Aim** – acquisition of required specific sequence of genomic DNA without previous cloning
- **Principle** – multiple replications
  - 25 to 35 cycles
  - depends on temperature of reaction mixture
  - amount of replicated DNA grows exponentially (2^n)

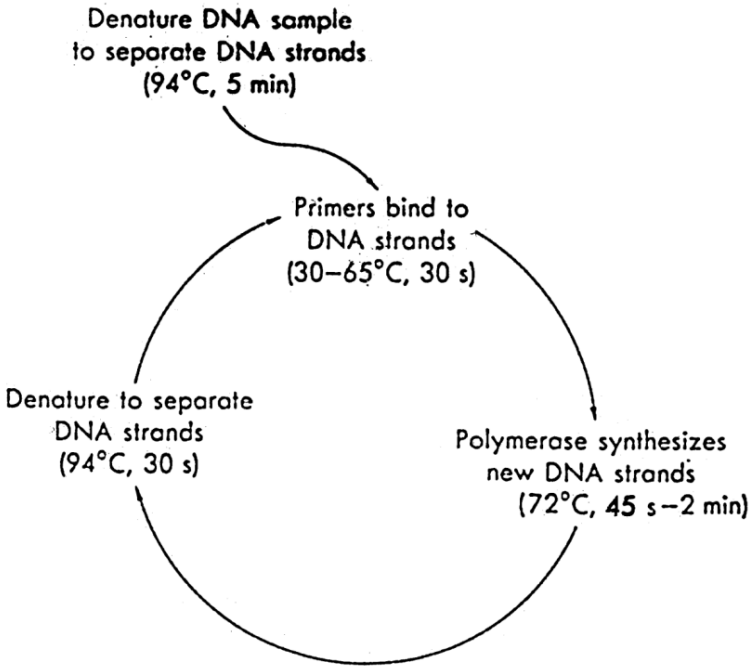
- Thermocycler

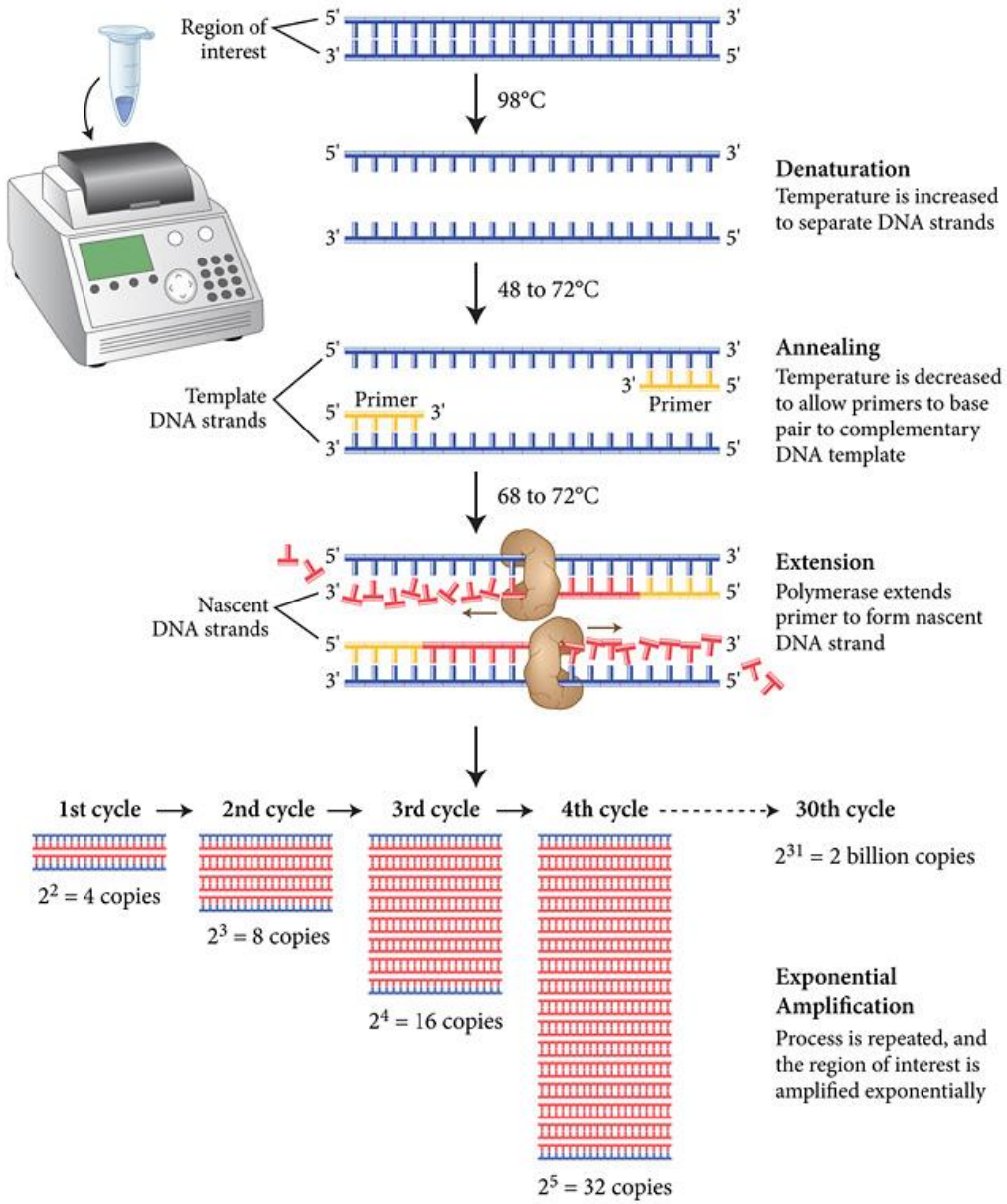


PCR

- **Multiple *in vitro* replication in a tube**
- Chain reaction based on DNA replication

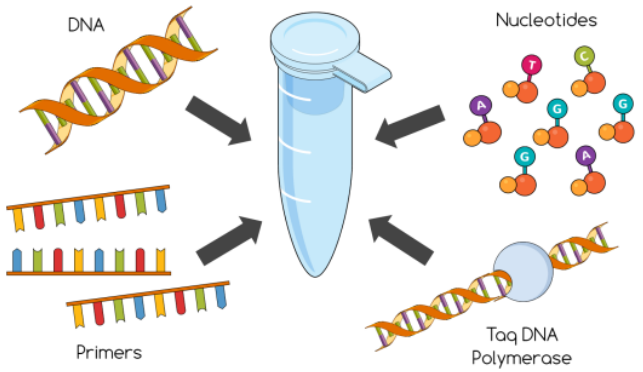
- Repeating cycles:
  - denaturation (separation of dsDNA) – 96 °C
  - annealing – primer binding – 50–65 °C
  - elongation – synthesis of a new DNA strand – 72 °C



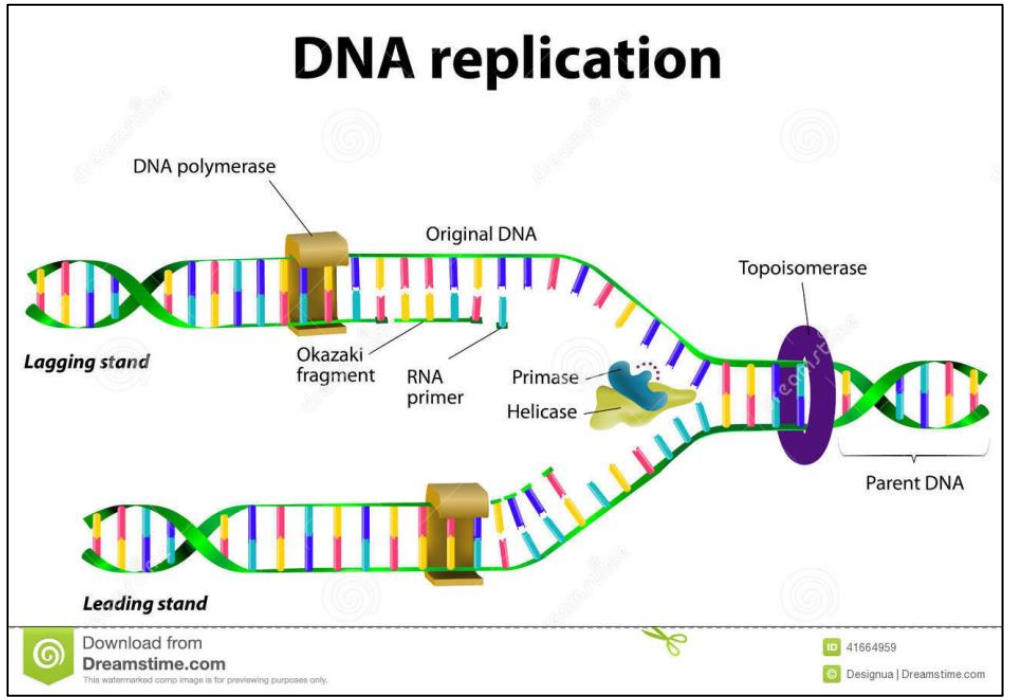


PCR

- **DNA replication – *in vitro* (PCR)**
  - **DNA polymerase**
    - thermostable (resists to temperatures up to 98 °C)
    - Taq (*Thermus aquaticus*), Tth (*Thermus thermophilus*)
  - **primer**
    - short specific segments of DNA
    - oligonucleotide 20–25 pb
    - limiting the region for DNA amplification
  - **Mg^{2+} ions**
    - affect activity and precision of polymerase
  - **template DNA**
  - **dNTP**
  - **buffer** (pH=8)
  - **temperature**



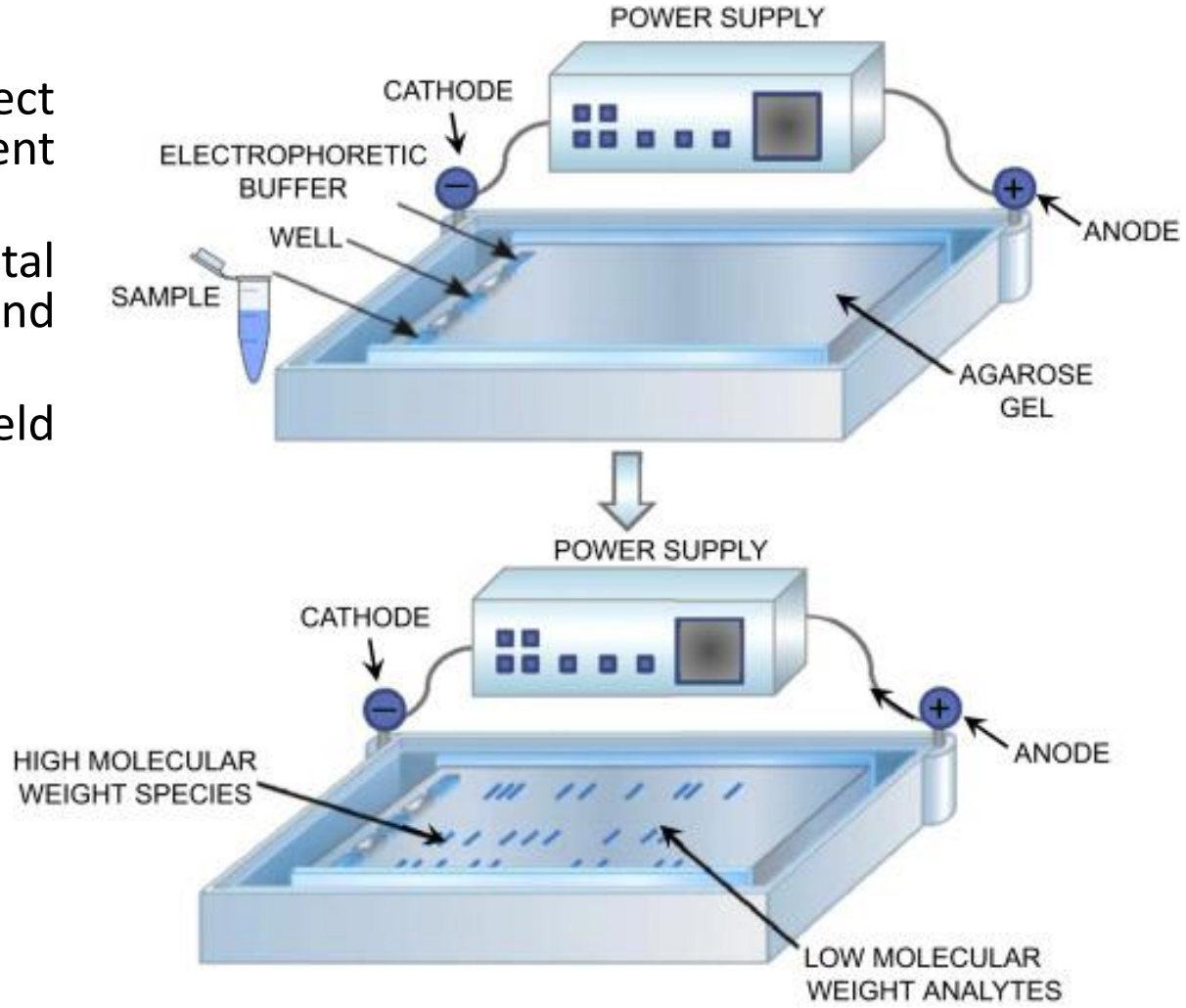
- **DNA replication – *in vivo***
  - **enzymes** – helicase, primase, DNA polymerase, ligase…



Video: https://www.youtube.com/watch?v=matsiHSuoOw and https://www.youtube.com/watch?v=oqeV72oYfD0

Gel Electrophoresis

- separation method
- **principle** – movement of charged molecules in direct current field (separation of molecules with different molecular weight)
- speed of movement depends on the size of the total surface charge, size and shape of the molecule and its concentration in solution
- DNA has uniformly negative charge → in electric field moves **from cathode to anode**
- parts of **equipment**
  - electrophoretic container
  - separation gel
  - buffer
  - direct current power supply
- **agarose** (produced by seaweed – agar)
- EtBr – intercalates between bases, makes DNA visible under UV

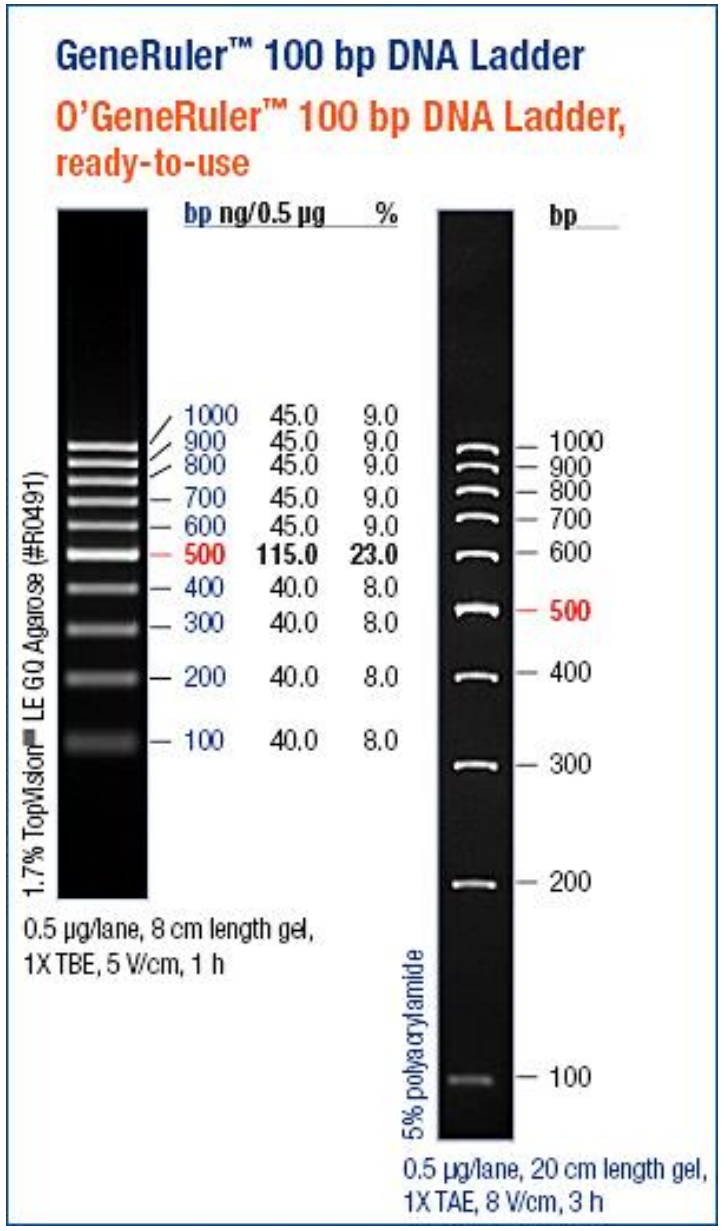


https://www.sciencedirect.com/topics/chemistry/agarose-gel-electrophoresis

Video: https://www.youtube.com/watch?v=vq759wKCCUQ

Gel Electrophoresis

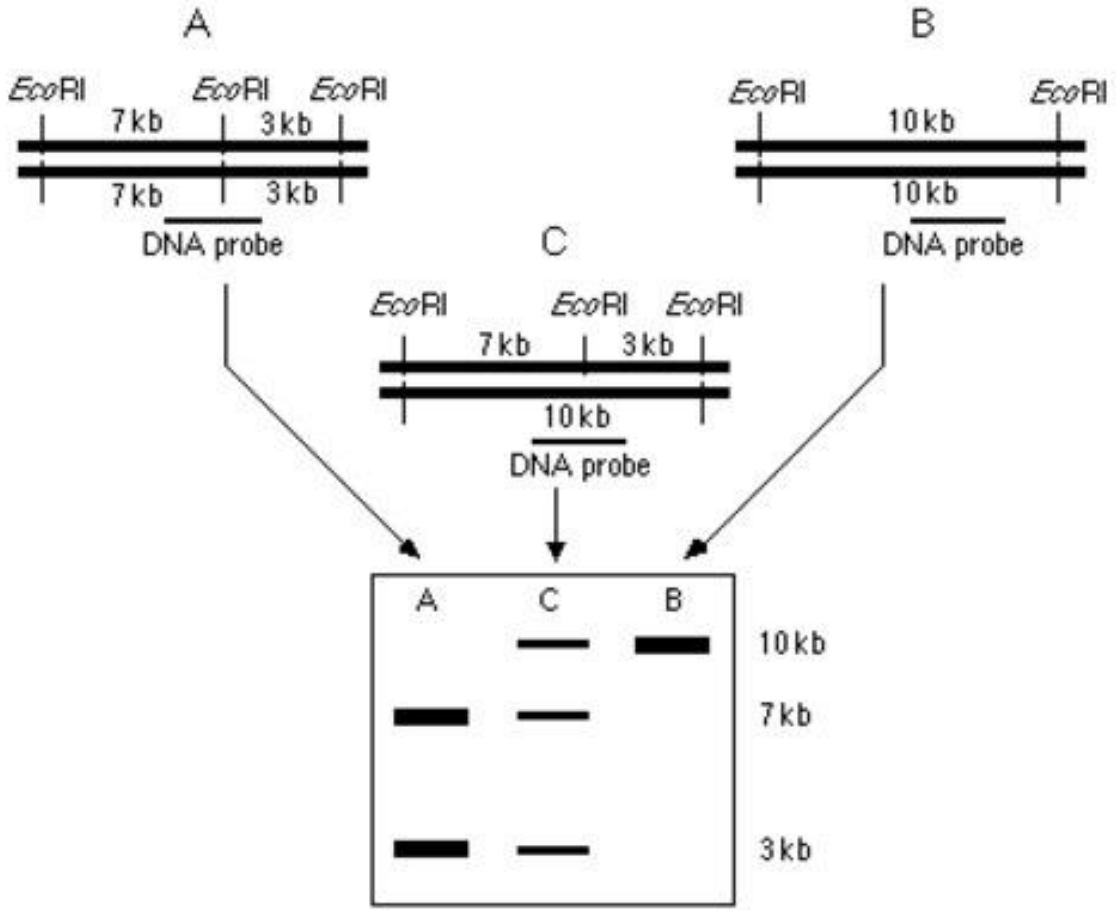
- Size of DNA fragments can be determined with molecular-weight ladders (= restriction fragments of plasmid molecules or genome of bacteriophages, size of which was determined *via* sequencing)



RFLP – Restriction Fragment Length Polymorphism

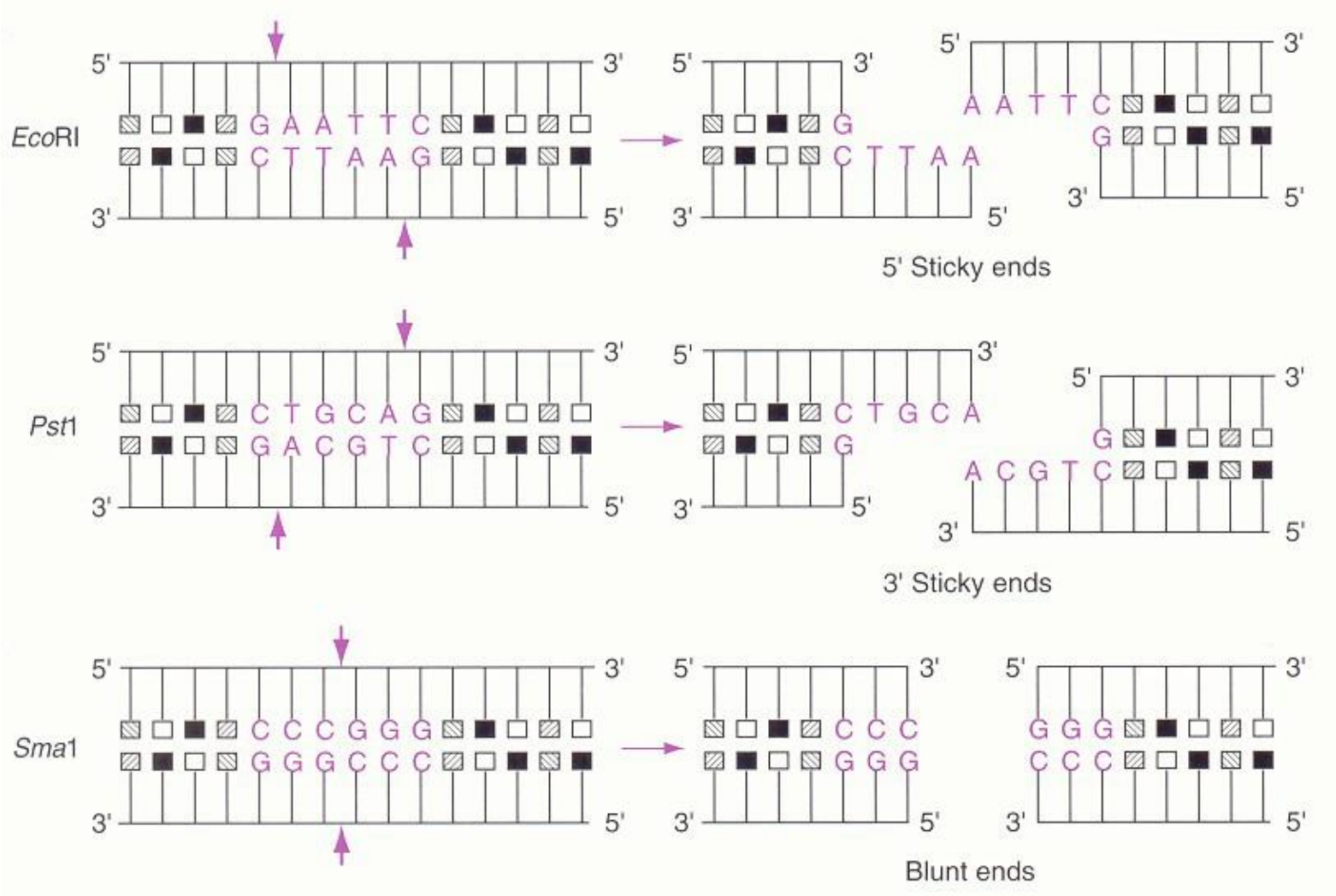
- Enzymatic cleavage of DNA in specific restriction site
- **Restriction endonucleases**
- Production of fragments with different lengths
- Created fragments separated *via* gel electrophoresis

- **Use**:
  - DNA mapping, analysis of DNA modifications, preparation of mutants
  - based on length and number of fragments we can observe differences in studied sequences, so called **polymorphisms** (polymorphisms are created by reconstruction of DNA strand, e.g. insertion, deletion, base substitution)
  - kinship analysis, determination of paternity, identification of persons



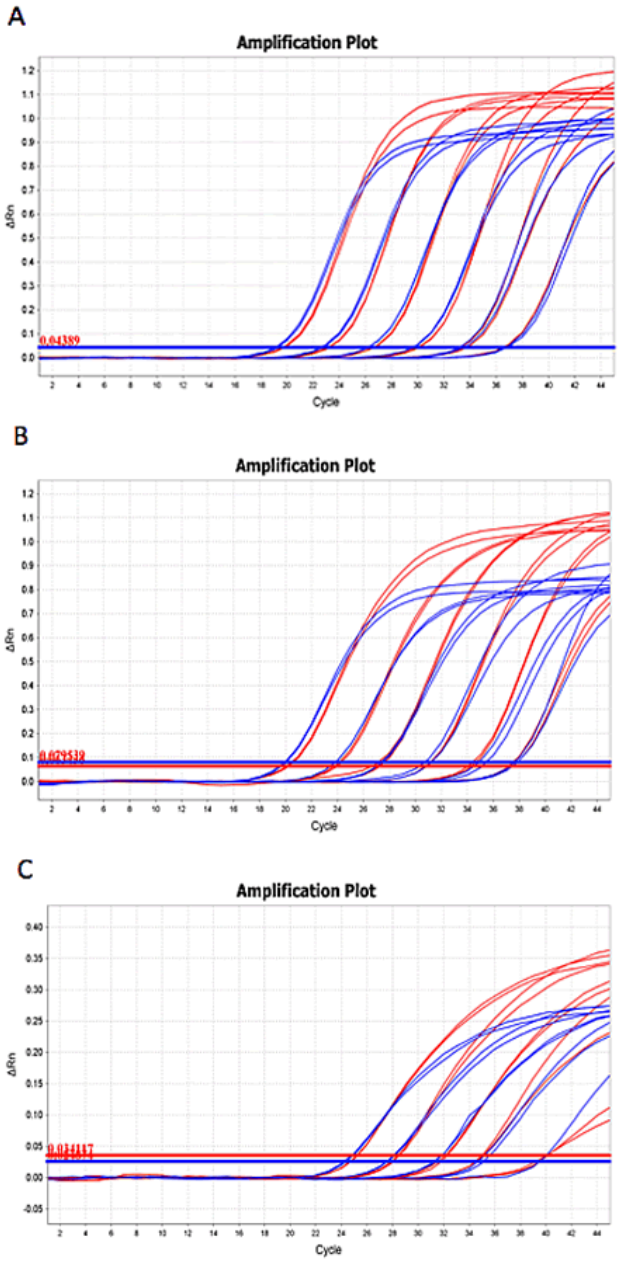
RFLP

- **Restriction endonuclease**
  - sequence specific endonucleases (originated from bacteria)
  - EcoRI (*Escherichia coli*), HindIII (*Haemophilus influenzae*)
  - blunt/sticky ends
  - function:
    - **recognition of specific dsDNA sequence and subsequent restriction** (hydrolysis of phosphodiester bonds)
  - recognition sequence
    - 4–8 bp long
    - character of **palindrome** = same sequence of bases in both directions



qPCR – Quantitative Real-time PCR

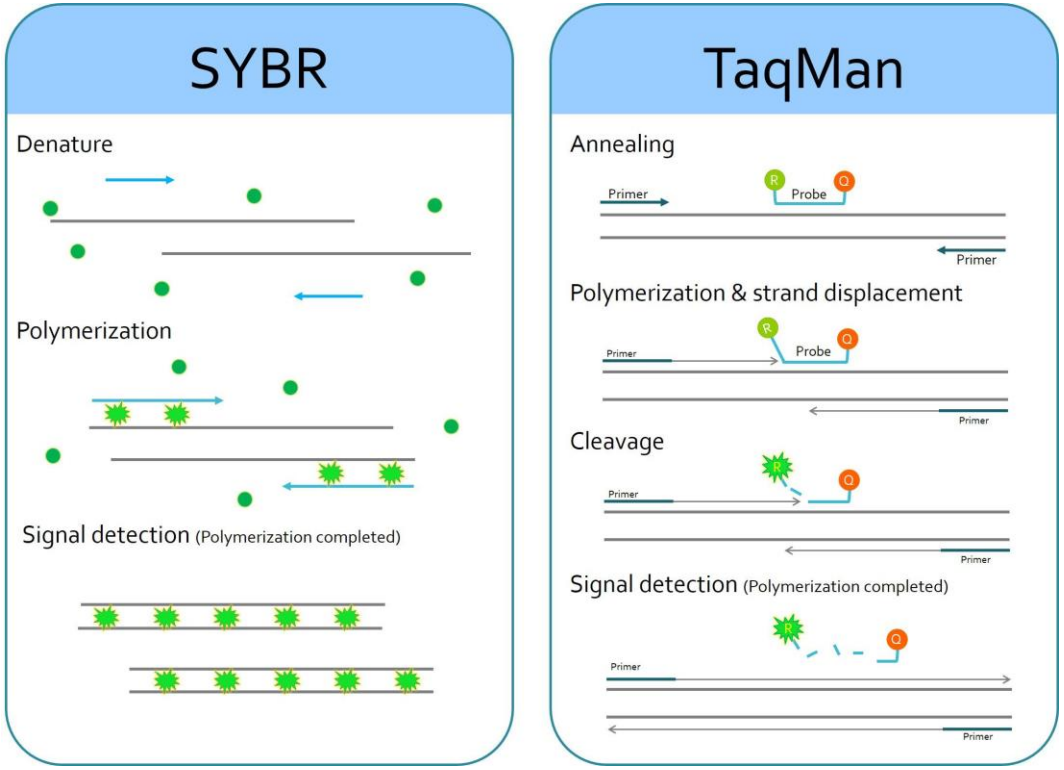
- Polymerase chain reaction monitored in real time
- **Quantification of DNA –** amount of DNA is monitored during each cycle
- Detection of the DNA amount enabled by the presence of **fluorescent substrate**
- Performed in a special cycler, which allows:
  - Cyclical changes of temperature
  - Fluorescence detection
  - Monitoring of PCR progress in real time without the need to detect PCR products *via* electrophoresis
- qPCR is usually performed in 96-well plates, level of fluorescence is monitored in each well
- Highly sensitive and specific method



qPCR

- Intensity of fluorescence is **directly proportional to the amount of product created in the reaction**

- Product detection:
  a) **intercalating dyes** – SYBR Green – non-specifically binds to dsDNA
  b) **sequence specific probe** – short oligonucleotide with a dye and a quencher (TaqMan) – after its breakdown during DNA synthesis – rise of fluorescent signal (uses 5'-3' exonuclease activity of DNA polymerase)



Video: https://youtu.be/YhXj5Yy4ksQ

DNA Sequencing

- determination of **primary structure of DNA** (sequence of nucleotides)

a) **chemical method –** degradation of nucleic acid chains *via* chemical agents (dimethyl sulfate, NaOH, hydrazine,..)

b) **enzymatic method –** specific inhibition of enzymatic synthesis of DNA

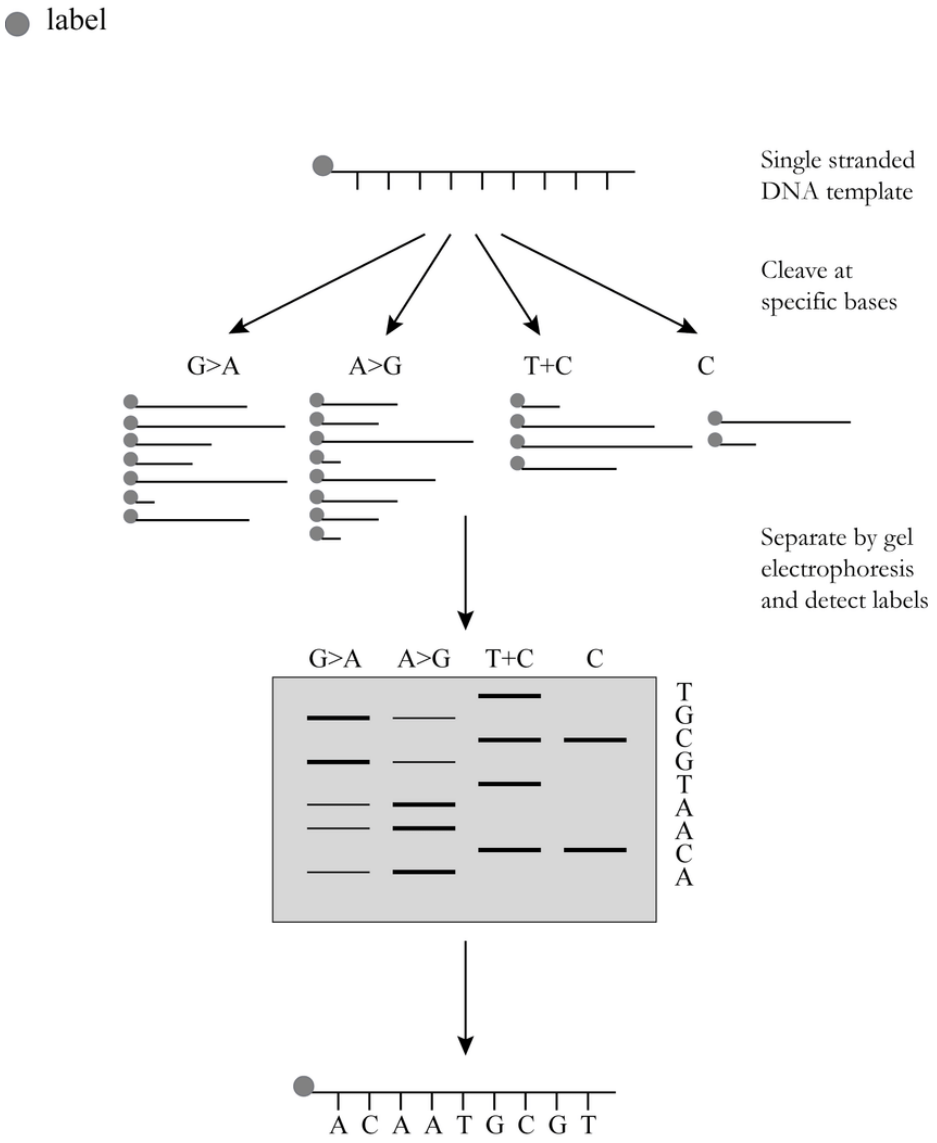
c) modern large format applications based on e.g. pyrosequencing (**next generation sequencing**)

- **Product** – strands of ssDNA, their relative sizes differing by one base (evaluation using ELPHO)

- **Input material** – fragment of DNA with both defined ends

Maxam-Gilbert Sequencing

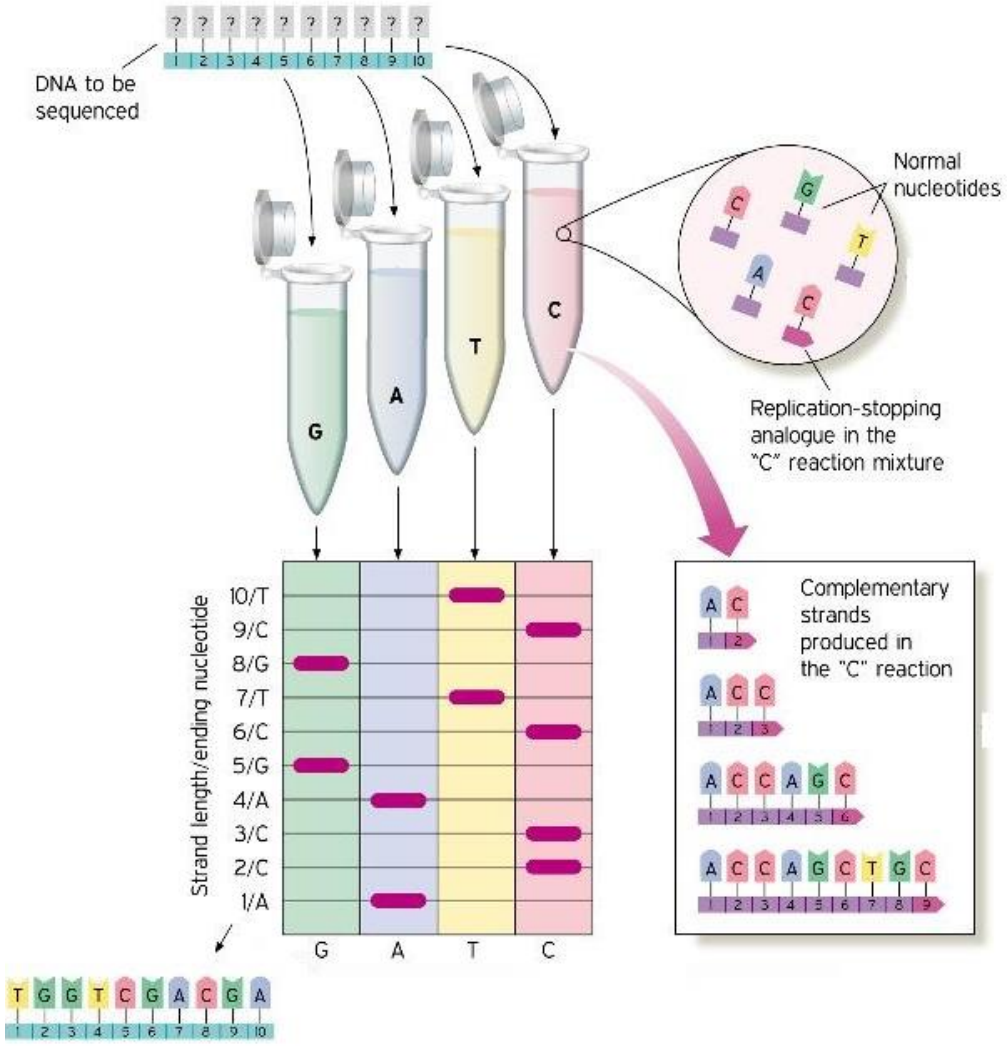
- Sequence is derived from a DNA molecule which chemically degrades to fragments in places with a base of a specific type. These are subsequently separated using ELPHO.
- **Chemical agents** – example:
  - piperidine breaks glycosidic bond of A and G (A + G)
  - hydrazine in the presence of NaCl reacts only with C
  - NaOH at 90 °C strongly cleaves A and weakly cleaves C (A > C)
- Requires radioactive labelling on one end of ssDNA.
- Reaction is done in 4 tubes – in each only some types of bases are cleaved.
- Mixture of differently long fragments ending in a place of a specific base is created ⟶ evaluation using ELPHO, determination of a sequence of a given section.



Video: https://www.youtube.com/watch?v=_B5Dj8PL4E0

Sanger Sequencing

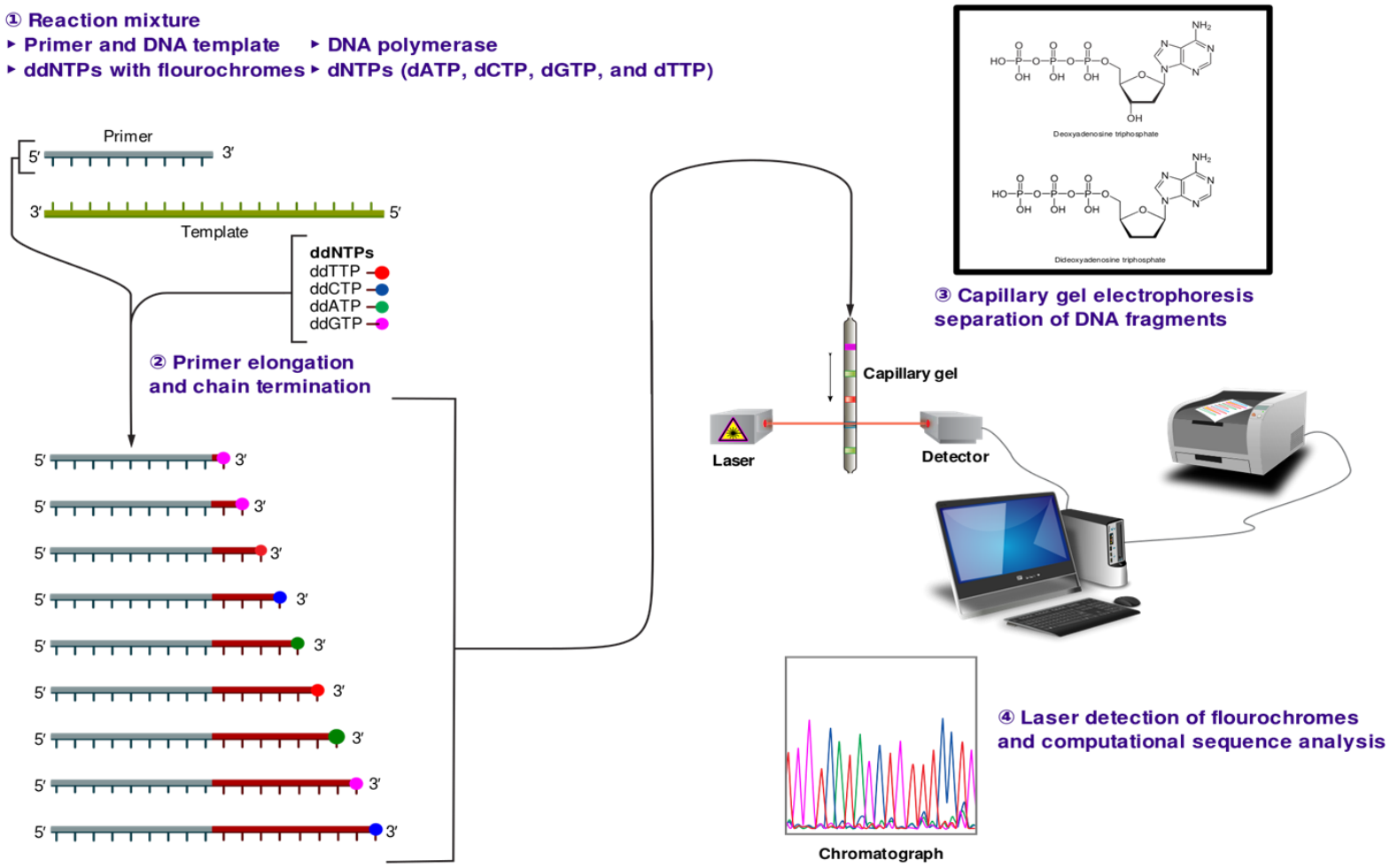
- Enzymatic method
- Based on **principle of replication** – end of DNA synthesis in the moment ddNTP is incorporated instead of dNTP
- **ddNTP** = analogue of dNTP, but lacks 3'-OH group on carbon
- ddNTP – terminator
- **Reaction mixture (4×)**
  - DNA template
  - primer
  - Taq DNA polymerase – synthesis of DNA from 5' to 3' end
  - buffer
  - dNTP – abundant (to get fragments of all possible lengths)
  - ddNTP – low concentration
- **Evaluation** – electrophoresis



Video pro názornost: https://www.youtube.com/watch?v=wdS3j0TgbjM

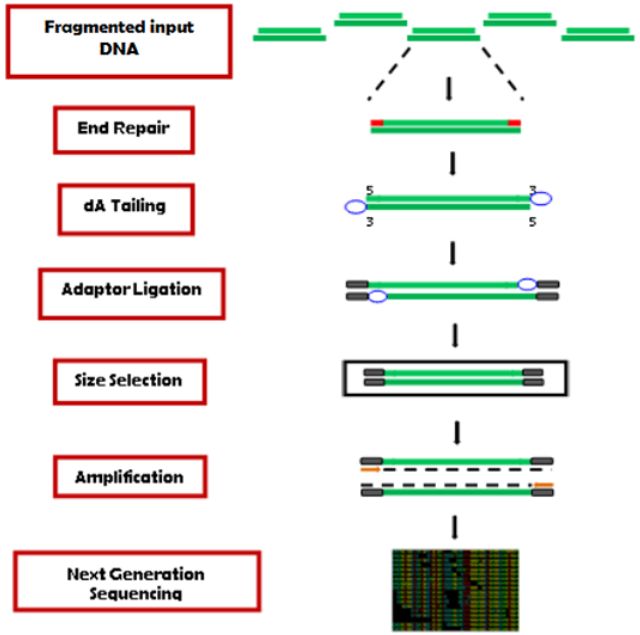
Sanger Sequencing

- Modification –> fluorescently labeled ddNTP (4 different color labels) – reaction performed in one tube
- Capillary sequencing of DNA with fluorescently labeled ddNTP



NGS – Next Generation Sequencing

- Sequencing of thousands to millions of sequences at the same time
- Template DNA are fragmented sections few hundred bases long
- Ends of fragments are enzymatically blunted and connected to oligonucleotides of specific sequence (= adapters)
- Single fragments are separately amplified *via* PCR and in the next step sequenced in parallel

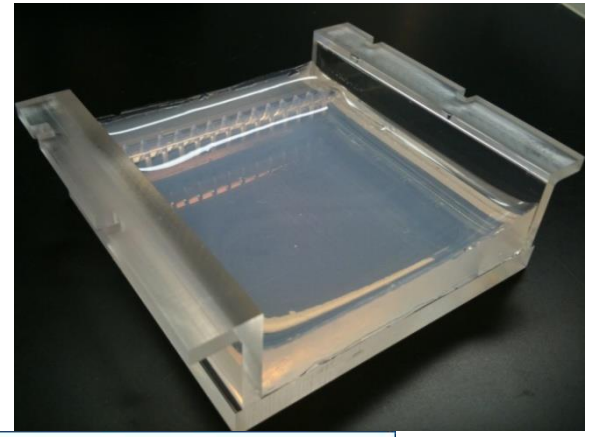


- Use:
  - whole genome sequencing
  - sequencing of chromosomes, plasmids, mt
  - study of genetic variability, mutational analysis
  - transcriptome analysis

Video:
https://www.youtube.com/watch?v=CZeN-IgjYCo
https://www.youtube.com/watch?v=fCd6B5HRaZ8

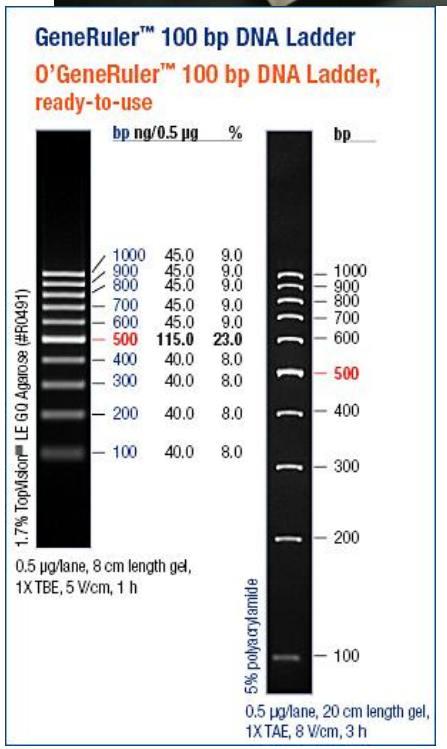
Practical implementation of ELPHO

1. **Prepare casting tray – combs and tape**
2. **Prepare gel (2%): weigh agarose and add it to Erlenmeyer flask, add TBE buffer (200 ml)**
3. **Weigh and boil in microwave.**
4. **After cooling to cca 40 °C add EtBr (1 µl/10 ml)**
5. **Cool down the gel (cca 30 min)**
6. **Remove combs and put into ELPHO container with TBE buffer (electrolyte)**



7. Add size standard ladder (DNA + dye)
8. Prepare drops of loading dye on paraffin paper and mix with DNA samples.
9. Load samples into wells (max 15 µl)
10. Connect to power supply
11. Track the progress of DNA through the gel (40 min)

12. Visualisation under UV and evaluation



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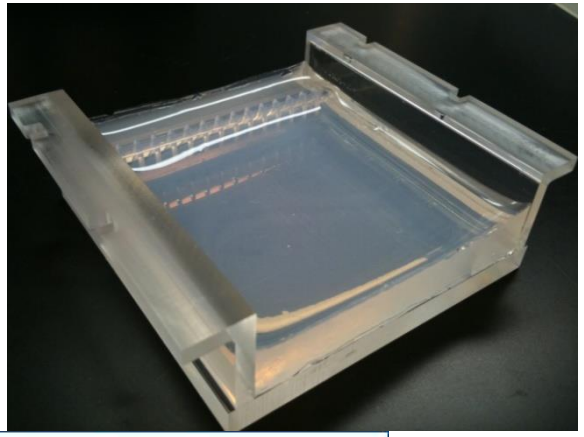

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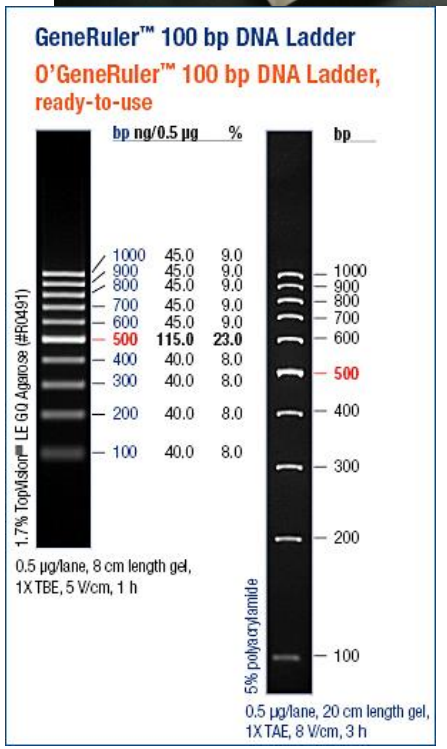
8. Prepare drops of loading dye on paraffin paper and mix with DNA samples.

9. Load samples into wells (max 15 µl)

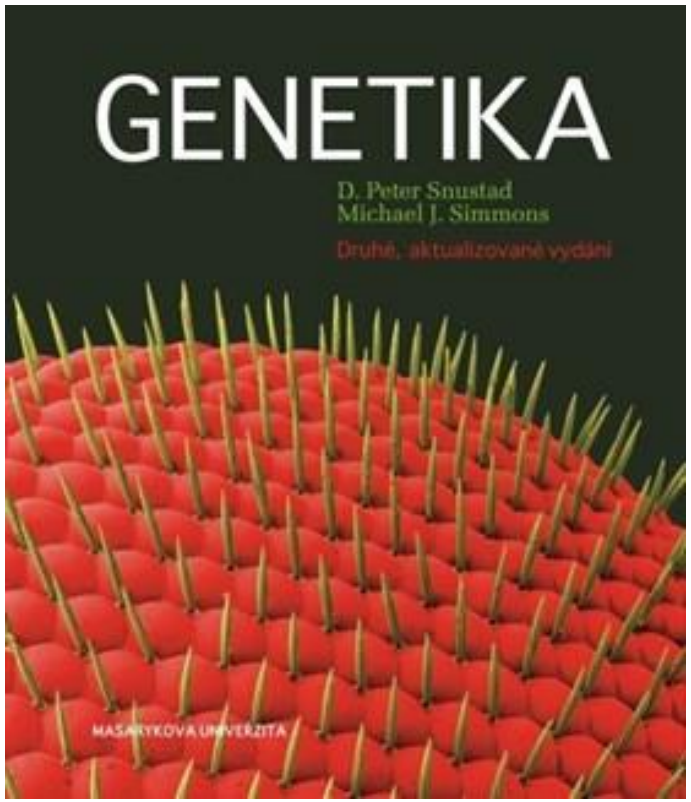
10. Connect to power supply

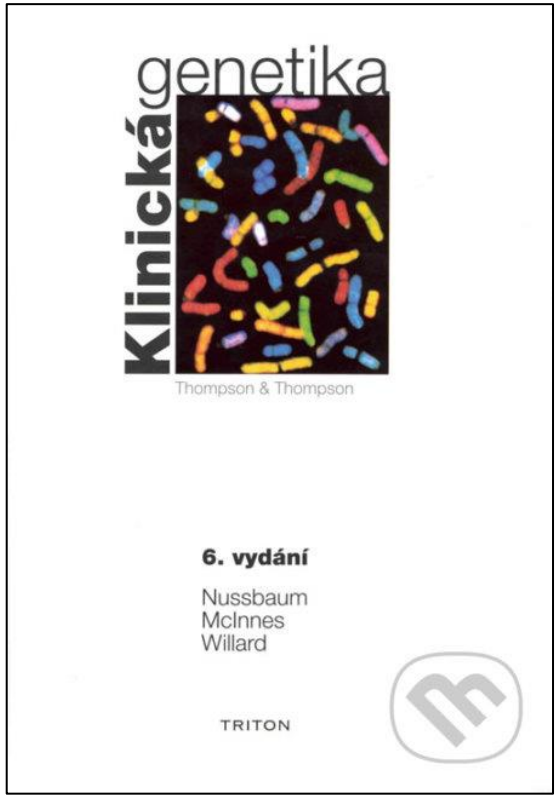
11. Track the progress of DNA through the gel (60 min)

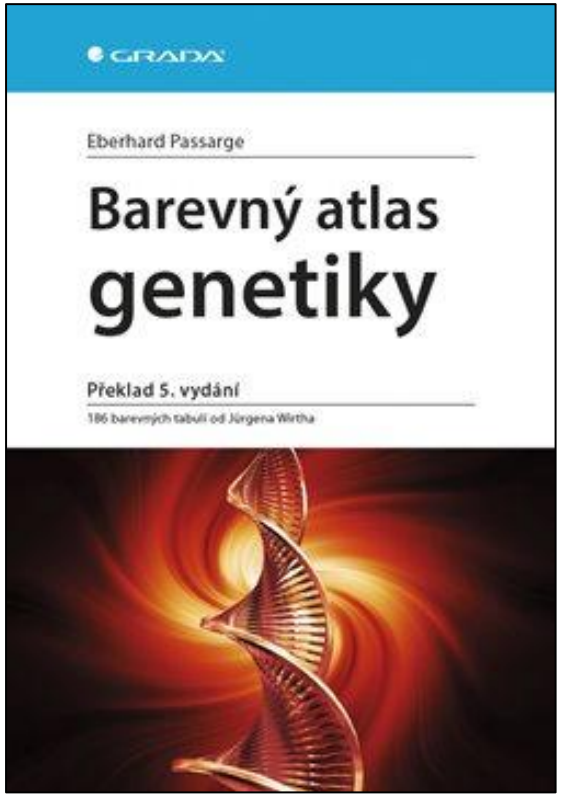
12. Visualisation under UV and evaluation

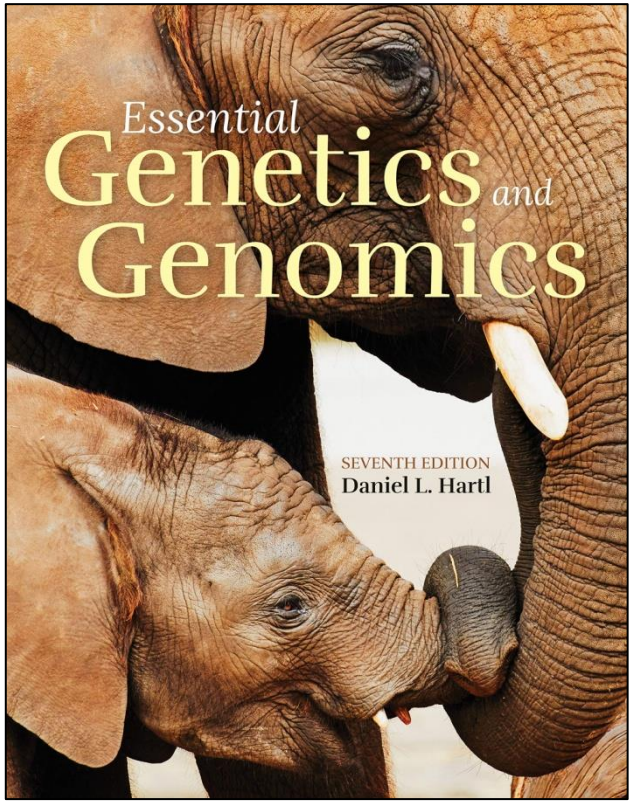


Recommended Literature for Self-study



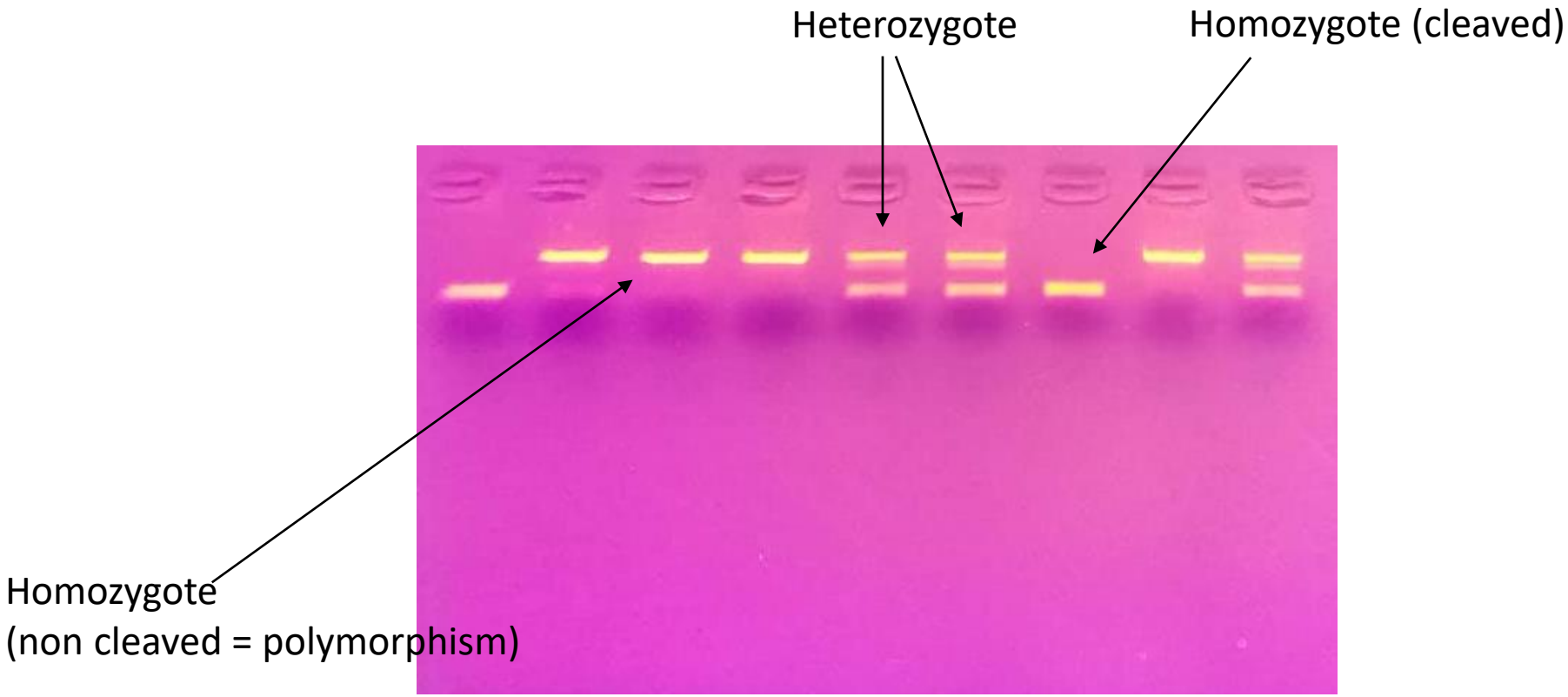


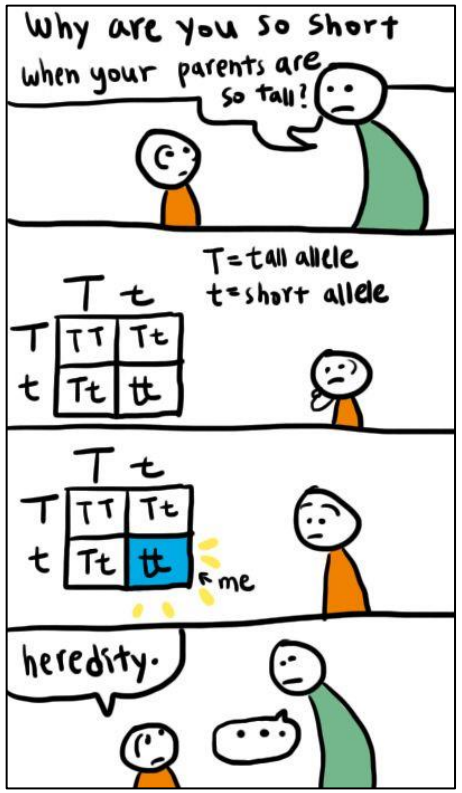


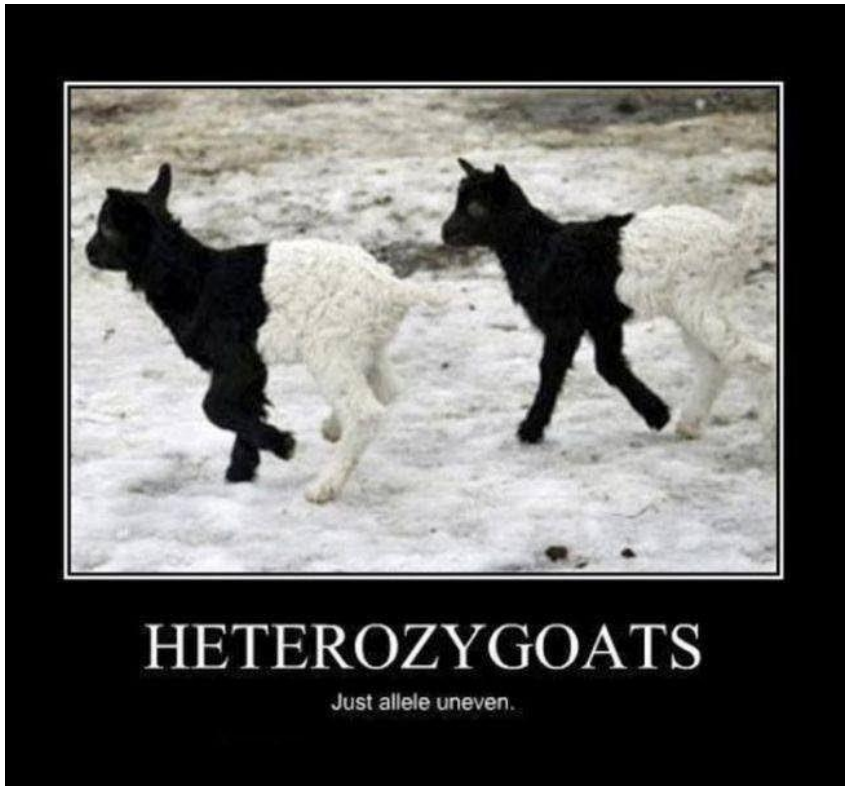


Practical implementation of ELPHO

Electrophoresis of restrictive fragments after enzymatic cleavage







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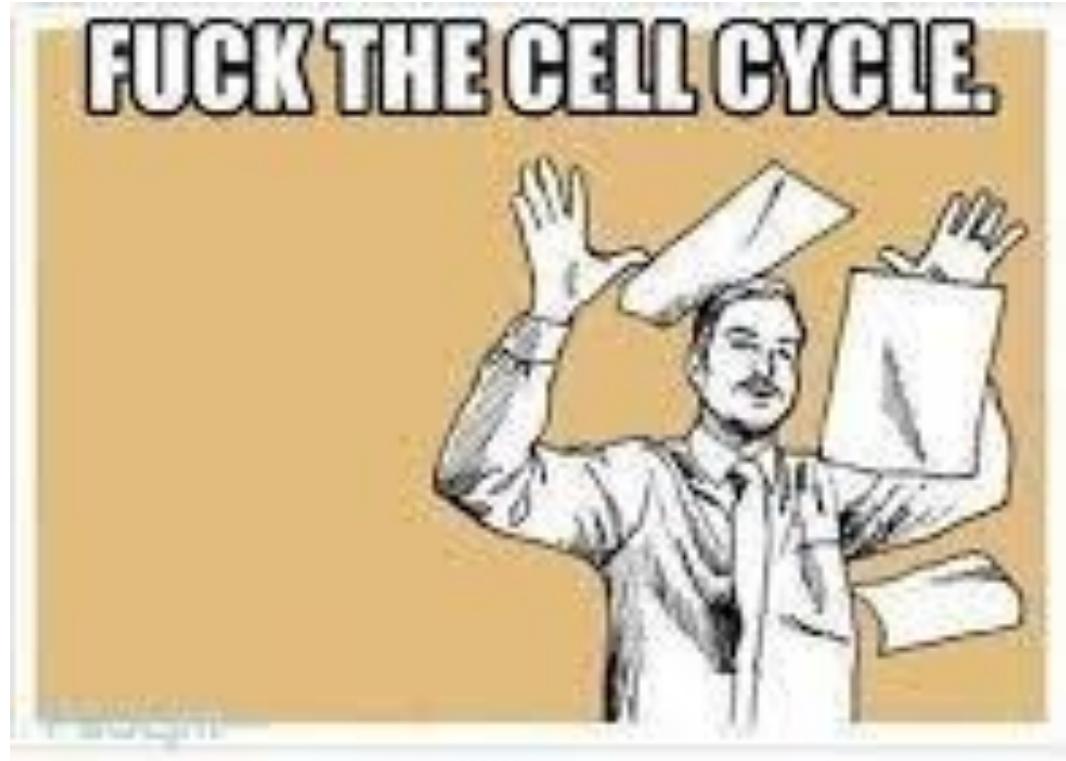
FUCK THE CELL CYCLE.


JUST KIDDING THE TEST IS NEXT WEEK...
memegenerator.net