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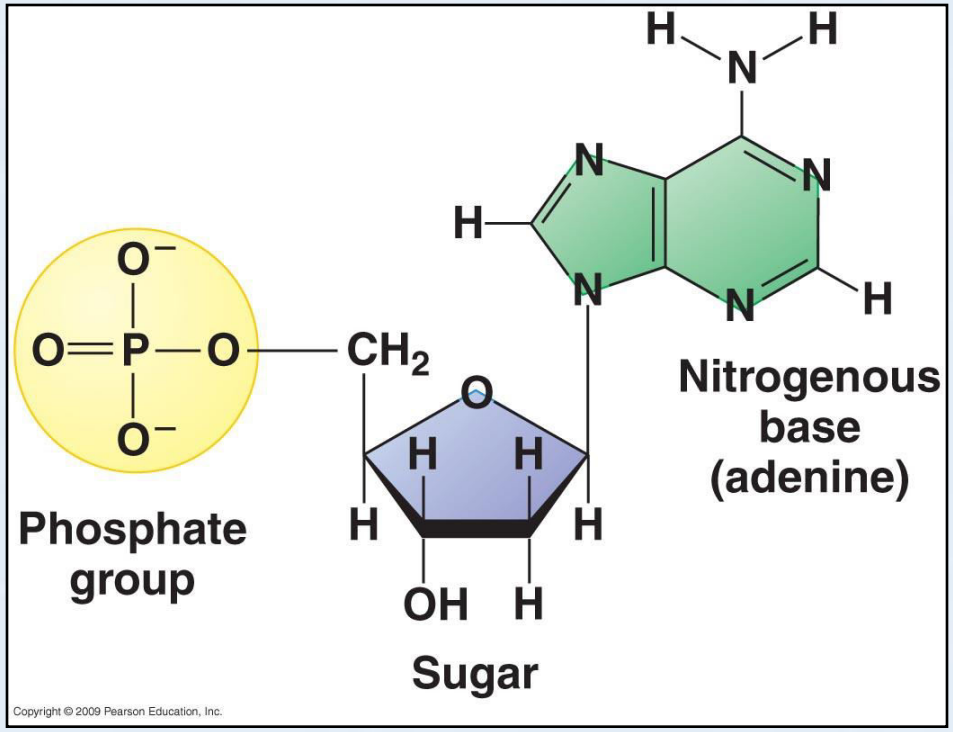
Genetic Analysis: A basic tool for the identification of food products

1. STRUCTURE AND FUNCTION OF DNA

STRUCTURE OF DNA

- Two long polynucleotide chains composed of four types of nucleotide subunits
- Nucleotides in DNA: five-carbon sugar + one phosphate group + a nitrogen-containing base
- Sugar is deoxyribose
- The bases may be either adenine (A), cytosine (C), guanine (G), or thymine (T)

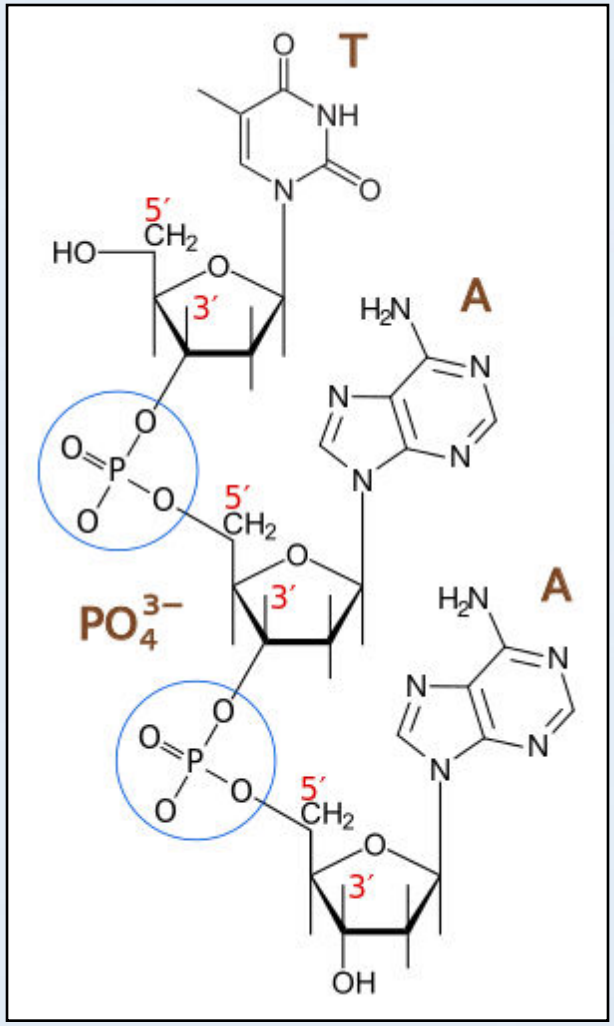
Nucleotide



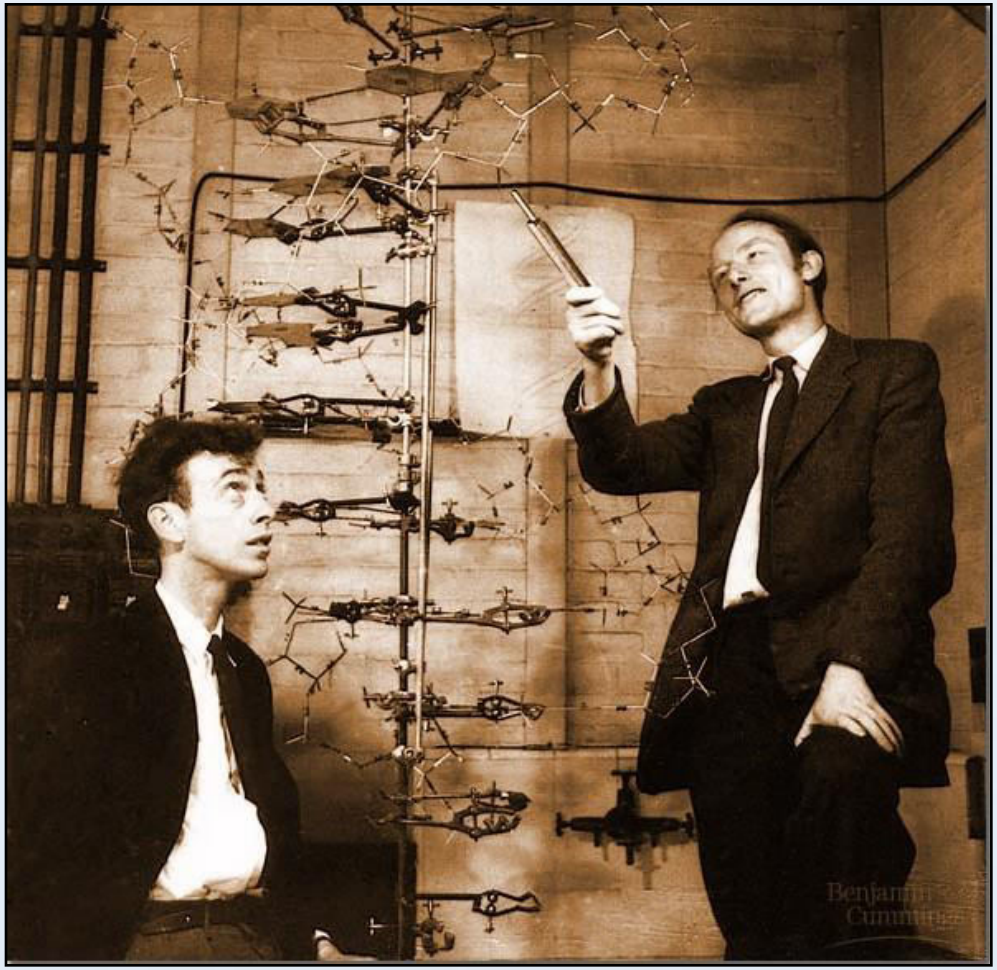
STRUCTURE OF DNA

- Phosphodiester bond links the nucleotides in DNA
- The phosphodiester bond is the linkage between the 3' carbon atom of one sugar molecule and the 5' carbon atom of another
- The two ends of the chain will be easily distinguishable, as one has the 3′ hydroxyl and the other the 5′ phosphate at its terminus
- This polarity in a DNA chain is indicated by referring to one end as the 3′ end and the other as the 5′ end

3´- 5´ phosphodiester bond



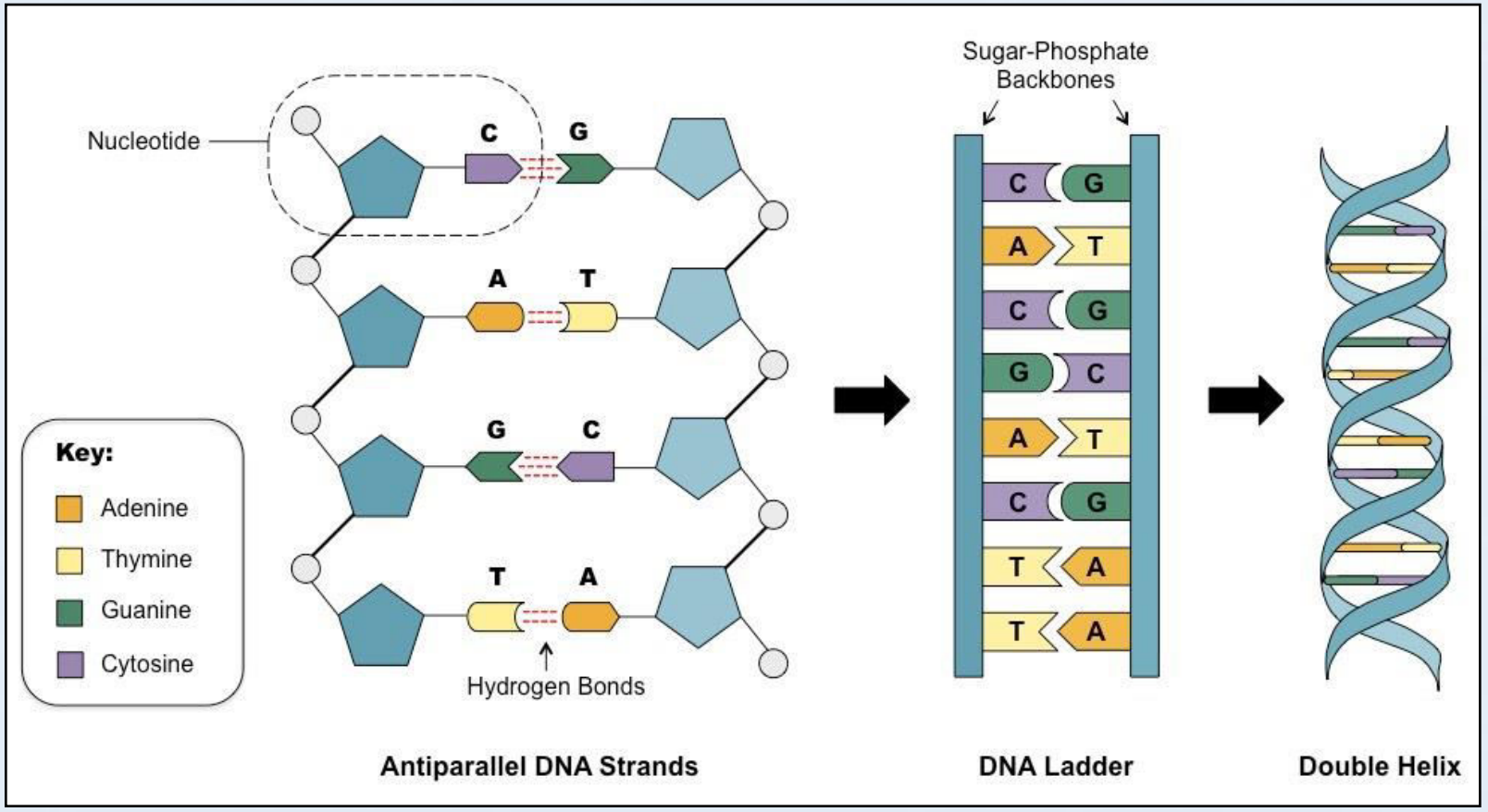
Watson and Crick (1953)



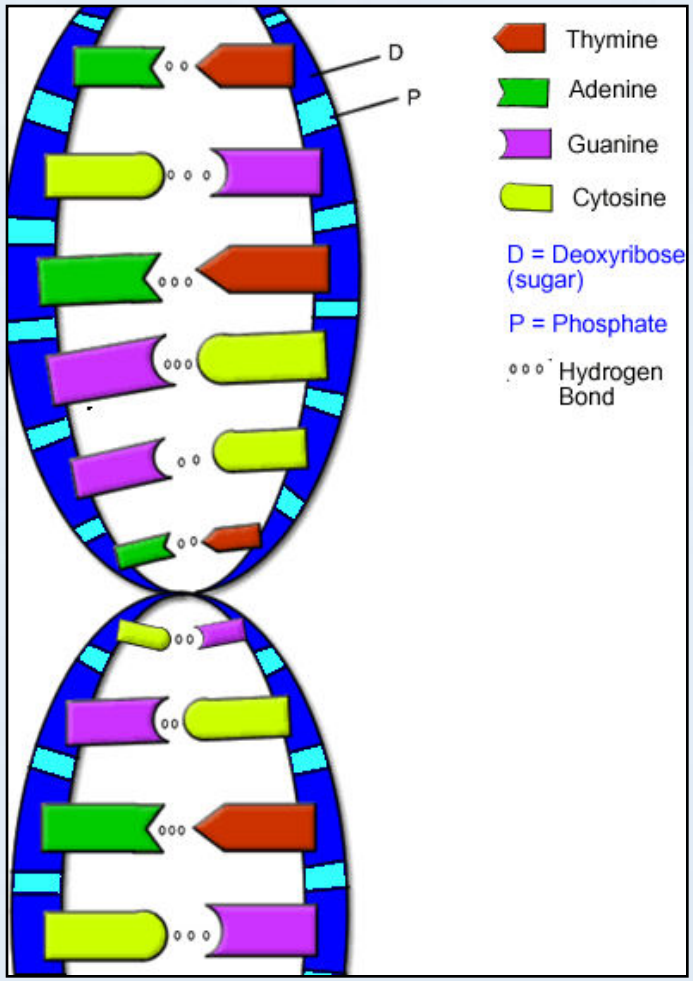
Watson and Crick, 1953

- Three-dimensional structure of DNA - the double helix
- The two chains are held together by hydrogen bonding between the bases on the different strands
- All the bases are on the inside of the double helix, and the sugar-phosphate backbones are on the outside
- Two hydrogen bonds form between A and T, while three form between G and C
- The bases can pair in this way, only if the two polynucleotide chains that contain them are antiparallel to each other

The DNA double helix



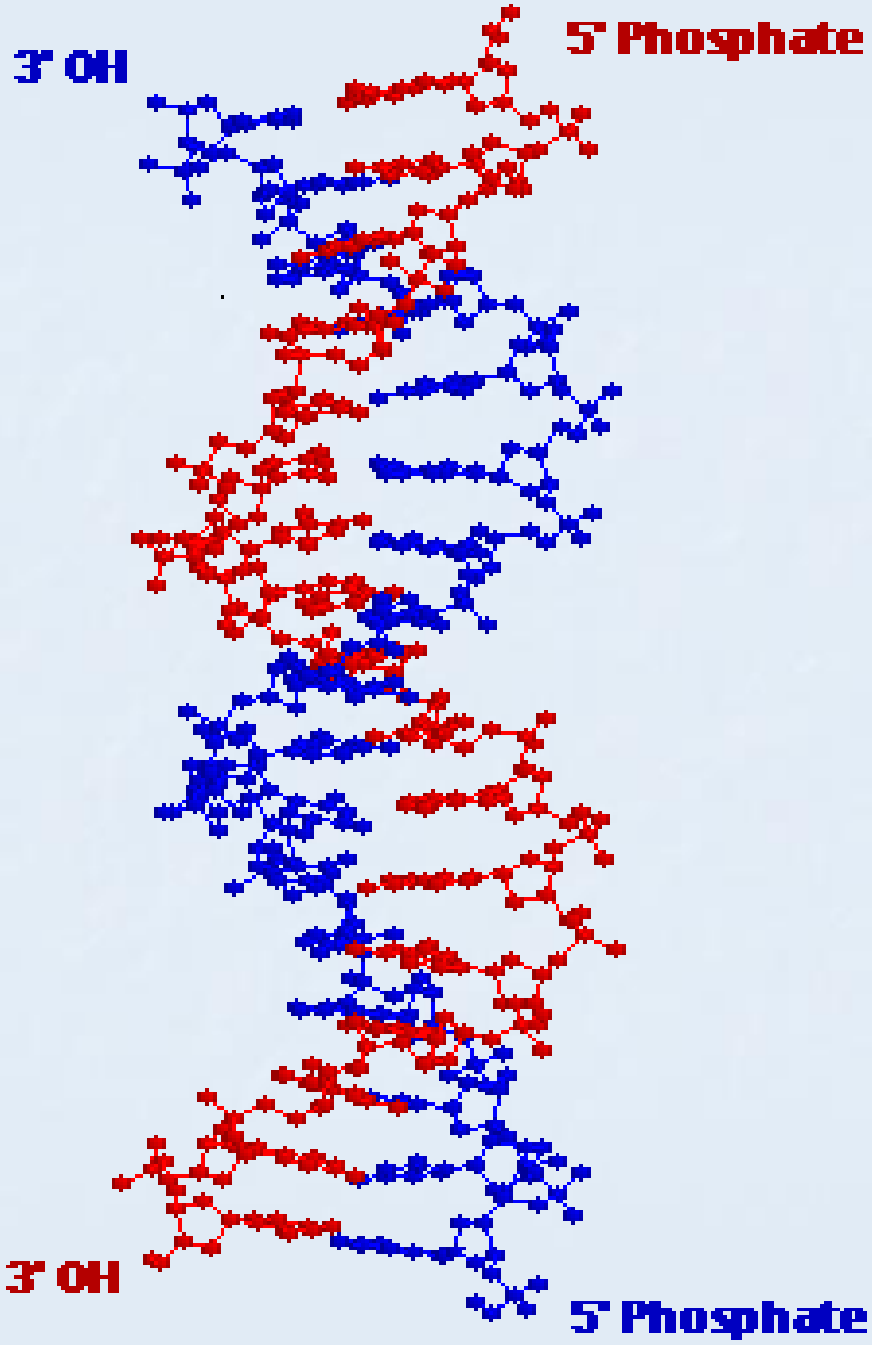
The DNA double helix



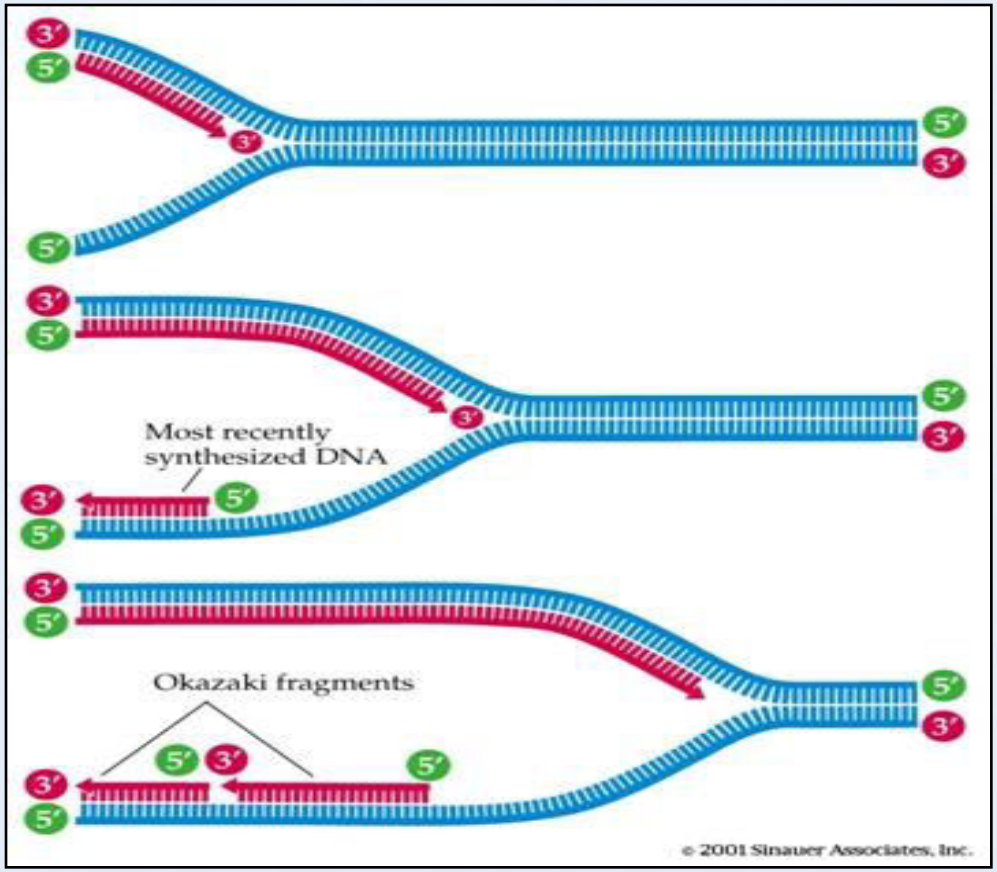
DNA REPLICATION

- The DNA double helix acts as a template for its own duplication
- DNA polymerase was discovered in 1957
- The addition of a deoxyribonucleotide to the 3′ end of a polynucleotide chain is the fundamental reaction by which DNA is synthesized
- Base-pairing between an incoming nucleotide and an existing strand of DNA guides the formation of the new strand of DNA, and causes it to have a complementary nucleotide sequence

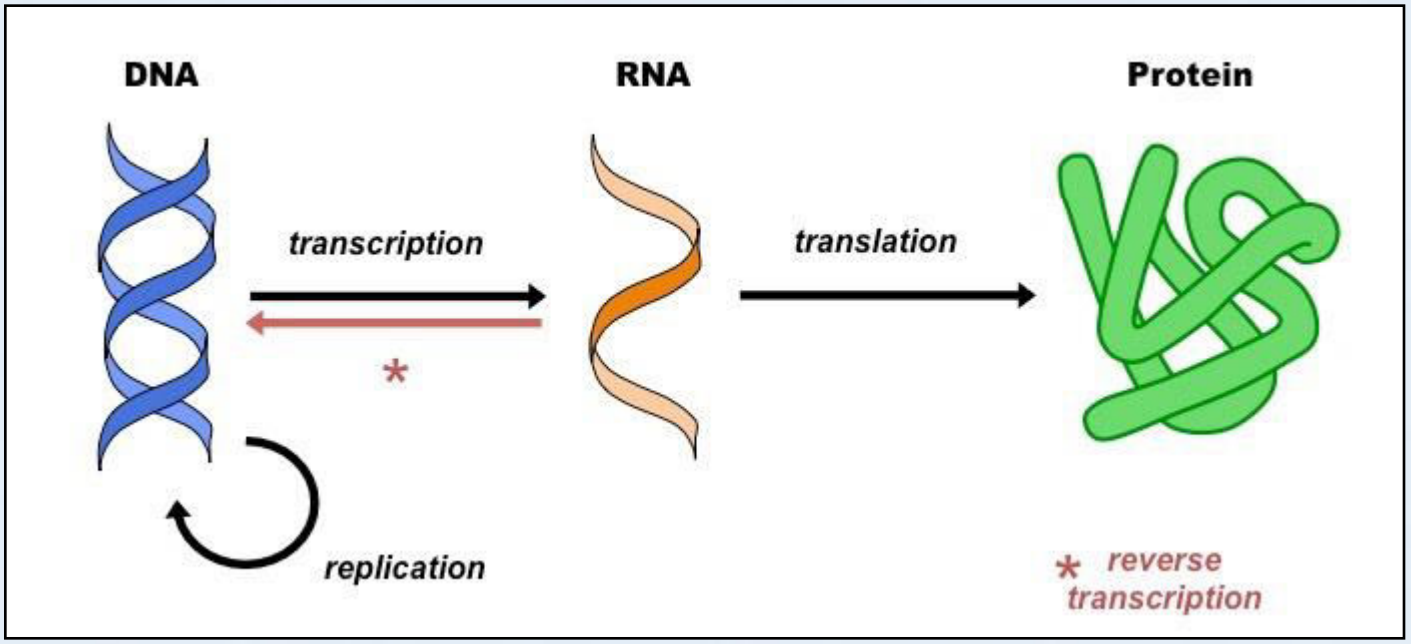
Ο διπλασιασμός του DNA


3' OH
5' Phosphate
3' OH
5' Phosphate

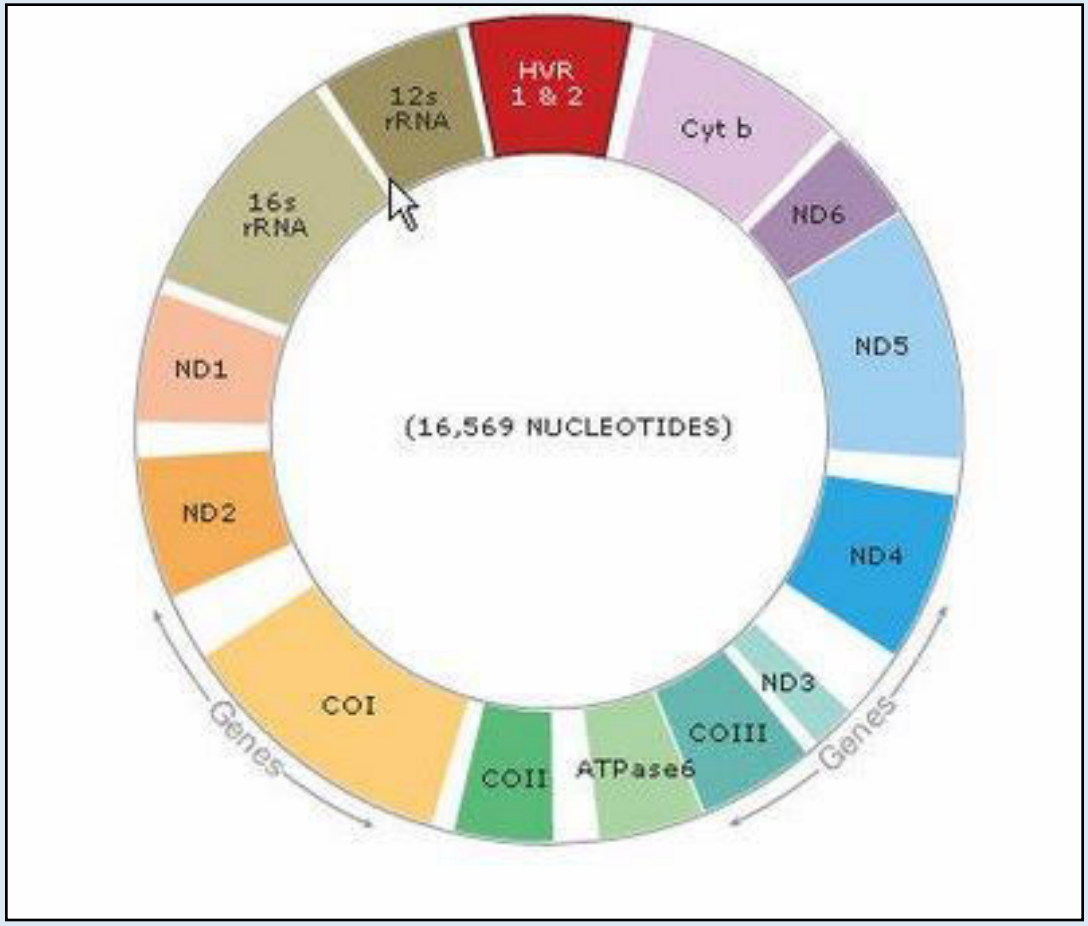
DNA REPLICATION



CENTRAL DOGMA OF MOLECULAR BIOLOGY



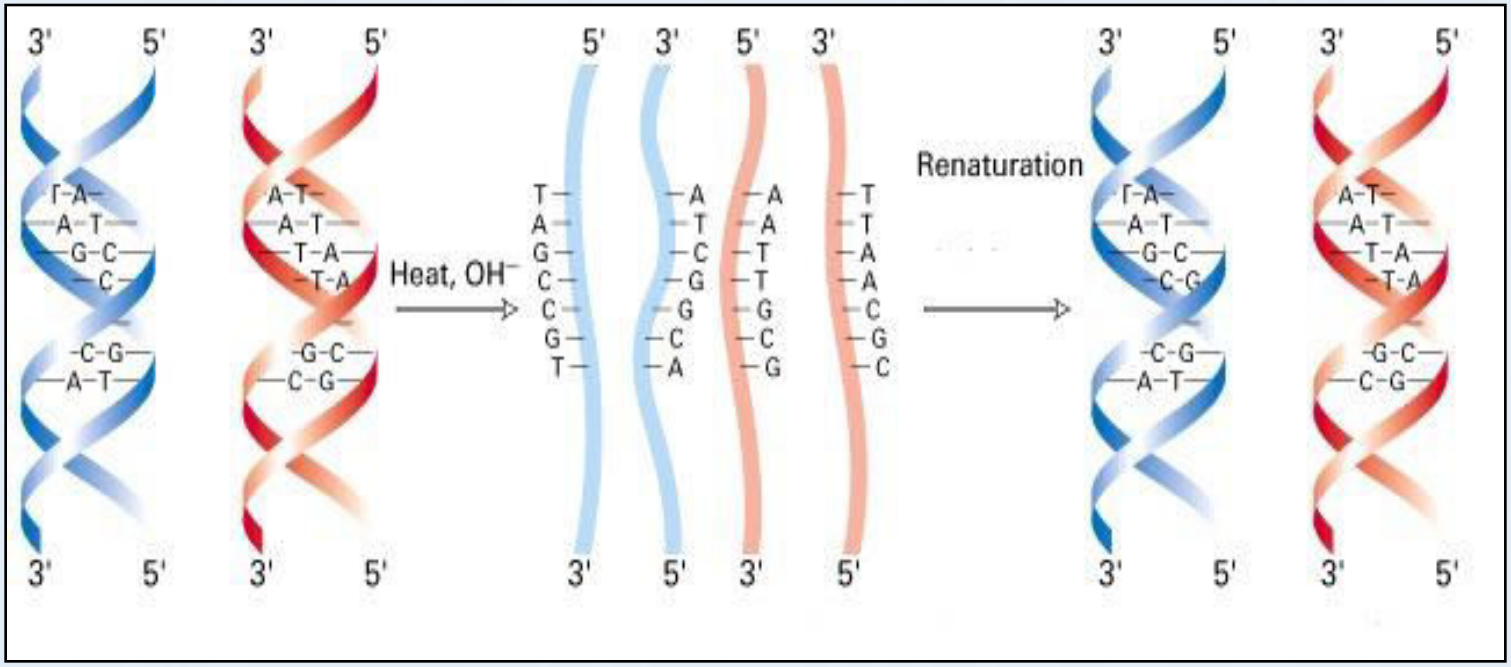
mtDNA


HVR
1 & 2
Cyt b
ND6
ND5
ND4
ND3
COIII
ATPase6
COII
COI
ND2
ND1
16s
rRNA
12s
rRNA
(16,569 NUCLEOTIDES)
Genes
Genes

DNA DENATURATION

- When a DNA solution is heated enough, the double-stranded DNA unwinds, and the hydrogen bonds that hold the two strands together weaken and finally break
- DNA denaturation or DNA melting
- Melting temperature (Tm): the temperature at which the DNA strands are half denatured
- Renaturation: If the heat-denatured DNA is then cooled slowly, the double stranded helix reforms. It involves reannealing and formation of hydrogen bonds between complementary base pairs

DENATURATION - RENATURATION

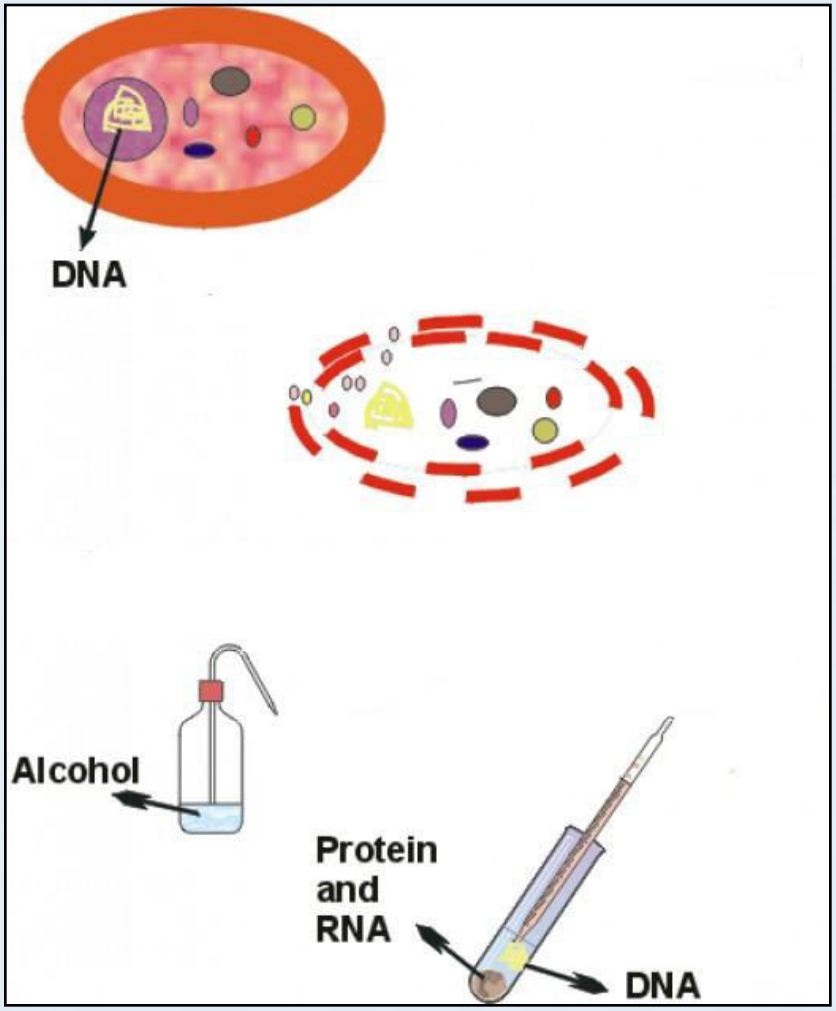


2. DNA EXTRACTION

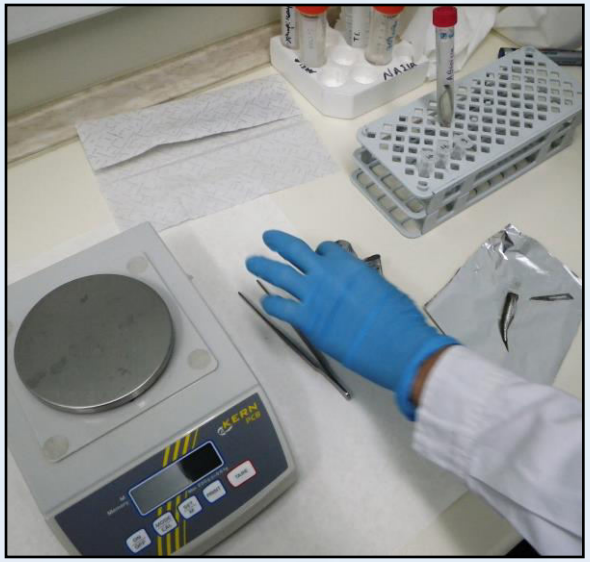
Laboratory of Genetic analysis

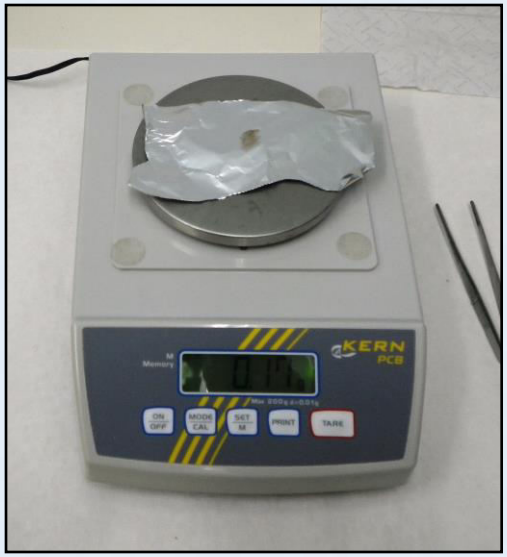

Στατώ
Μπέκ Tips


Scharlau
153
Invitrogen


DNA
Alcohol
Protein
and
RNA
DNA

70 mg of tissue in a 1.5 ml microcentrifuge tube





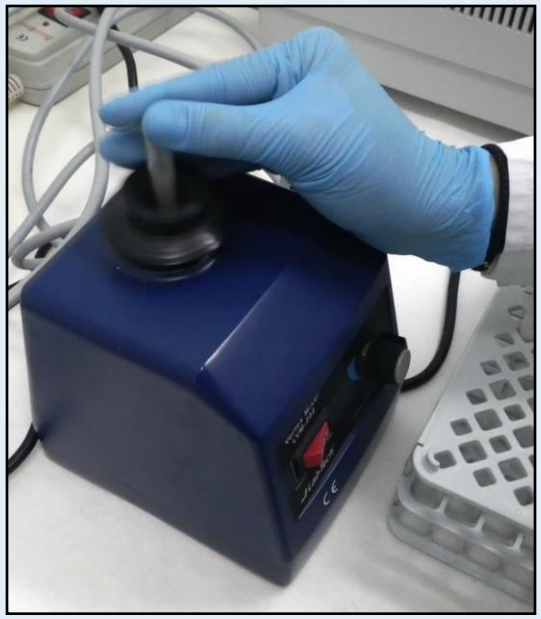
Add 500µl of CTAB solution. CTAB is a strong detergent and can break the plasma membrane, as the CTAB molecule can pull out the phospholipids and incorporate themselves.

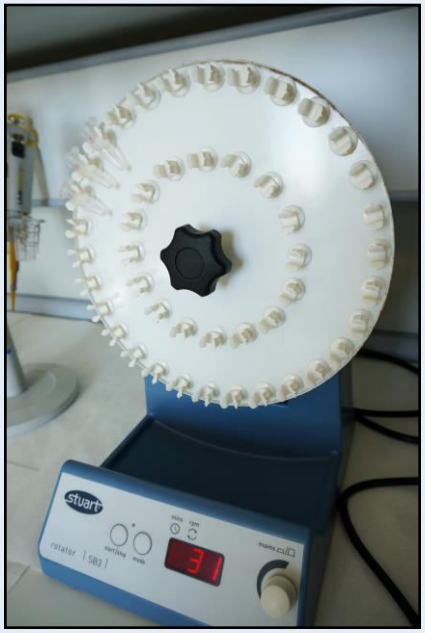
- Homogenize with pestle
- Add 5 µl of 10 mg/ml proteinase K solution. Proteinase K is an enzyme that cleaves the peptide bond in proteins
- Vortex briefly to mix
- Incubate at 55-65°C at least two hours to overnight
- Invert occasionally during incubation (once every 30 minutes for the first two hours)

Incubation at 55°C-65°C

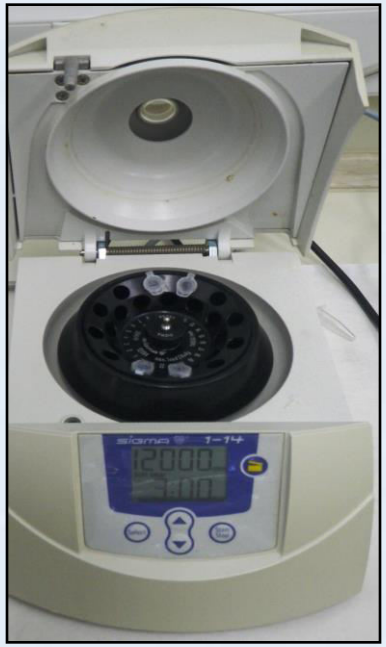
Add equal volume phenol:chloroform:isoamyl alcohol (25:24:1) which removes proteins

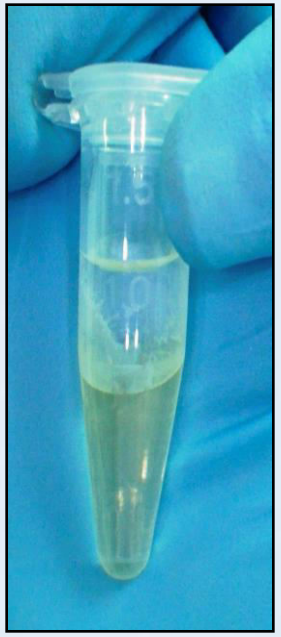
Vortex very well to mix. Then rotate for five minutes to mix better.



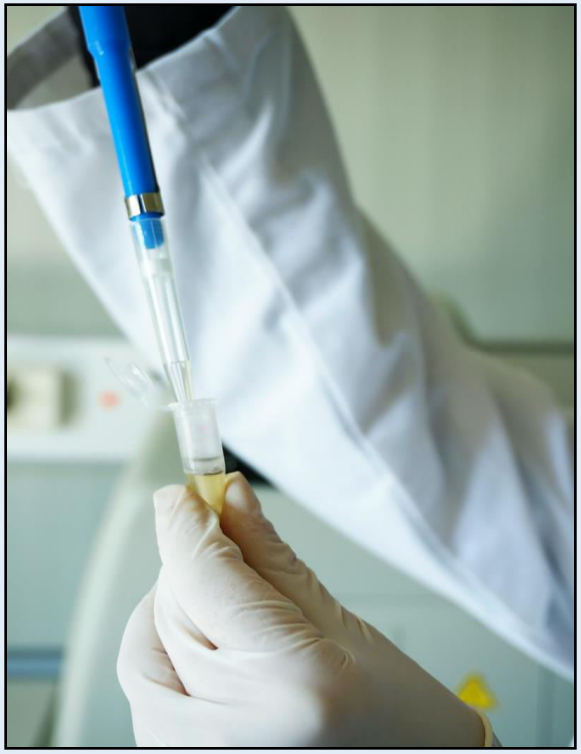


Spin at 12,000 rpms for 3 min





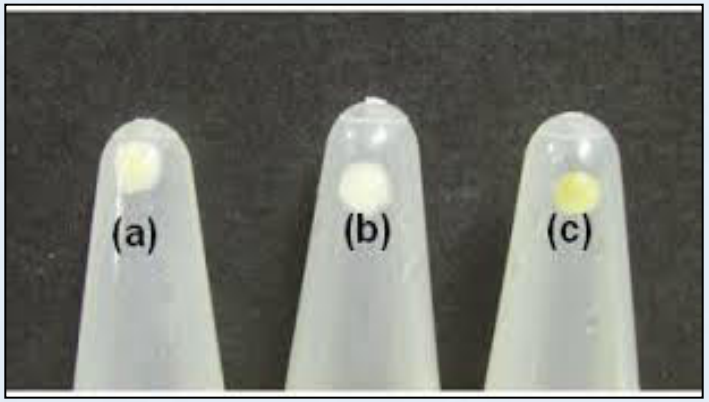
Transfer upper phase to fresh tube



Add 0,5 ml of chloroform. Rotate for five minutes to mix. Spin at 12,000 rpms for 3 min.

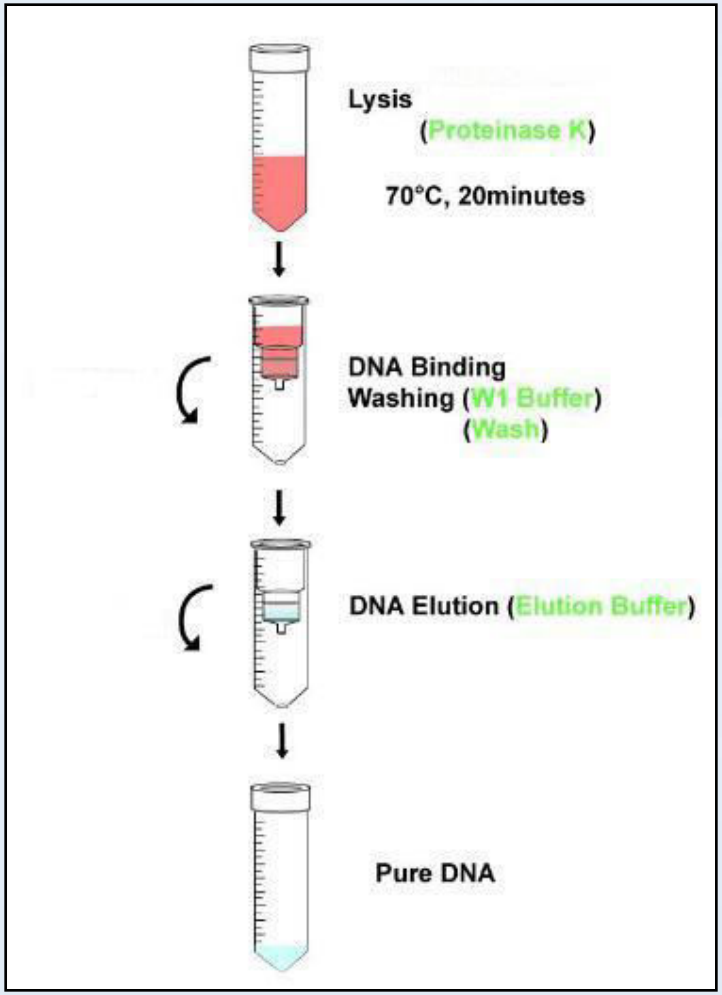
- Transfer upper phase to fresh tube
- Add 1000µl cold ethanol. The 100% ethanol is used for precipitation, so we get good amount of DNA
- Invert two or three times
- Spin for 2 min at 12,000 rpms
- Carefully decant liquid ethanol from DNA pellet

The DNA pellet



DNA EXTRACTION KIT

DNA ISOLATION WITH EXTRACTION KITS



3. AGAROSE GEL ELECTROPHORESIS

- An agarose is a polysaccharide polymer material, generally extracted from seaweed

- Agarose gel electrophoresis is a method of gel electrophoresis, used to separate macromolecules such as DNA fragments, in a matrix of agarose

- Biomolecules are separated by applying an electric field, to move the charged molecules through an agarose matrix

- 1% gels is often used for a standard electrophoresis

- The separated DNA may be viewed with Ethidium Bromide stain, most commonly under UV light
- The EtBr molecules are tiny, and slip in between the DNA base pairs
- When you expose EtBr to Ultra Violet light, it fluoresces with an orange/pink color, that is easily seen by the naked eye
- It is used because upon binding of the molecule to the DNA and illumination with a UV light source, the DNA banding pattern can be visualized

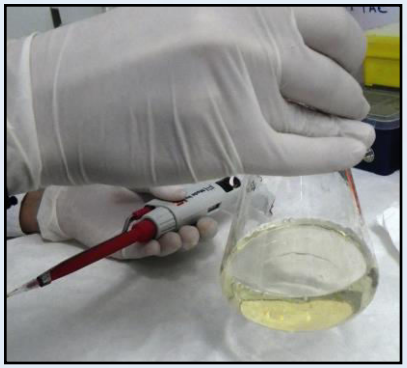
3a. PREPARATION OF AGAROSE GEL

Add 1 gram of agarose in 100 ml of 1XTBE solution

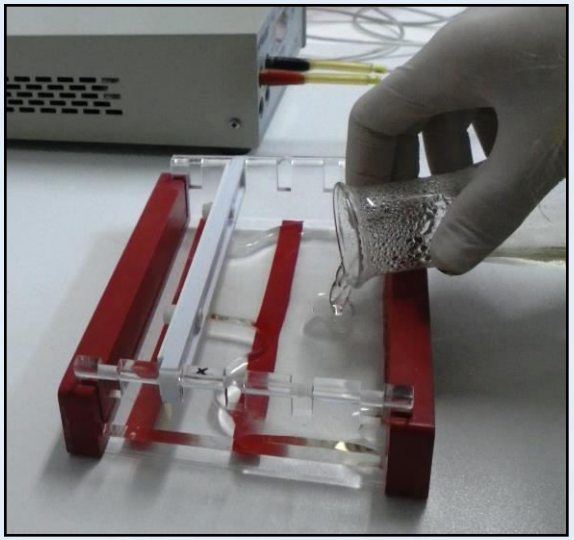
Place the mix in a microwave oven and heat using 20-60 second intervals, until the agarose is completely dissolved

Cool the solution to 50-60°C

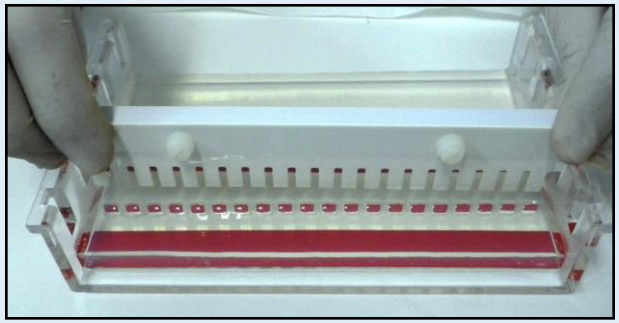
Add 7.5 µl of 1% ethidium bromide and mix well



We prepare the cast, where we pour the melted agarose. A comb is placed in the cast, to create wells for loading the samples.



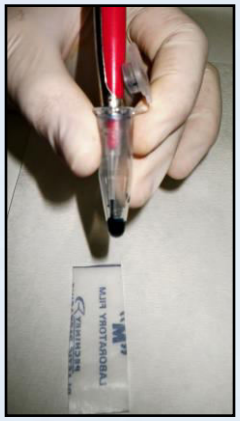
After 20 minutes the agarose gel is solid

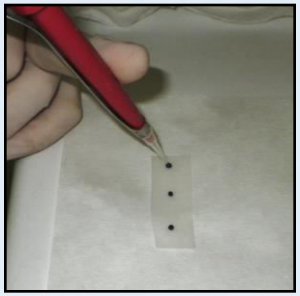


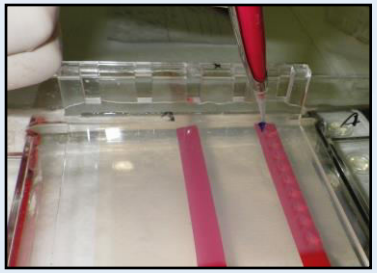
Once the gel has set, the comb is removed leaving wells where DNA samples can be loaded

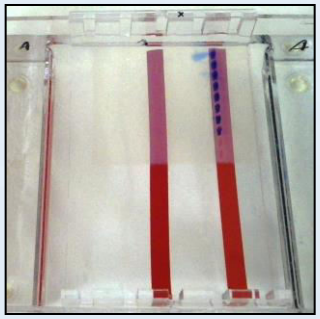
3b. AGAROSE GEL ELECTROPHORESIS

The agarose gel is placed in the electrophoresis tank, with 700 ml of 1XTBE solution. Mix 2 µl DNA solution with 3 µl loading buffer (glycerol and bromophenol blue). The DNA samples are loaded.

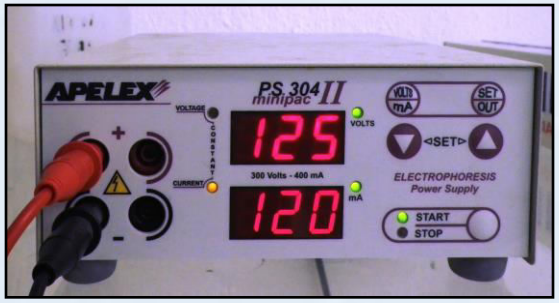


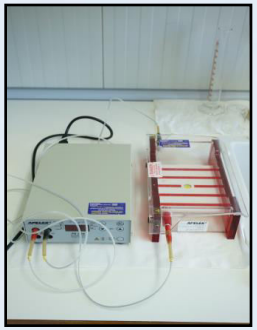


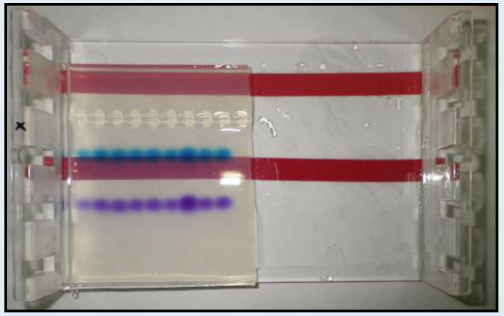


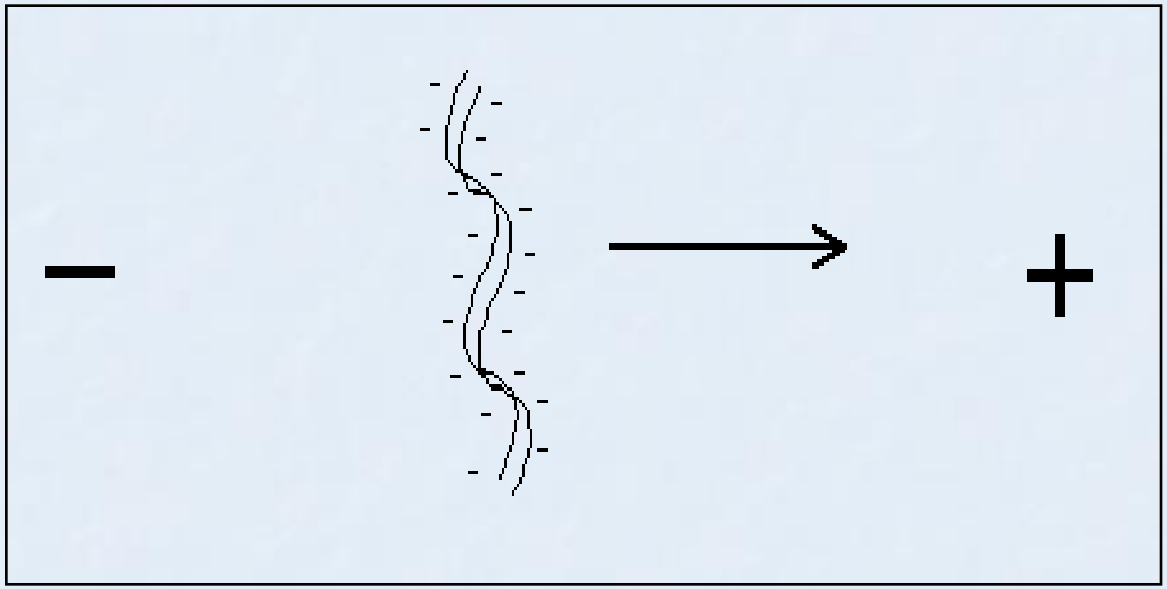


The tank is connected with the power supply unit (120 Volts, 90 mAmpere). As the DNA molecule has a negative charge, it will move towards the anode. The progress of the electrophoresis is monitored with bromophenol blue.



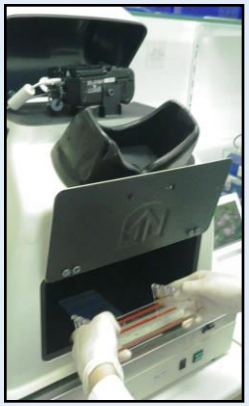


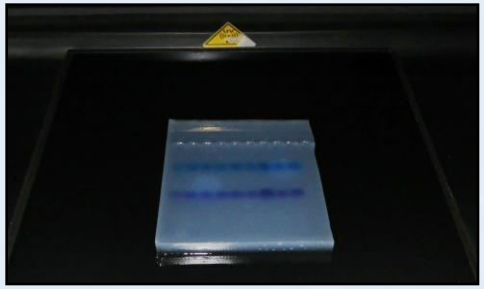


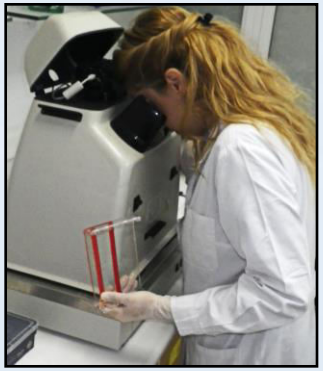


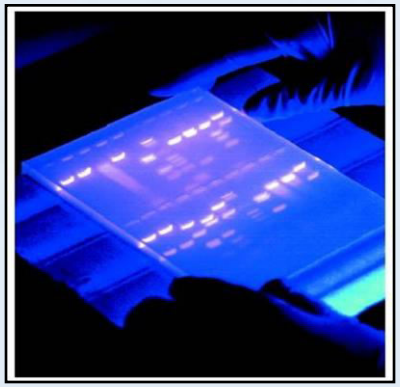
3c. OBSERVATION OF THE AGAROSE GEL

The gel is removed from the tank and it is placed in the UV transilluminator. If the DNA isolation is successful, DNA appears as orange bands on the gel.

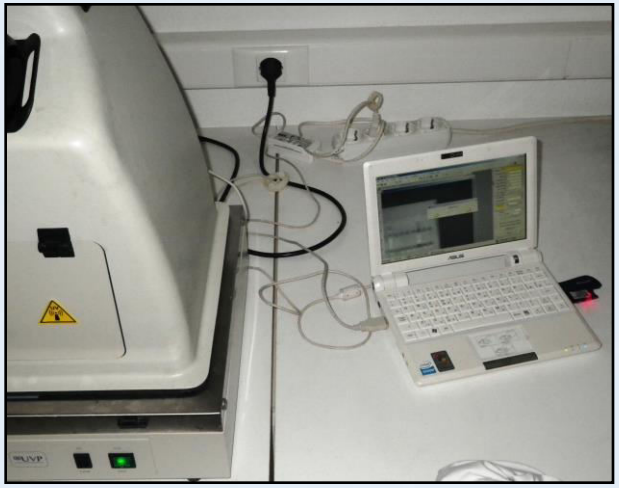


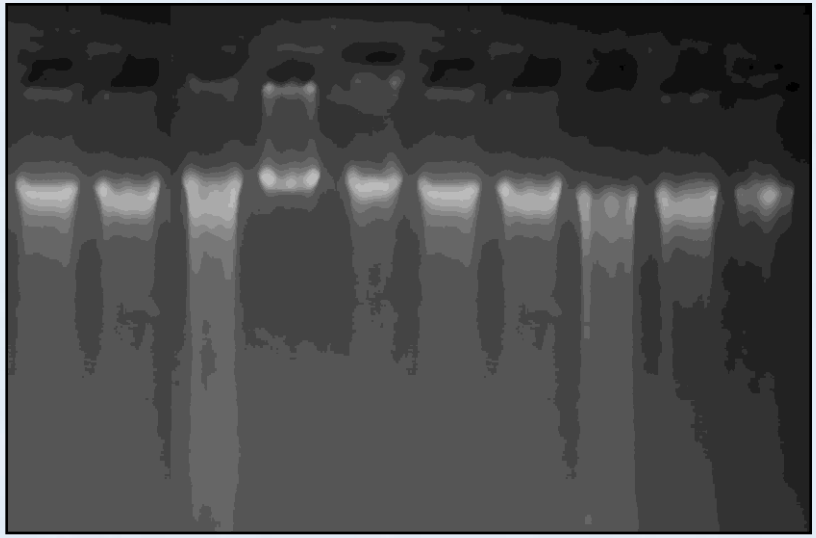






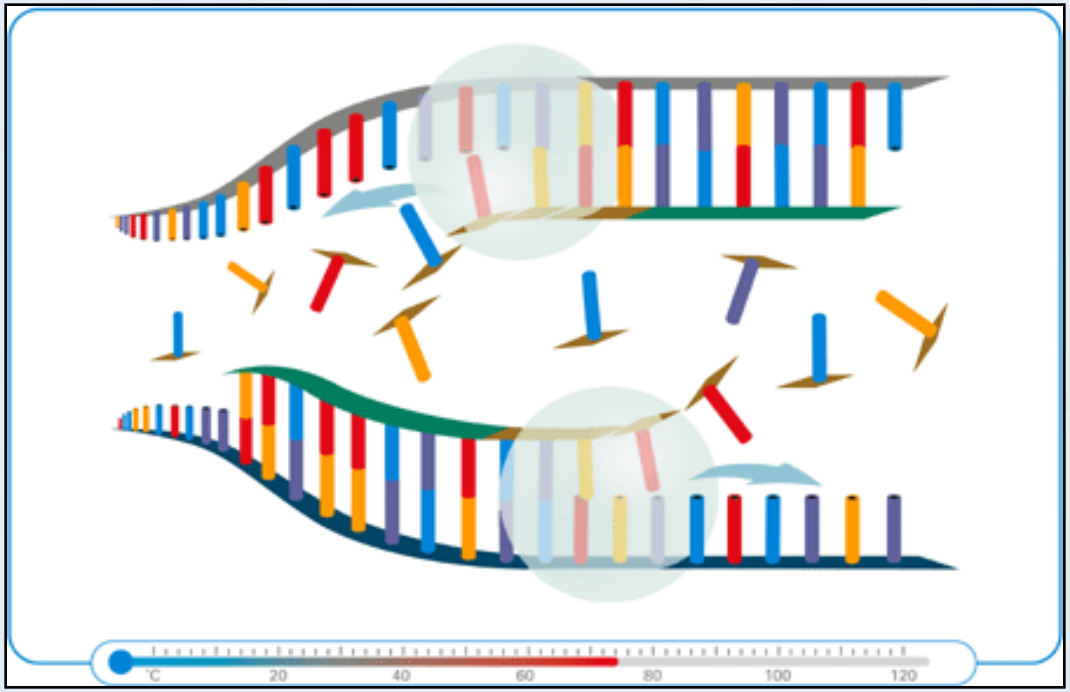
The transilluminator apparatus is connected with an image capture device such as a digital camera, that allows an image of the gel to be taken. The whole system is connected with a computer. An image of the gel is taken, saved in the computer and then is processed.



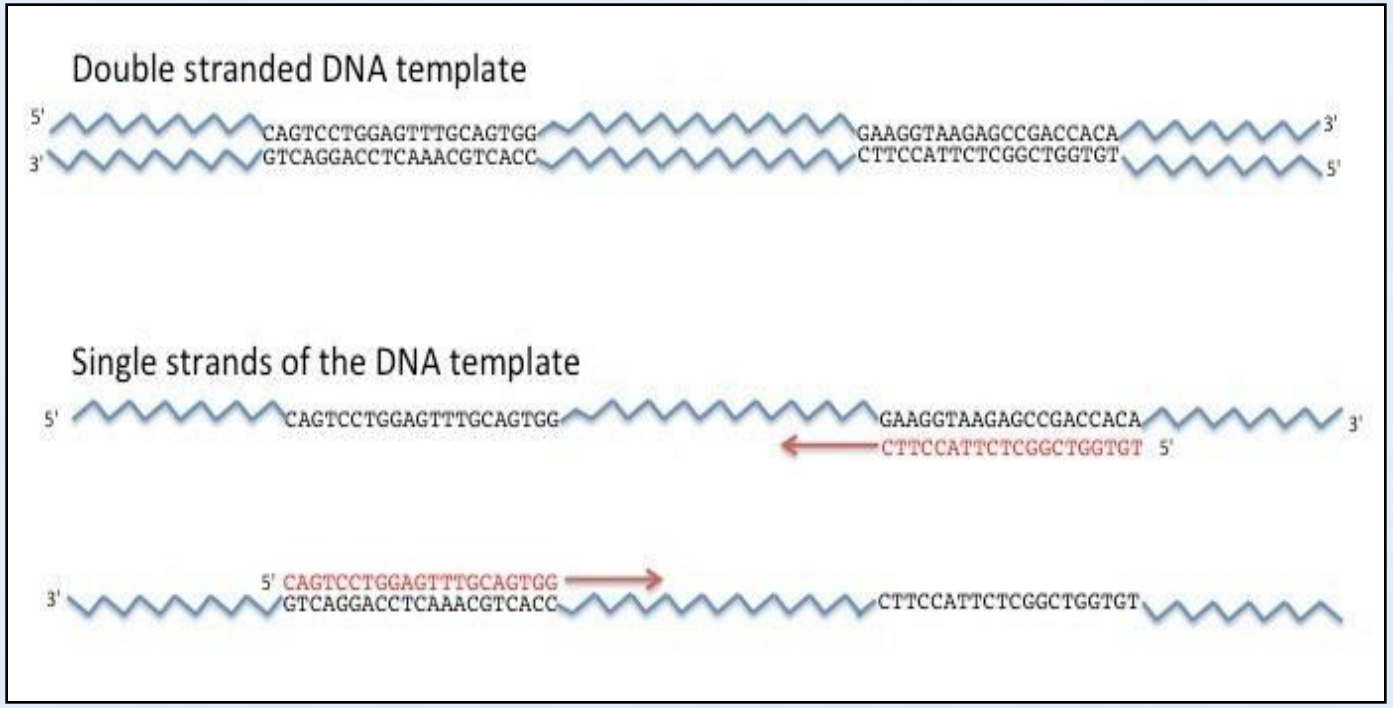


4. POLYMERASE CHAIN REACTION (PCR)

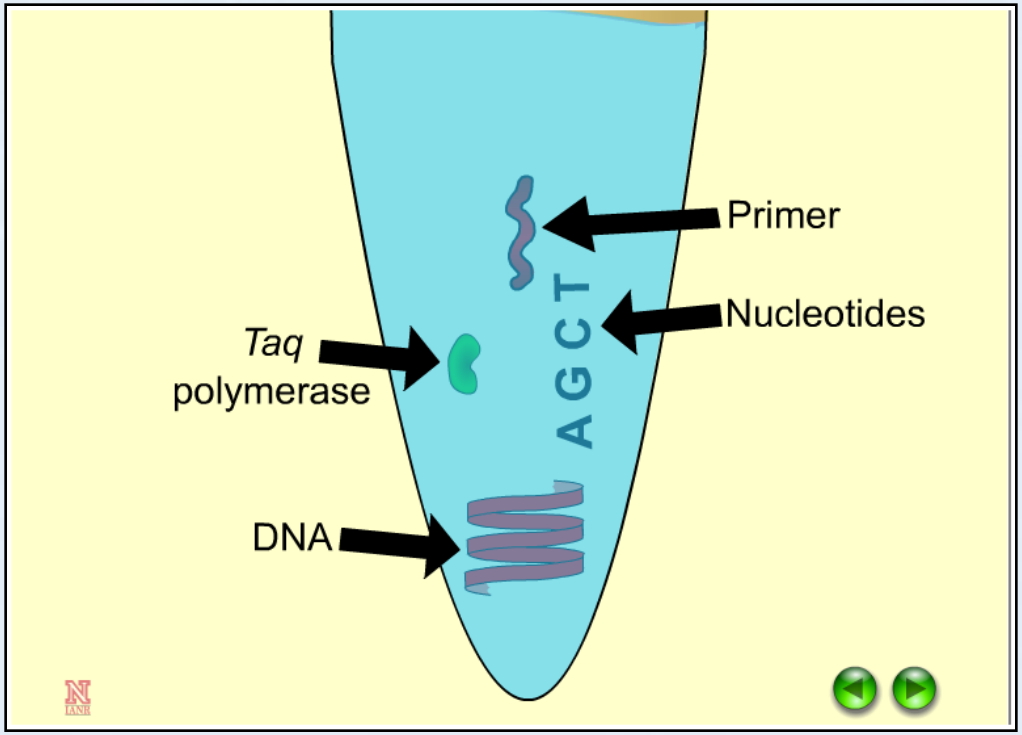
PCR is based on using the ability of DNA polymerase to synthesize new strand of DNA, complementary to the offered template strand – *Taq polymerase*



DNA polymerase can add a nucleotide only onto a preexisting 3'-OH group, so it needs a primer to which it can add the first nucleotide. Two primers are needed, complementary to the 3'ends of each of the sense and the anti sense strand of the DNA target.



Components of the PCR reaction

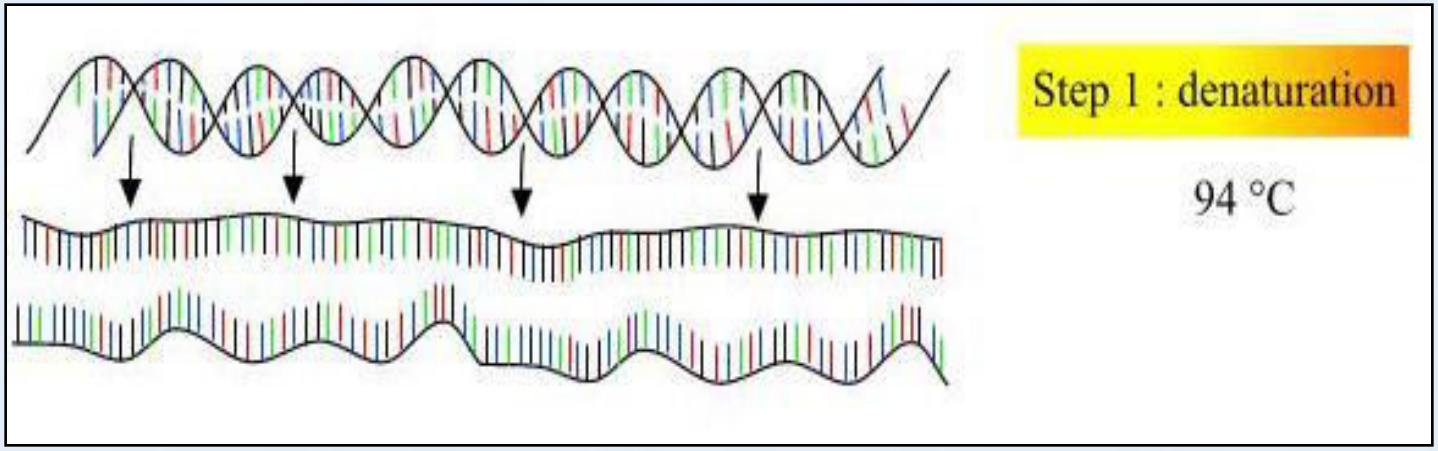


Components of the PCR reaction

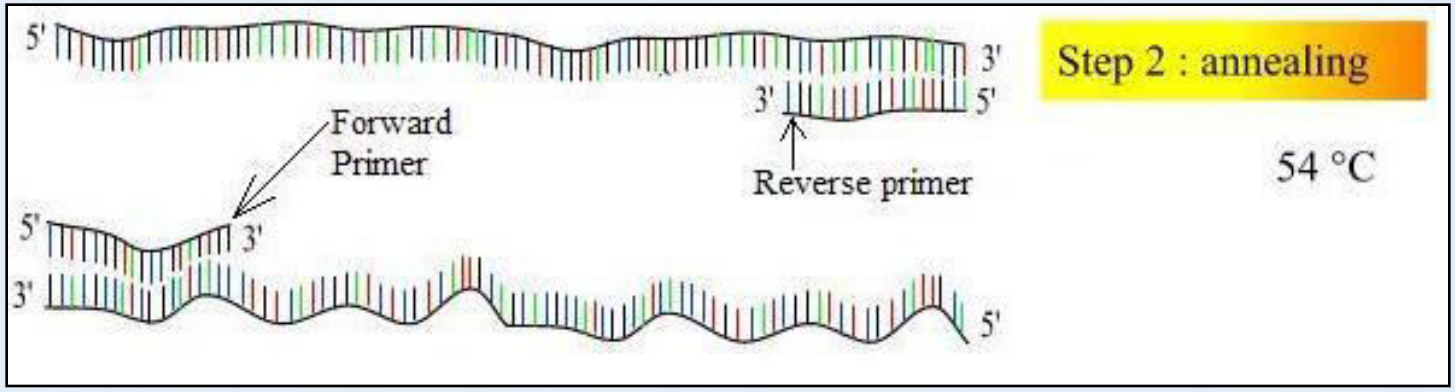
PCR reaction

- PCR consists of a series of 20–40 repeated temperature changes, called cycles, with each cycle commonly consisting of three discrete temperature steps
- The cycling is often preceded by initialization: it consists of heating the reaction chamber to a temperature of 94–96°C, which is then held for 1–10 minutes
- The cycling followed by final elongation which is performed at 72°C for 5–15 minutes after the last PCR cycle, to ensure that any remaining single-stranded DNA is fully elongated

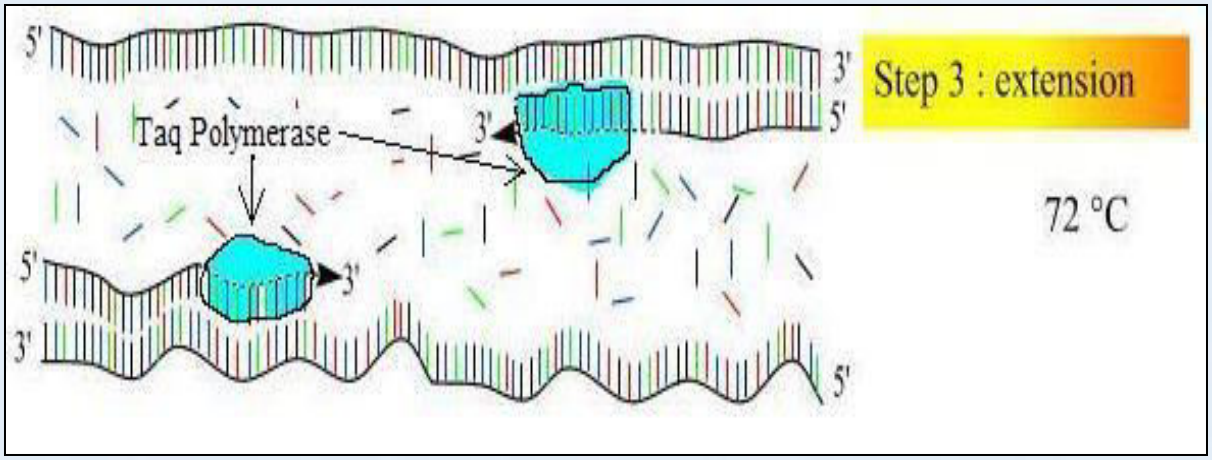
Denaturation: 94–96°C for 20–30 seconds



Annealing: 50–65°C for 20–40 seconds

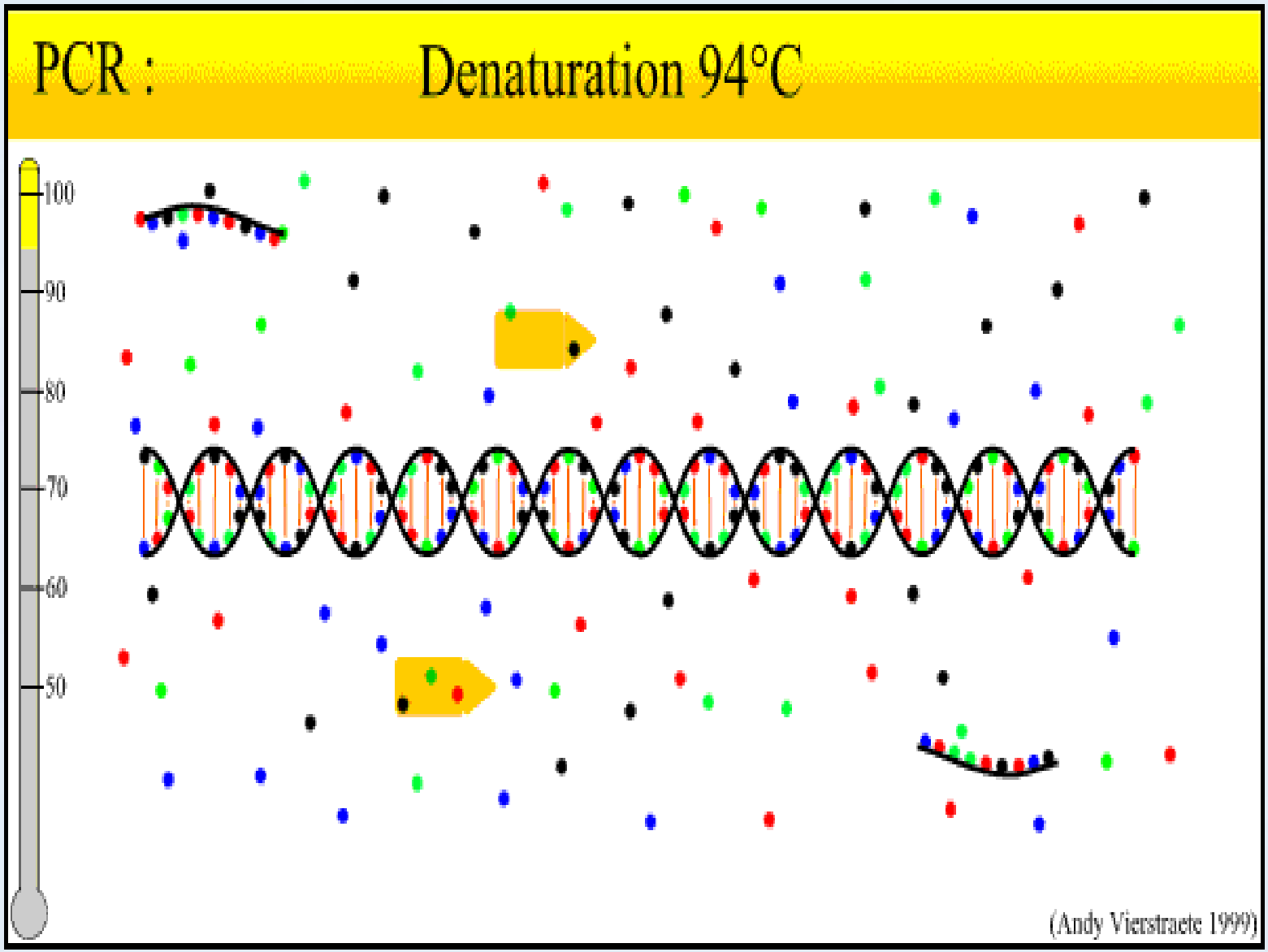


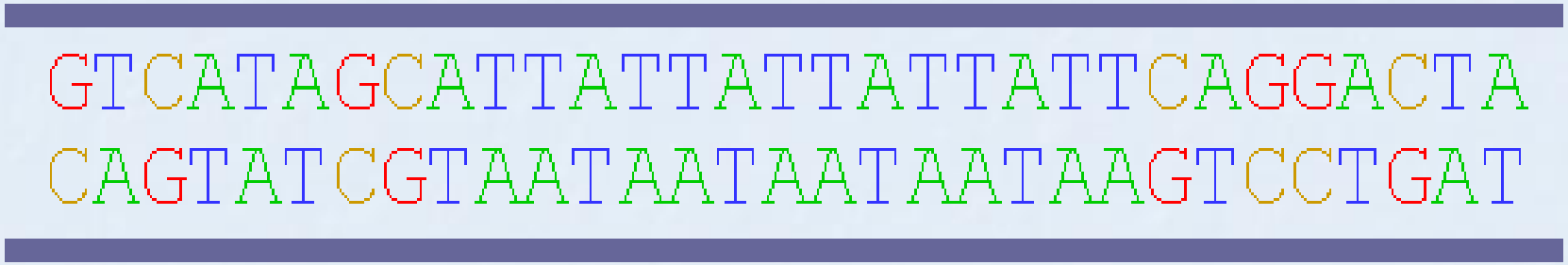
Extension/elongation: 72°C for…



PCR reaction

- The processes of denaturation, annealing and elongation constitute a single cycle
- Multiple cycles are required to amplify the DNA target to millions of copies
- The formula used to calculate the number of DNA copies formed after a given number of cycles is 2^n, where n is the number of cycles
- Thus, a reaction set for 30 cycles results in 2^{30}, or 1,073,741 copies of the original double-stranded DNA target region


PCR : Denaturation 94°C
100
90
80
70
60
50
(Andy Vierstraete 1999)



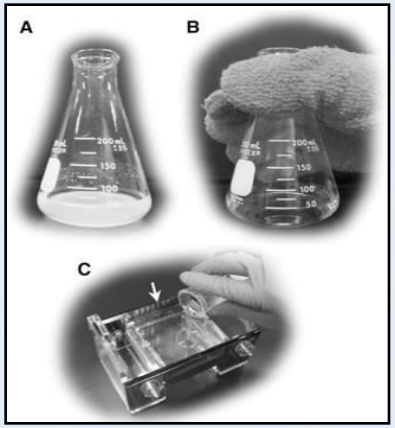
A template sequence with 5 ATT repeats.

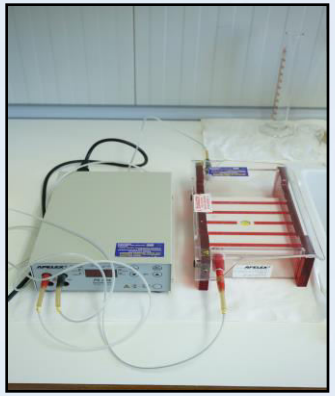
PCR reaction

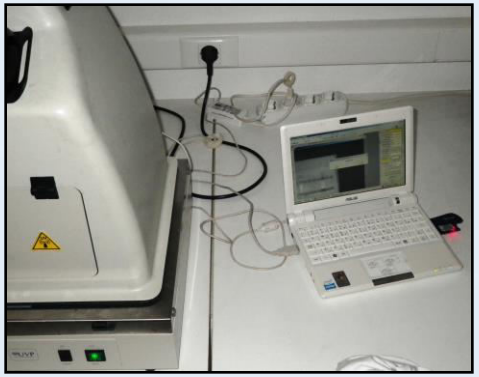
- The reaction is commonly carried out in a volume of 10–100 µl, in small reaction tubes (0.2–0.5 ml volumes), in a thermal cycler
- The thermal cycler heats and cools the reaction tubes, to achieve the temperatures required at each step of the reaction
- Thin-walled reaction tubes permit favorable thermal conductivity, to allow for rapid thermal equilibration
- Most thermal cyclers have heated lids, to prevent condensation at the top of the reaction tube

PCR thermal cycler

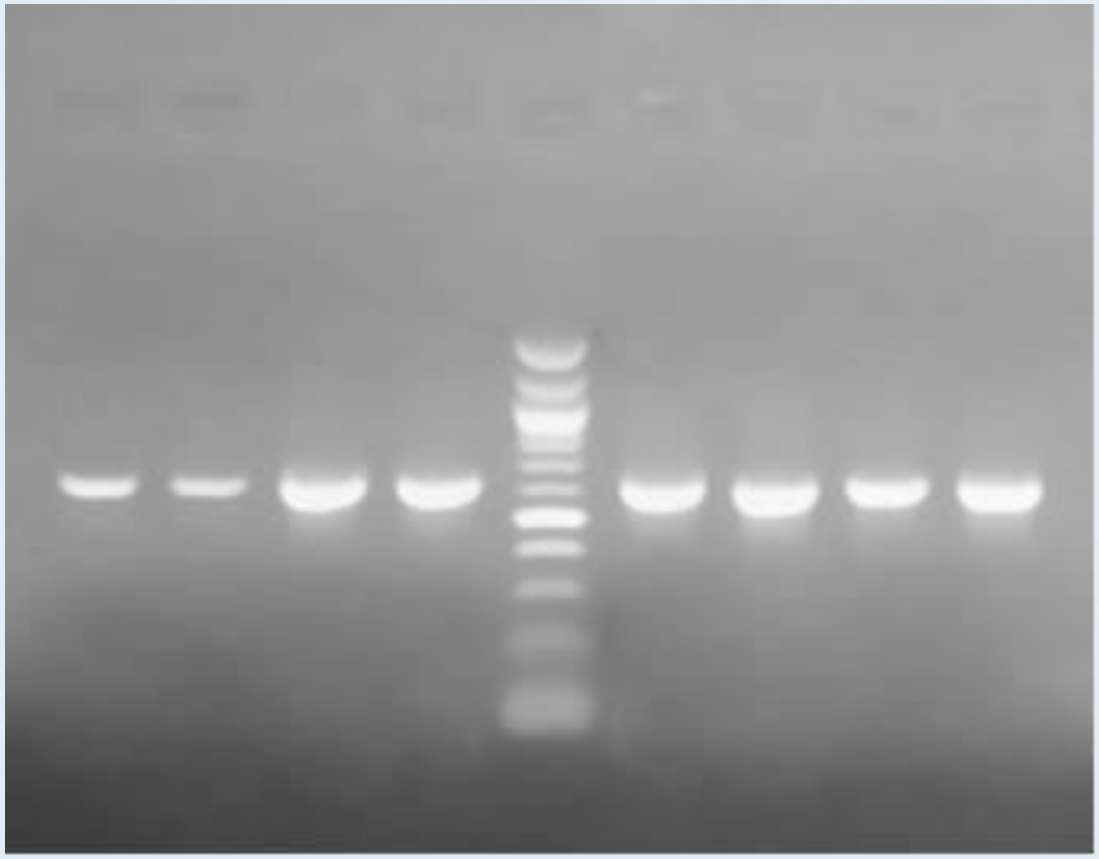
Agaroze gel electrophoresis



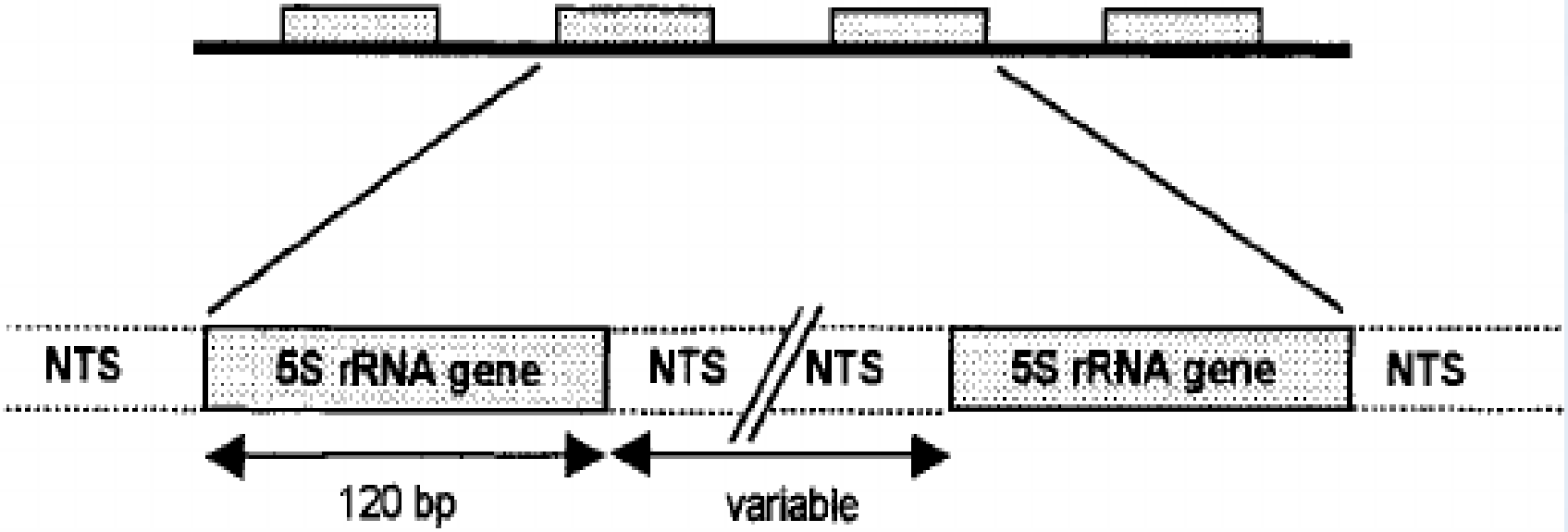




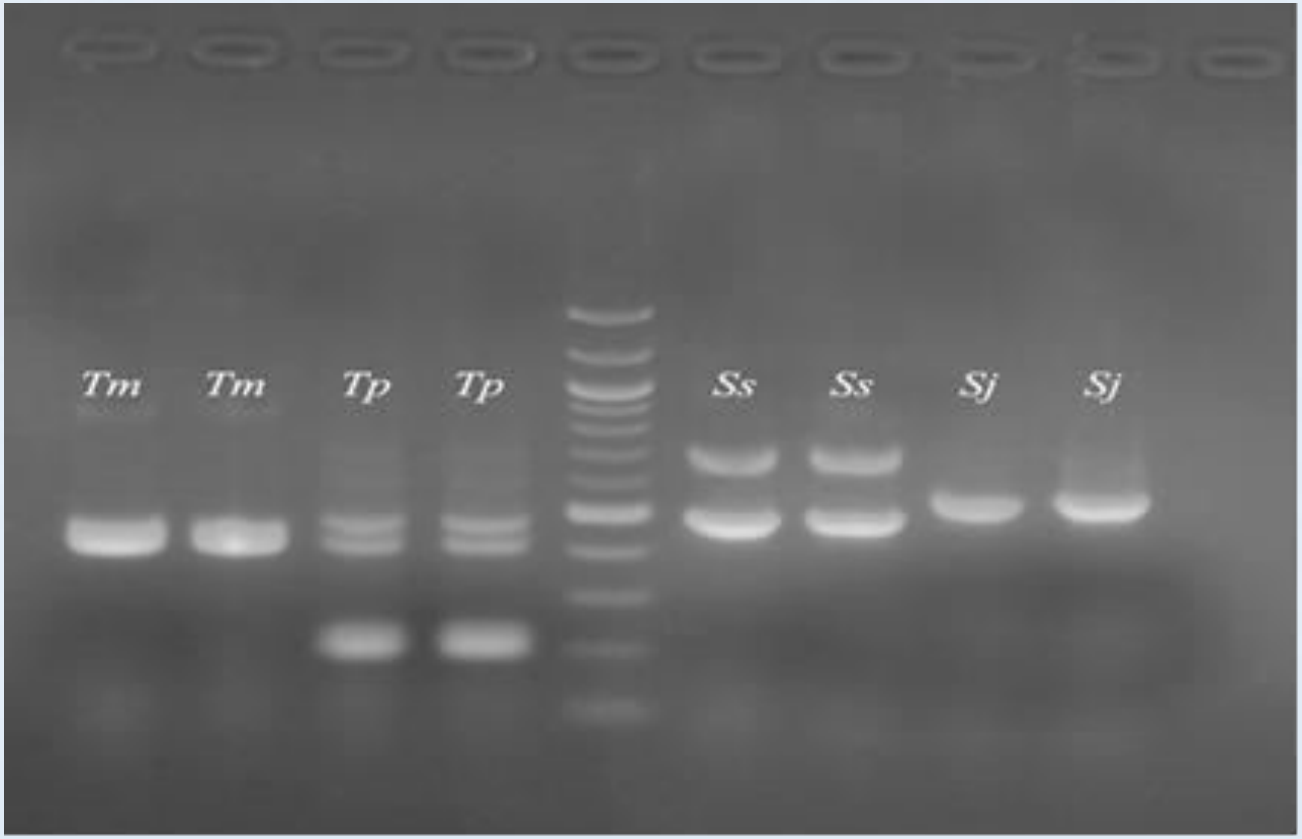
Agarose gel electrophoresis with the PCR products of the amplified 16S rDNA gene. The marker used is the 100 bp DNA ladder. Two species: *Liza ramada*, *Liza saliens*



The structure of the 5S rRNA



Agarose gel electrophoresis with the PCR products of the amplified 5S rDNA gene.The marker used is the 100 bp DNA ladder. Four species: Tm=*Trachurus mediterraneus*, Tp=*Trachurus picturatus*, Ss=*Scomber scombrus*, Sj=*Scomber japonicus*



5. REAL TIME PCR

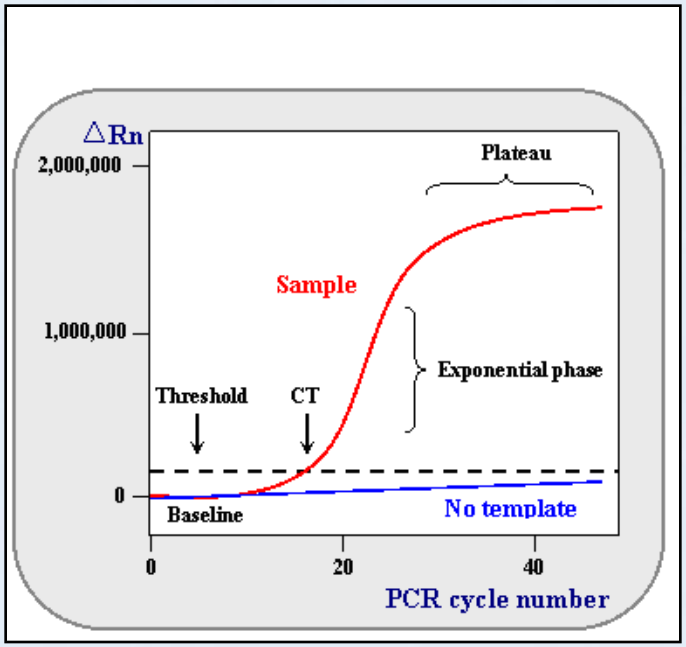
PRINCIPLES OF THE METHOD

- Quantitative polymerase chain reaction (qPCR). It monitors the amplification of a targeted DNA molecule during the PCR, in real-time
- At the same time, a relatively small amount of PCR product can be quantified
- In the beginning, the PCR product is not detectable, as it is found in a very small quantity
- An exponential phase follows, where the quantity of PCR product is doubled in every step
- If there are more target DNA molecules in the original sample, less PCR cycles will be needed for the exponential phase to begin

PRINCIPLES OF THE METHOD

- Real Time PCR is based on the detection of the fluorescence produced by a reporter molecule, which increases as the reaction proceeds
- This occurs due to the accumulation of the PCR product with each cycle of amplification
- These fluorescent reporter molecules include dyes that bind to the double-stranded DNA (SYBR Green), or sequence specific probes (molecular beacons or TaqMan probes)

A real time quantitative PCR plot

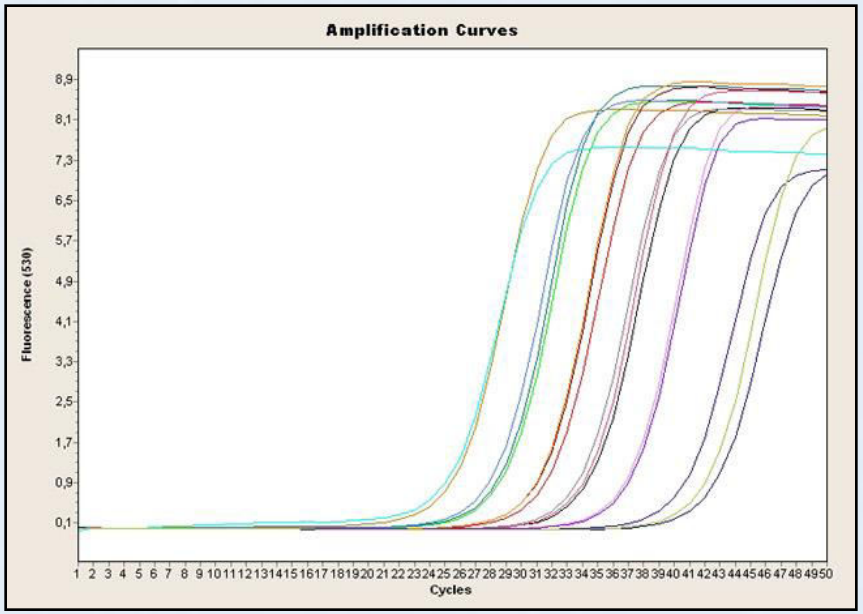


- **Baseline** is defined as the PCR cycles in which the reporter fluorescent signal is accumulating, but is beneath the limits of detection of the instrument
- **ΔRn** is an increment of fluorescent signal at each time point
- **Threshold** is a level of fluorescence chosen on the basis of the baseline variability. **A signal** that is detected **above** the threshold is considered a real signal, that can be used to define the threshold cycle (Ct) for a sample
- **Ct** is defined as the fractional PCR cycle number at which the reporter fluorescence is greater than the threshold

Ct values

- The Ct is a basic principle of real time PCR, and is an essential component in producing accurate and reproducible data
- The Ct values are inversely proportional with the amount of DNA target molecules found in the original sample
- Lower values of Ct denote bigger amount of DNA target molecules
- When Ct ≤ 29, the reaction is strongly positive, with a big amount of DNA target molecules in the original sample
- When Ct ≤ 30-37, the reaction is positive, with moderate amount of DNA target molecules
- When Ct=38-40, we have weak reactions with minimum amounts of DNA target molecules, which probably denote an environmental contamination

Real-time RCR reactions

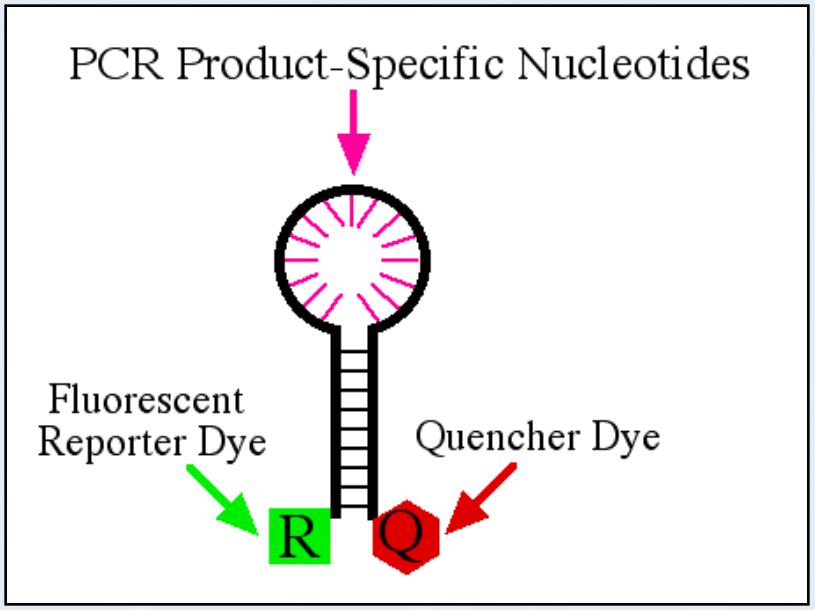


- Comparison of the cycle number required for the advent of the exponential phase in different reactions
- We can determine the original amount of DNA matrix molecules, used in these reactions

DETECTION OF PCR PRODUCT USING TARGET SPECIFIC PROBES

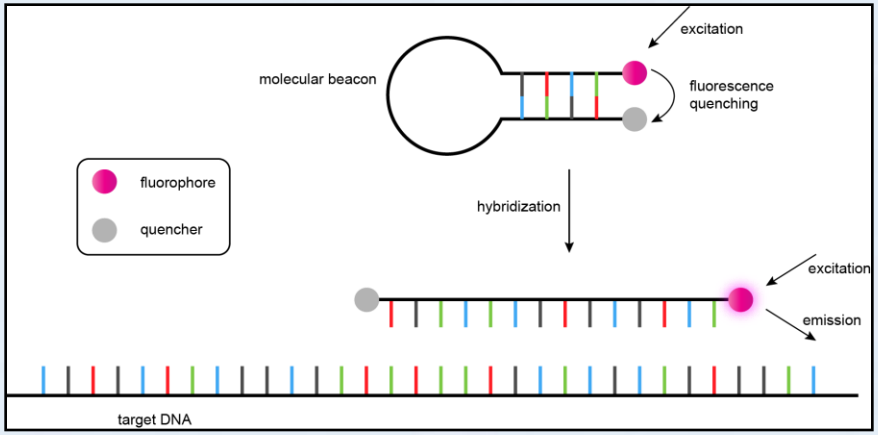
- Specific detection of real time PCR is done with some oligonucleotide probes
- They are labeled with both a reporter fluorescent dye and a quencher dye
- Probes based on different chemistries are available for real time detection, and these include molecular beacons and TaqMan probes

Molecular beacon



- the **loop** is the 18-30 base pair region of the molecular beacon, which is complementary to the target sequence
- the **stem** which lies on both the ends of the loop, is typically 5-8 bp long, and the sequences at both the ends are complementary to each other
- the **5' fluorophore** which is a dye and fluoresces in presence of a complementary target
- the **3' quencher dye** attached to the 3' end and when the beacon is in closed loop shape, prevents the fluorophore from emitting light

Operation of a molecular beacon in a RT PCR reaction



- In the absence of a complementary target sequence, the beacon remains closed and there is no appreciable fluorescence
- In the presence of a complementary target, the stem portion of the beacon separates, resulting in the probe hybridizing to the target
- When the beacon unfolds, the fluorophore is no longer quenched, and the molecular beacon fluoresces
- The fluorescence is easily detected in a thermal cycler

Role of the fluorescence in a Real time reaction

- The amount of fluorescence at any given cycle, or following cycling, depends on the amount of specific product
- For quantitative PCR, molecular beacons bind to the amplified target following each cycle of amplification
- So, the resulting signal is proportional to the amount of template
- Fluorescence is monitored and reported during each annealing step, when the beacon is bound to its complementary target
- This information is then used during the experiment to quantify initial copy number

REAL TIME PCR THERMAL CYCLERS

APPLICATIONS OF REAL-TIME PCR IN FOOD SECTOR

- Quantification of microbial load in foods or on vegetable material
- Detection of GMOs (genetically modified organisms)
- Quantification of different species in complex meat products

Table 1. Real-time PCR analysis of commercial products using the duck group assay. Hird et al. (2005).

Product	Declared meat species or animal content	Average Ct value ± SD
Roasted duck in plum sauce	Roasted duck	12.03±0.09
Smoked duck slices	Smoked Barbary duck	11.13±0.09
Duck pate	19% Pork, pork fat, 19% duck liver, milk, 11% chicken liver, duck fat	12.09 ± 0.07
Whiskers duck cat food (pouch)	Meat and animal derivatives including min 4% duck	30.67 ± 0.59
Friskies duck cat biscuits	Min 4% duck	19.81 ± 0.14

Table 2. Composition of reference sausages "Sukuk" (KalA – KalE) ranging from 1 to 55% proportion for each species in total meat ingredient

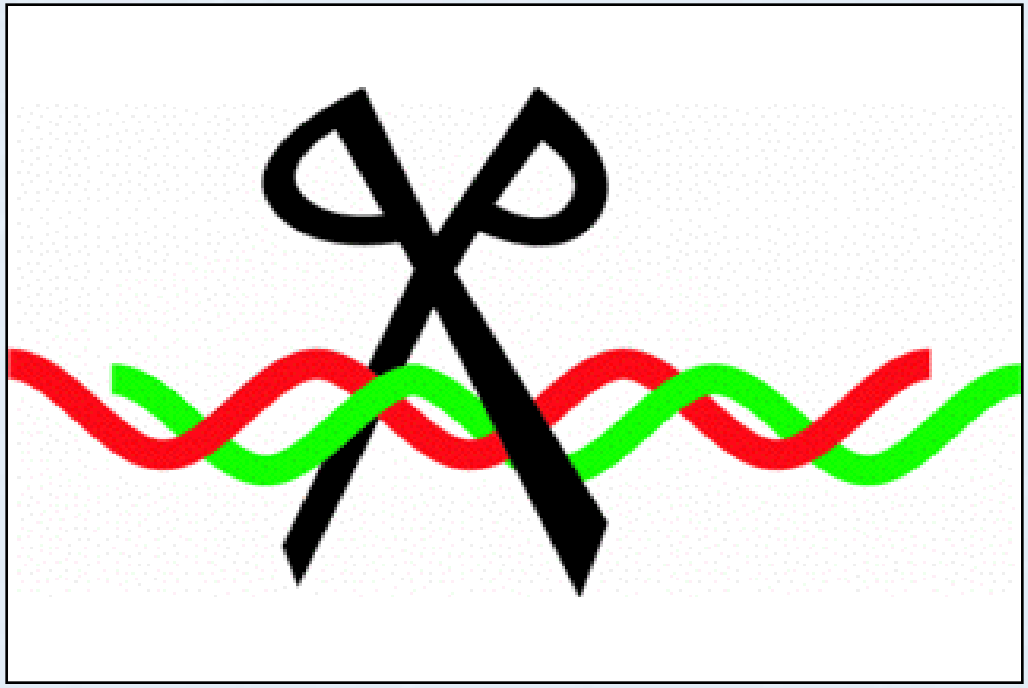
Reference sausage composition (Portion of beef, horse, pork and sheep in total meat ingredient)	Beef (%) Beta-actin-gene	Sheep (%) Beta-actin-gene	Pork (%) Growth hormone receptor	Horse (%) Prolactin receptor
KalA	0.35	8.9	39.3	51.5
KalB	23	1.5	51	24.4
KalC	34	25.8	18.8	21.4
KalD	29.9	61.7	7.6	0.9
KalE	51.2	37.2	1	10.5

6. RESTRICTION FRAGMENT LENGTH POLYMORPHISMS (RFLPs)

RESTRICTION ENZYMES

- Enzymes that cleaves DNA into fragments, at or near specific recognition sites within the molecule, known as restriction sites
- To cut DNA, all restriction enzymes make two incisions, once through each sugar-phosphate backbone (each strand) of the DNA double helix
- These enzymes are found in bacteria and archaea and provide a defence mechanism against invading viruses
- Restriction enzymes are named from the bacterium from which are isolated
- For example EcoRI was isolated from ***E****scherichia* ***co****li*, BamHI was isolated from ***B****acillus* **am**yloliquefaciens and HaeIII was isolated from ***H****aemophilus* ***ae****gyptius*

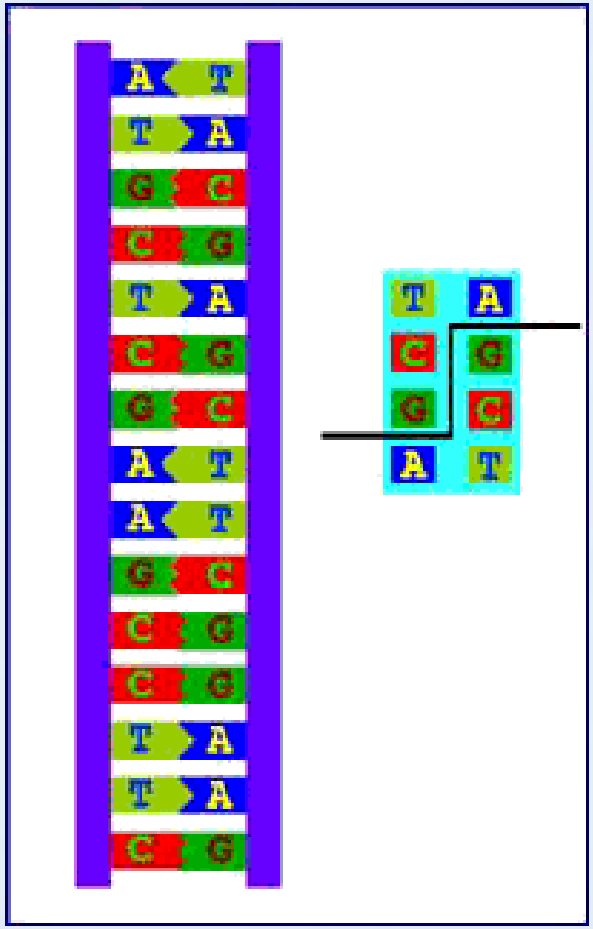
Restriction enzyme



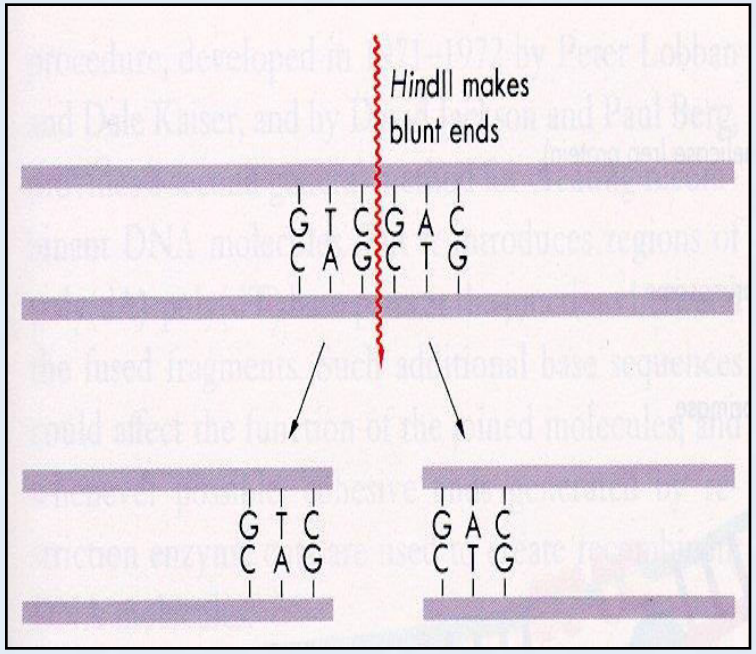
Restriction enzymes and their properties

Enzyme	Source	Recognition sequence	Cut	Product
EcoRI	***E****scherichia* ***co****li*	5´GAATTC 3´ 3´CTTAAG 5´	5'---G **AATTC**---3‘ 3'---**CTTAA** G---5'	Sticky ends of 4 bases
BamHI	***B****acillus* ***am****yloliquefaciens*	5'GGATCC 3´ 5´CCTAGG 3´	5'---G **GATCC**---3‘ 3'---**CCTAG** G---5'	Sticky ends of 4 bases
Hae III	***H****aemophilus* ***ae****gyptius*	5´ GGCC 3´ 3´ CCGG 5´	5'---**GG CC**---3‘ 3'---**CC GG**---5'	Blunt ends of 2 bases

Sticky ends

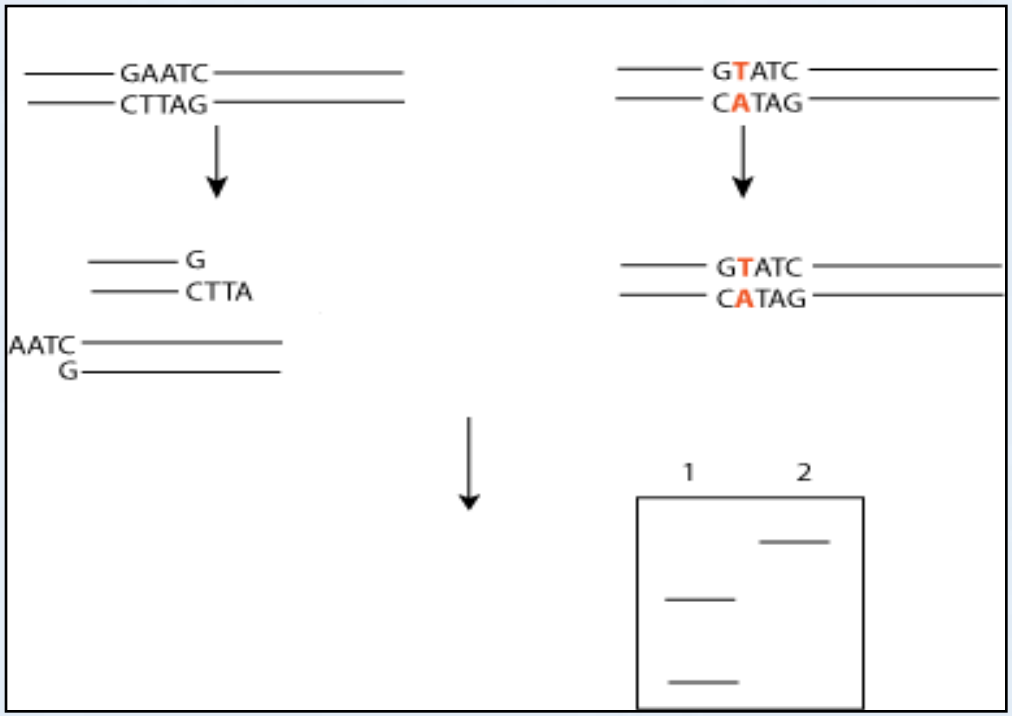


Blunt ends



- When the enzyme cuts the two strands of the double helix in the same position…
- In a blunt-ended molecule both strands terminate in a base pair and there are no unpaired nucleotides

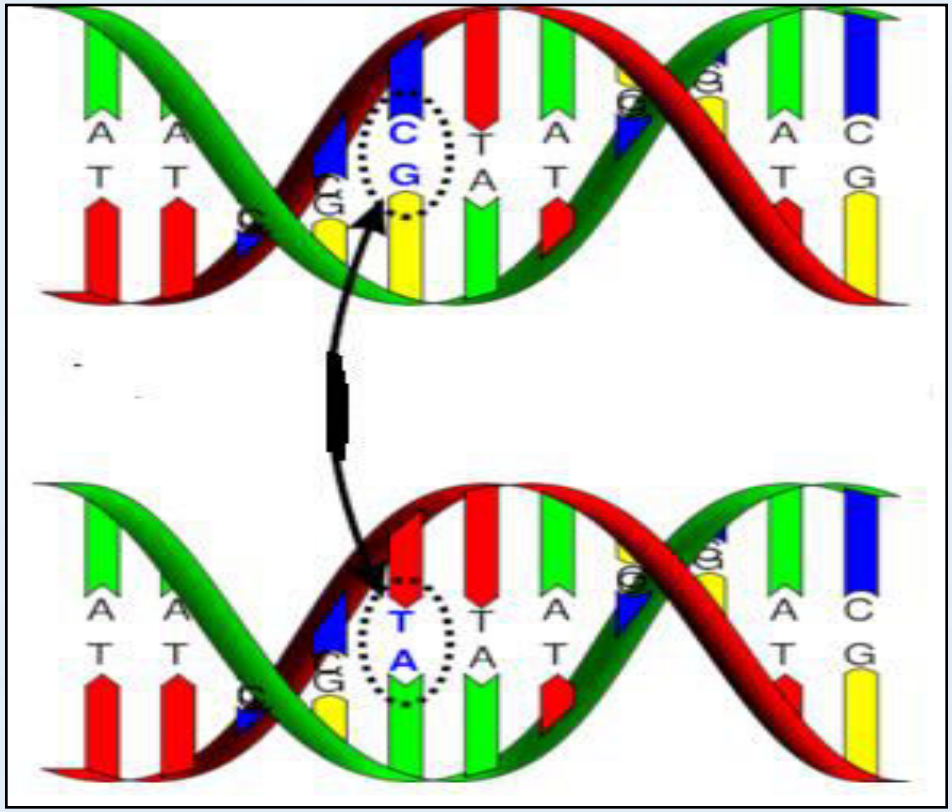
RESTRICTION FRAGMENT LENGTH POLYMORPHISMS (RFLPs)



RESTRICTION FRAGMENT LENGTH POLYMORPHISMS (RFLPs)

- Restriction endonucleases cut the DNA sample inside a specific recognition sequence and generate a restriction pattern with specific number of fragments
- Differences in the number or the size of the fragments (polymorphisms) could occur after nucleotide substitutions inside the recognition sites, sequence rearrangements, insertions or deletions
- Each of these mutations results to differences in the fragment pattern
- Nucleotide substitutions inside the recognition sites lead to creation or loss of the recognition sites

Loss of a restriction site



Restriction Enzymes (Molecular Scissors)

EXPERIMENTAL PROCEDURE

- I prepare 8 eppendorf tubes for the eight different samples. Each tube should contain:
  - **3 µl** PCR product (16S rDNA gene, 600 bp) of each sample
  - **1 µl** buffer of Bpm I enzyme (10X)
  - 6 units of Bpm I enzyme, corresponding to **2,4 µl** of enzyme
  - **0,1 µl** BSA solution (100X)
  - **3,5 µl** distilled water

- The final volume of each reaction should be 10 µl

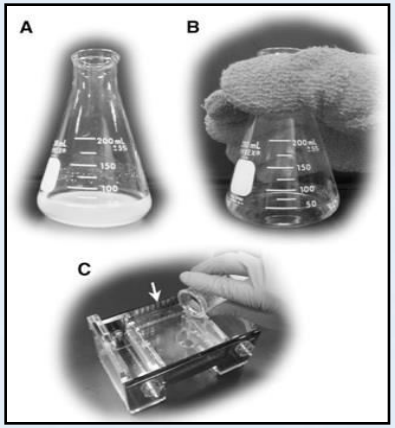
- Then I prepare a total mix for the eight reactions, multiplying all the component quantities X n = 8

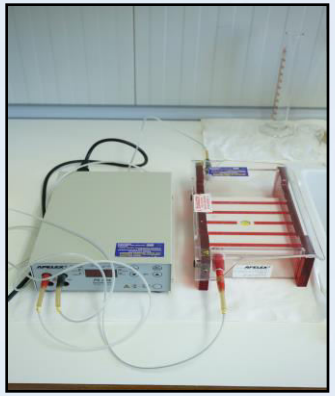
- I place 3 µl of the PCR product, in each of the eight eppendorf tubes. Then, in each tube I add 7 µl from the reaction mix.

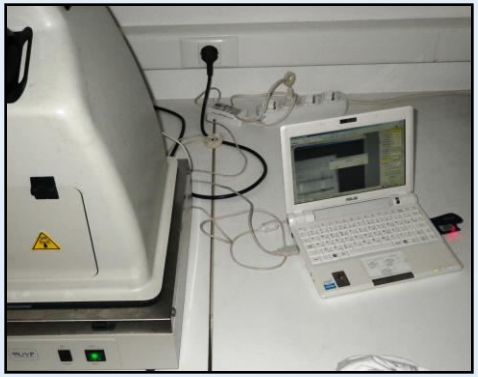
- The tubes are incubated at 37ºC for sixteen hours

Incubation of the samples

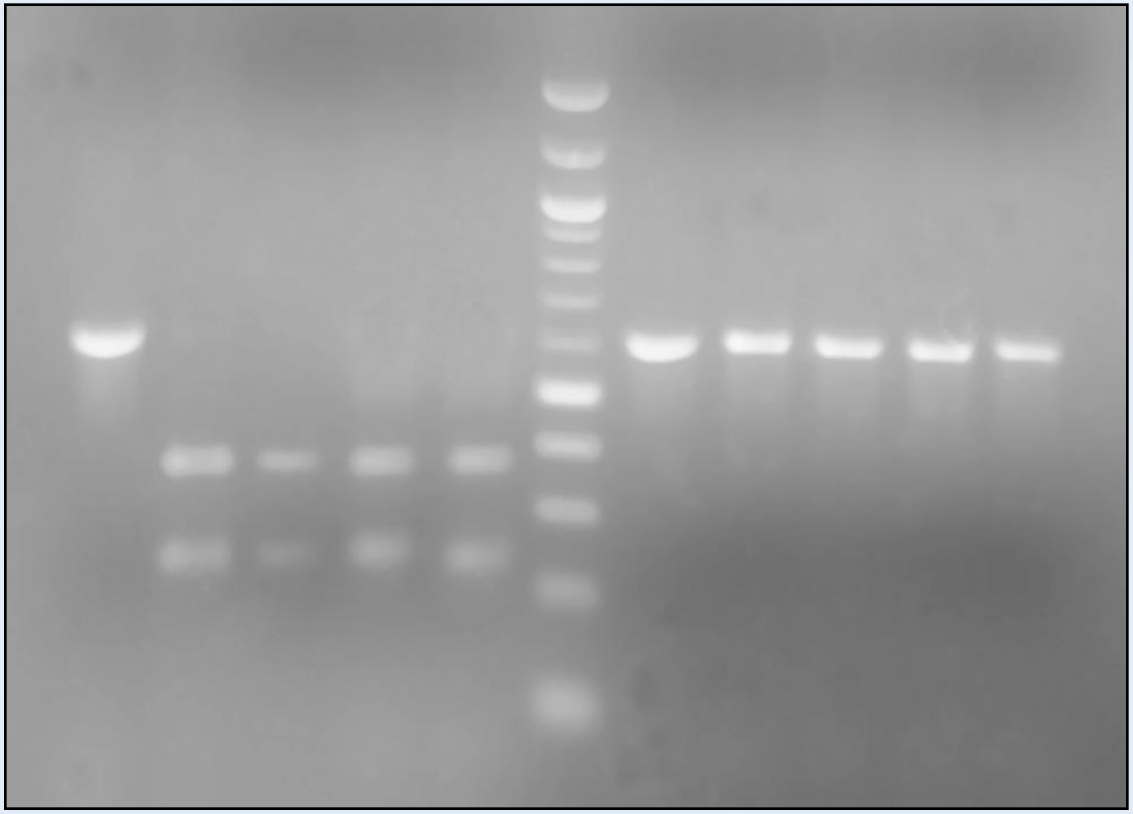
Agaroze gel electrophoresis







Restriction patterns arisen for eight different fish samples (two species: *Liza ramada, Liza saliens*) after digestion of the amplified 16S rDNA gene with enzyme Bpm I. Haplotypes: **A** = 220\380 bp, **B** = 600 bp. The marker used is the 100bp DNA ladder.



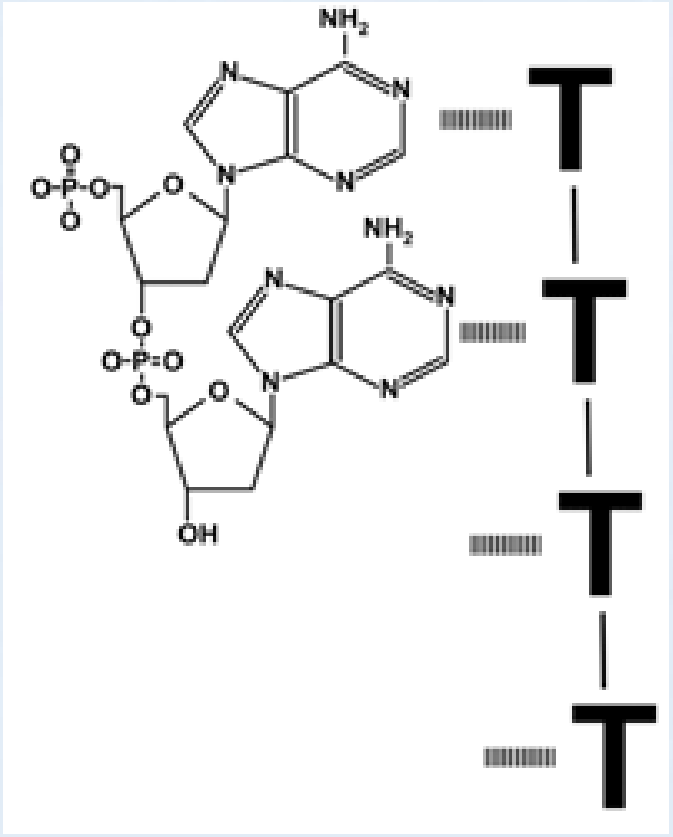
7. SANGER SEQUENCING ANALYSIS

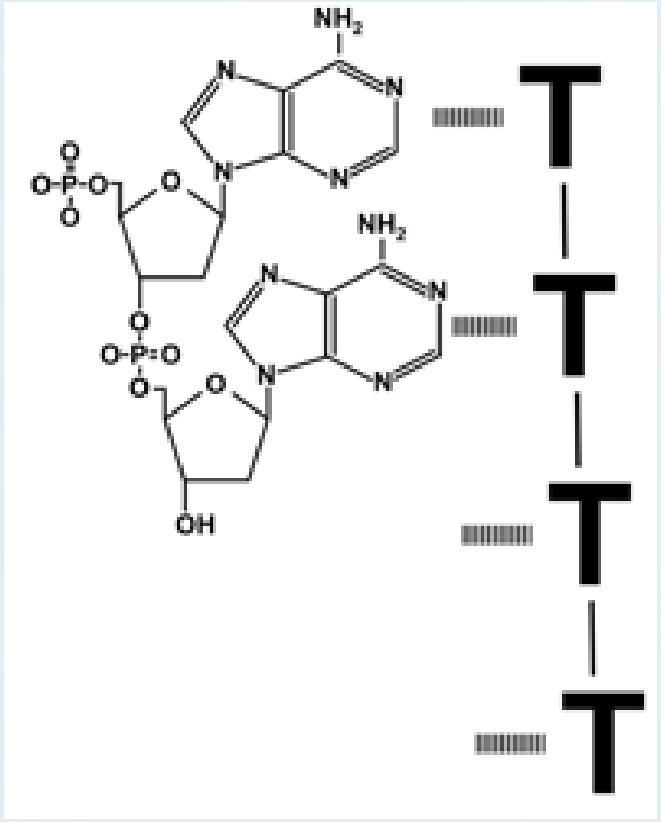
- DNA sequencing is the process of determining the precise order of nucleotides within a DNA molecule
- Maxam-Gilbert sequencing technology (1977). Chemical modification of DNA and subsequent cleavage at specific bases
- Chain-termination method (1977). The chain-termination method developed by Frederick Sanger and coworkers in 1977
- It is the most widely used sequencing method for approximately 40 years

SANGER SEQUENCING

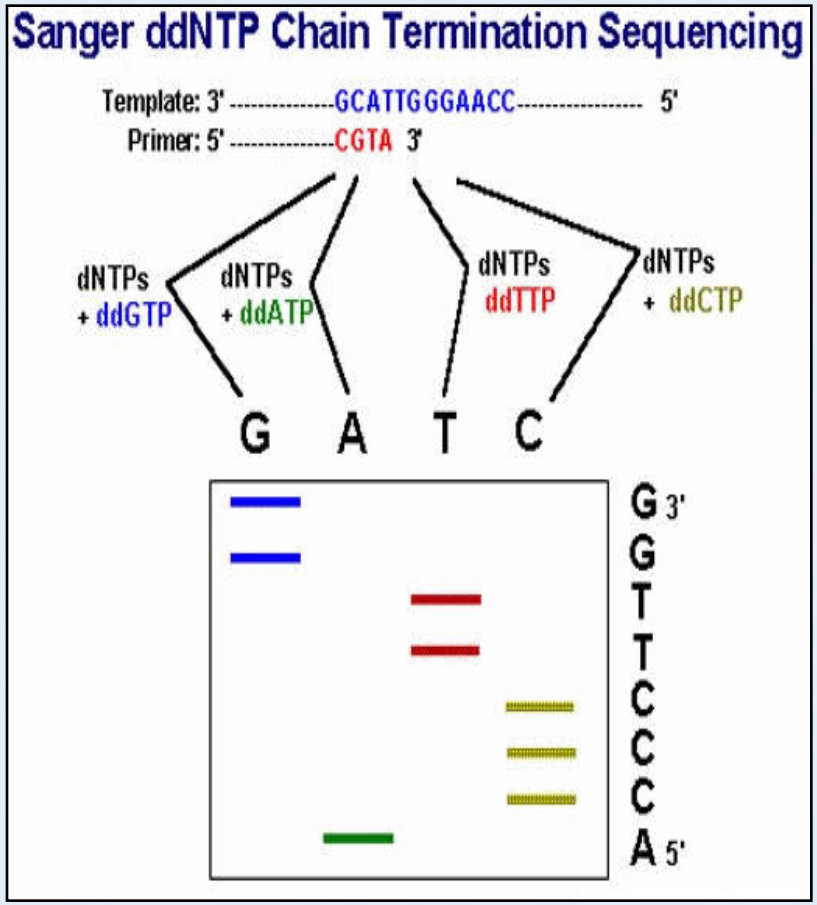
- It requires a single-stranded DNA template, a DNA primer, a DNA polymerase and the four DNA nucleotides (dATP, dTTP, dCTP, dGTP)
- It also contains dideoxy or chain-terminating versions of all four nucleotides (ddATP, ddTTP, ddCTP, ddGTP), each labeled with a different color of dye
- Dideoxy nucleotides lack a hydroxyl group on the 3´ carbon of the sugar ring
- Once a dideoxy nucleotide has been added to the chain, there is no hydroxyl available and no further nucleotides can be added
- The chain ends with the dideoxy nucleotide, which is marked with a particular color of dye depending on the base (A, T, C or G) that it carries

SANGER SEQUENCING





The procedure



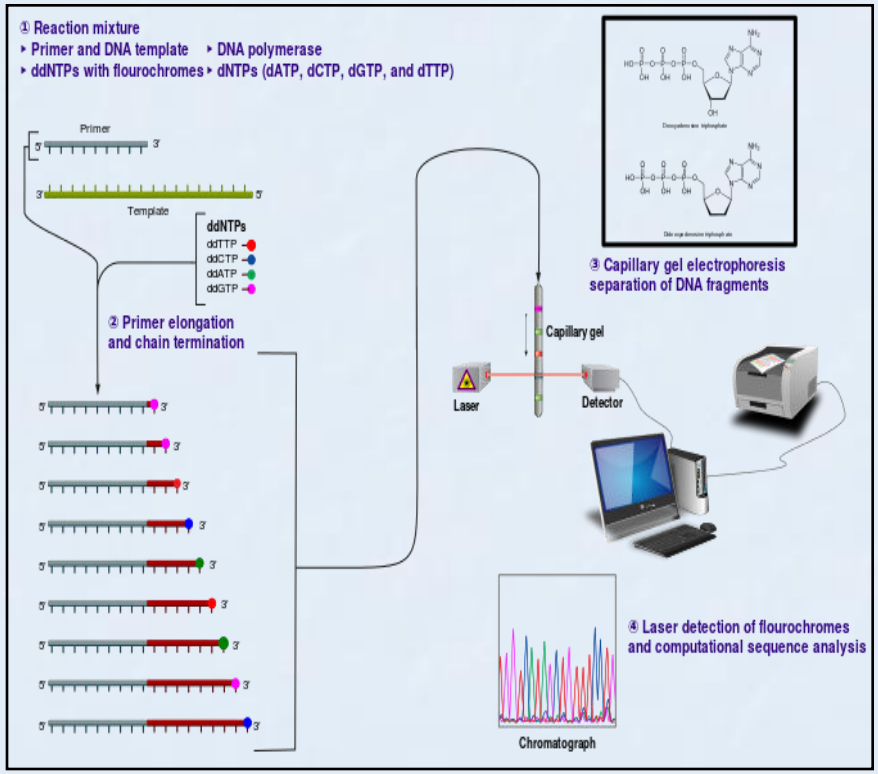
- The four dideoxy nucleotides are added but in much bigger amounts than the ordinary nucleotides
- The strand will end with the dideoxy nucleotide
- By the time the cycling is complete, it's guaranteed that a dideoxy nucleotide will have been incorporated at every single position of the target DNA, in at least one reaction
- The tube will contain fragments of different lengths
- The ends of the fragments will be labeled with dyes that indicate their final nucleotide (dideoxy nucleotide)

EXPERIMENTAL PROCEDURE OF AUTOMATED SANGER SEQUENCING METHODOLOGY

- **1**. PCR amplification of the COI gene
- **2**. Purification of the PCR product
- **3**. Preparation of the second PCR reaction (in a special plate: purified COI PCR product, COI Forward primer, DNA polymerase, dNTPs, ddNTPs)
- **4**. Purification of the second PCR product
- **5**. Electrophoresis in ABI Automated Sequence analyzer

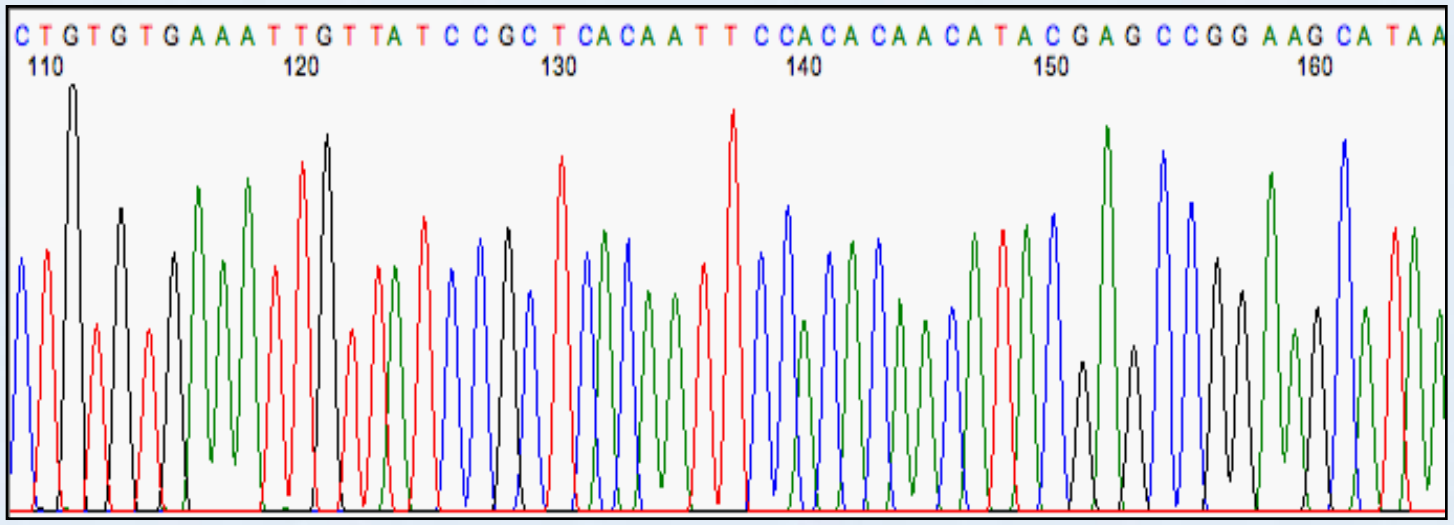
Automated DNA Sequence Analyzer of Applied Biosystems (ABI 3500)

The operation of the Automated DNA Sequence Analyzer



- The fragments are run through a long, thin tube, containing a gel matrix, in a process called capillary gel electrophoresis
- Each fragment is illuminated by a laser, allowing the attached dye to be detected
- From the colors of dyes registered one after another on the detector, the sequence of the original piece of DNA can be built up one nucleotide at a time

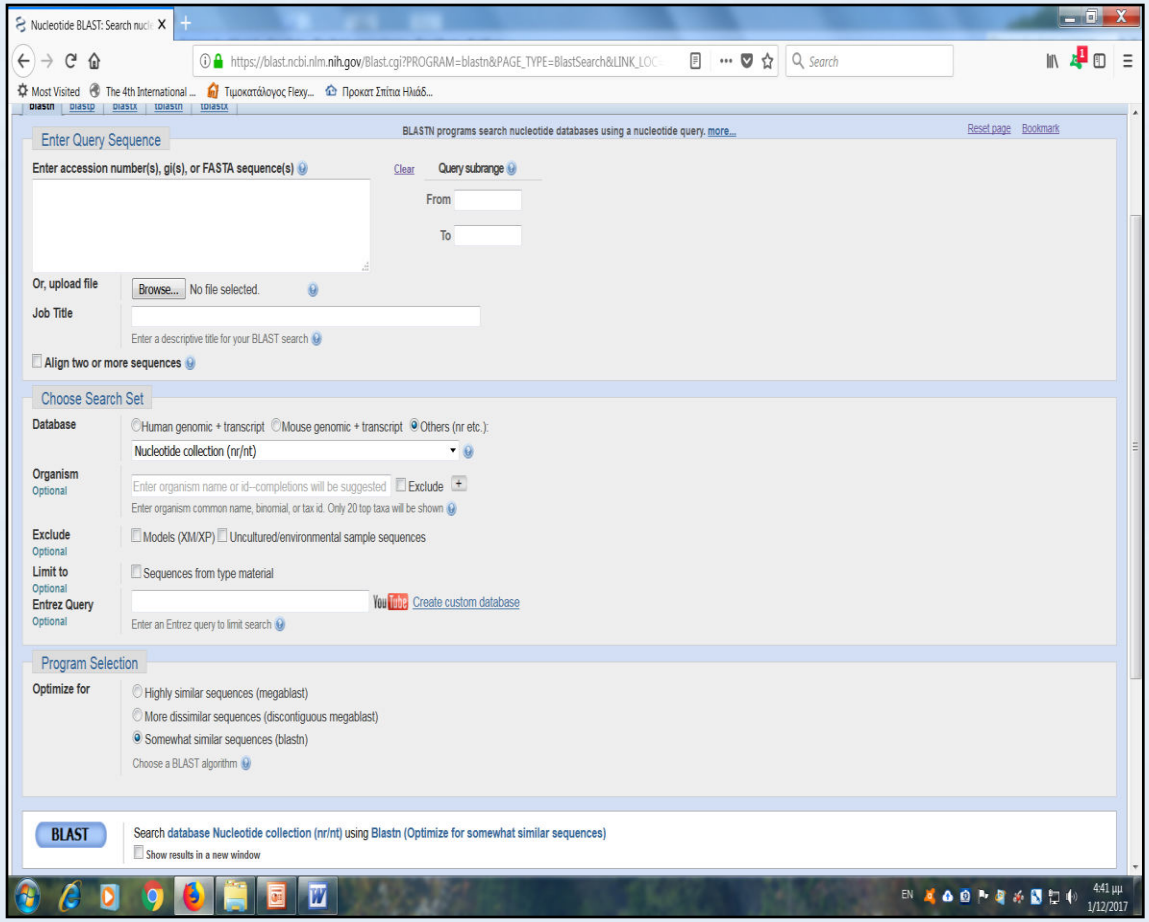
A series of peaks in fluorescence intensity → chromatogram



APPLICATIONS IN FOOD ANALYSIS

- 680 base pairs at the 5´ end of the COI gene were sequenced for 87 fish fillets, labelled with the common Greek name "galeos"

Basic Local Alignment Search Tool (BLAST)



- BLAST finds regions of similarity between biological sequences

Basic Local Alignment Search Tool (BLAST)

Sequences producing significant alignments:

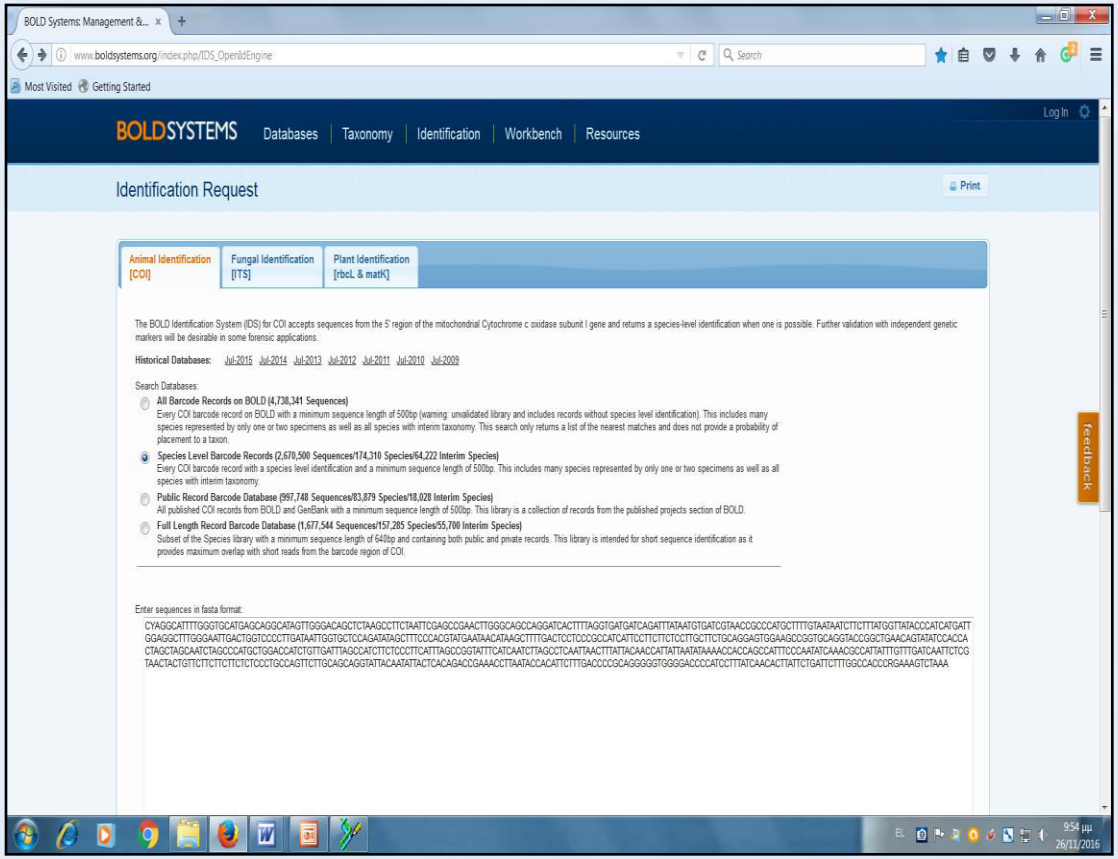
Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Squalus acanthia complete mitochondrial genome	1040	1040	96%	0.0	99%	Y18134.1
Squalus acanthias voucher NRM:46690 16S ribosomal RNA gene, partial sequence; mitochondrial	1022	1022	94%	0.0	99%	KJ128912.1
Squalus acanthias 16S ribosomal RNA gene, partial sequence; mitochondrial	1018	1018	95%	0.0	99%	KU577283.1
Squalus acanthias voucher WTU:047291 16S ribosomal RNA gene, partial sequence; mitochondrial	1018	1018	94%	0.0	99%	EF119240.1
Squalus acanthias voucher NRM:48733 16S ribosomal RNA gene, partial sequence; mitochondrial	1011	1011	94%	0.0	99%	KJ128913.1
Squalus acanthias voucher WTU:047709 16S ribosomal RNA gene, partial sequence; mitochondrial	1007	1007	94%	0.0	99%	EF119335.1
Squalus megalops isolate s05 tRNA-Phe gene, partial sequence; 12S ribosomal RNA and tRNA-Val genes, complete sequence; and 16S ribosomal RNA gene, partial seq	985	985	96%	0.0	98%	GU130625.1
Squalus montalbani mitochondrion, complete genome	979	979	96%	0.0	98%	KT459334.1
Squalus formosus mitochondrion, complete genome	974	974	96%	0.0	97%	KU951280.1
Cirrhigaleus australis mitochondrion, complete genome	974	974	96%	0.0	97%	KJ128289.2
Squalus cubensis mitochondrial partial 16S rRNA gene, specimen voucher Pi20	959	959	94%	0.0	98%	FN431789.1
Centrophorus squamosus isolate s08 tRNA-Phe gene, partial sequence; 12S ribosomal RNA and tRNA-Val genes, complete sequence; and 16S ribosomal RNA gene, part	944	944	96%	0.0	97%	GU130628.1
Squalus cubensis 16S ribosomal RNA gene, partial sequence; mitochondrial gene for mitochondrial product	942	942	92%	0.0	98%	AF288199.1

- The program compares nucleotide sequences to sequence databases and calculates the genetic identity

Barcode Of Life Data system (BOLD)



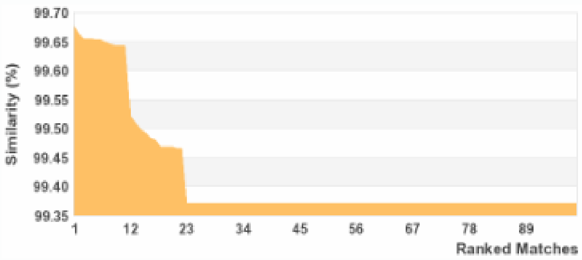
- BOLD is a cloud-based data storage and analysis platform, which accepts sequences from the 5' region of the COI gene, and returns a species-level identification when one is possible

Barcode Of Life Data system (BOLD)

Identification Summary:

Taxonomic Level	Taxon Assignment	Probability of Placement (%)
Phylum	Chordata	100
Class	Elasmobranchii	100
Order	Squaliformes	100
Family	Squalidae	100
Genus	Squalus	100
Species	Squalus acanthias	99.7

Similarity Scores of Top 99 Matches:

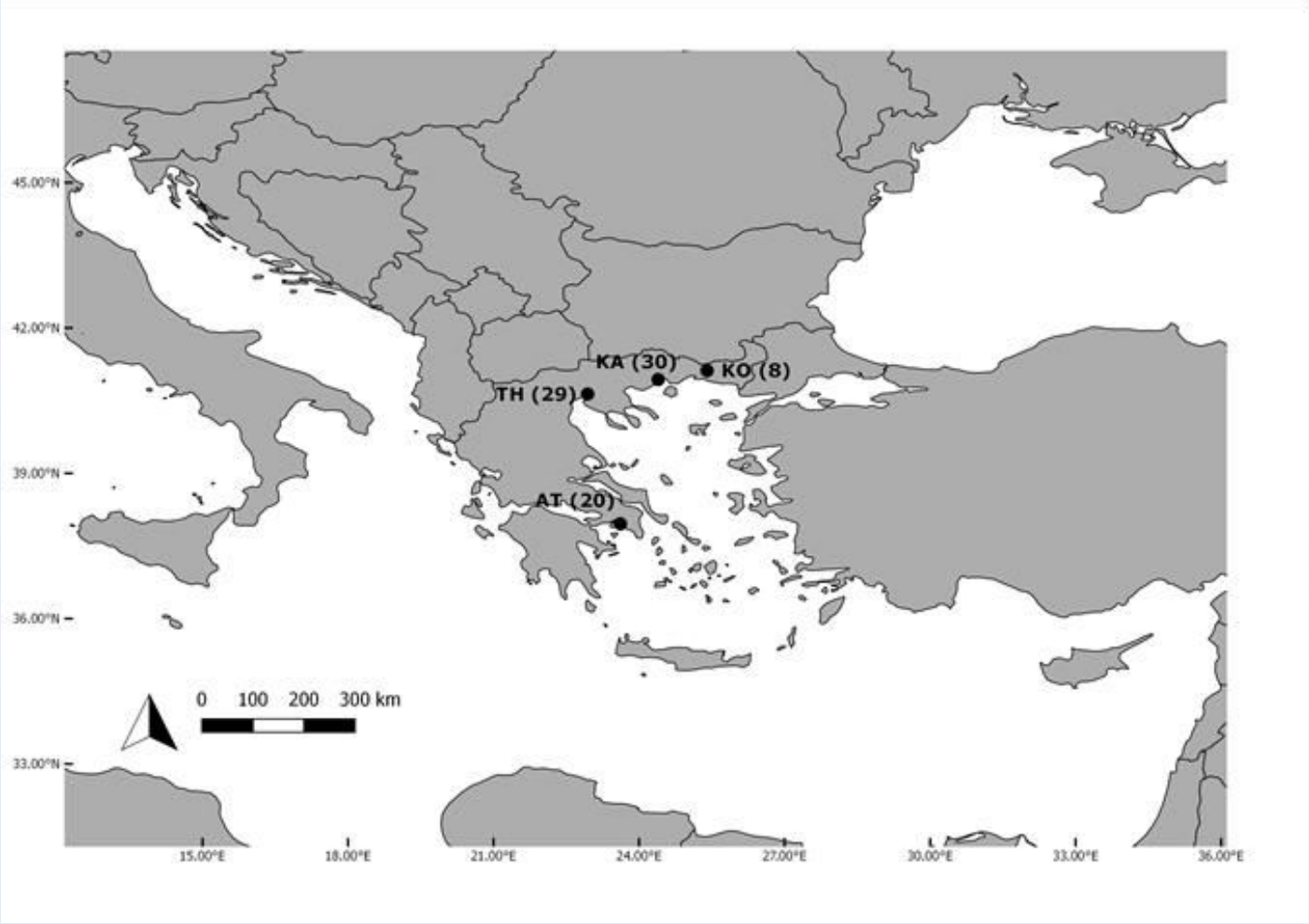


TOP 10 Matches :

Display option: Top 10

Phylum	Class	Order	Family	Genus	Species	Similarity (%)	Status
Chordata	Elasmobranchii	Squaliformes	Squalidae	Squalus	*acanthias*	99.68	Published
Chordata	Elasmobranchii	Squaliformes	Squalidae	Squalus	*acanthias*	99.66	Published
Chordata	Elasmobranchii	Squaliformes	Squalidae	Squalus	*acanthias*	99.65	Published
Chordata	Elasmobranchii	Squaliformes	Squalidae	Squalus	*acanthias*	99.65	Published
Chordata	Elasmobranchii	Squaliformes	Squalidae	Squalus	*acanthias*	99.65	Published
Chordata	Elasmobranchii	Squaliformes	Squalidae	Squalus	*acanthias*	99.65	Published
Chordata	Elasmobranchii	Squaliformes	Squalidae	Squalus	*acanthias*	99.65	Published
Chordata	Elasmobranchii	Squaliformes	Squalidae	Squalus	*acanthias*	99.65	Published
Chordata	Elasmobranchii	Squaliformes	Squalidae	Squalus	*acanthias*	99.64	Published
Chordata	Elasmobranchii	Squaliformes	Squalidae	Squalus	*acanthias*	99.64	Published

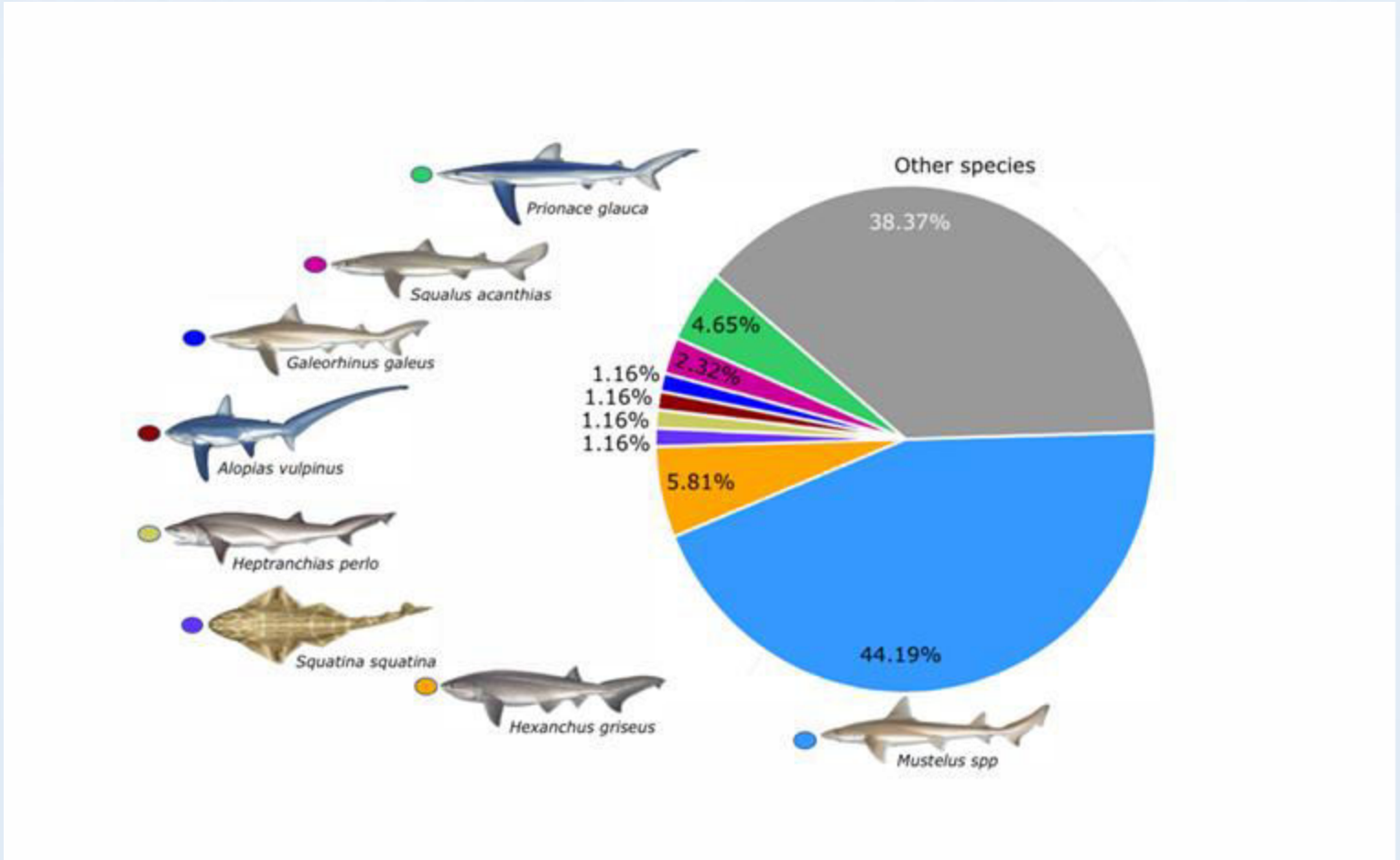
Sampling locations of collected shark tissue used in the present study across Greece. AT: Athens; TH: Thessaloniki; KA: Kavala; KO: Komotene.



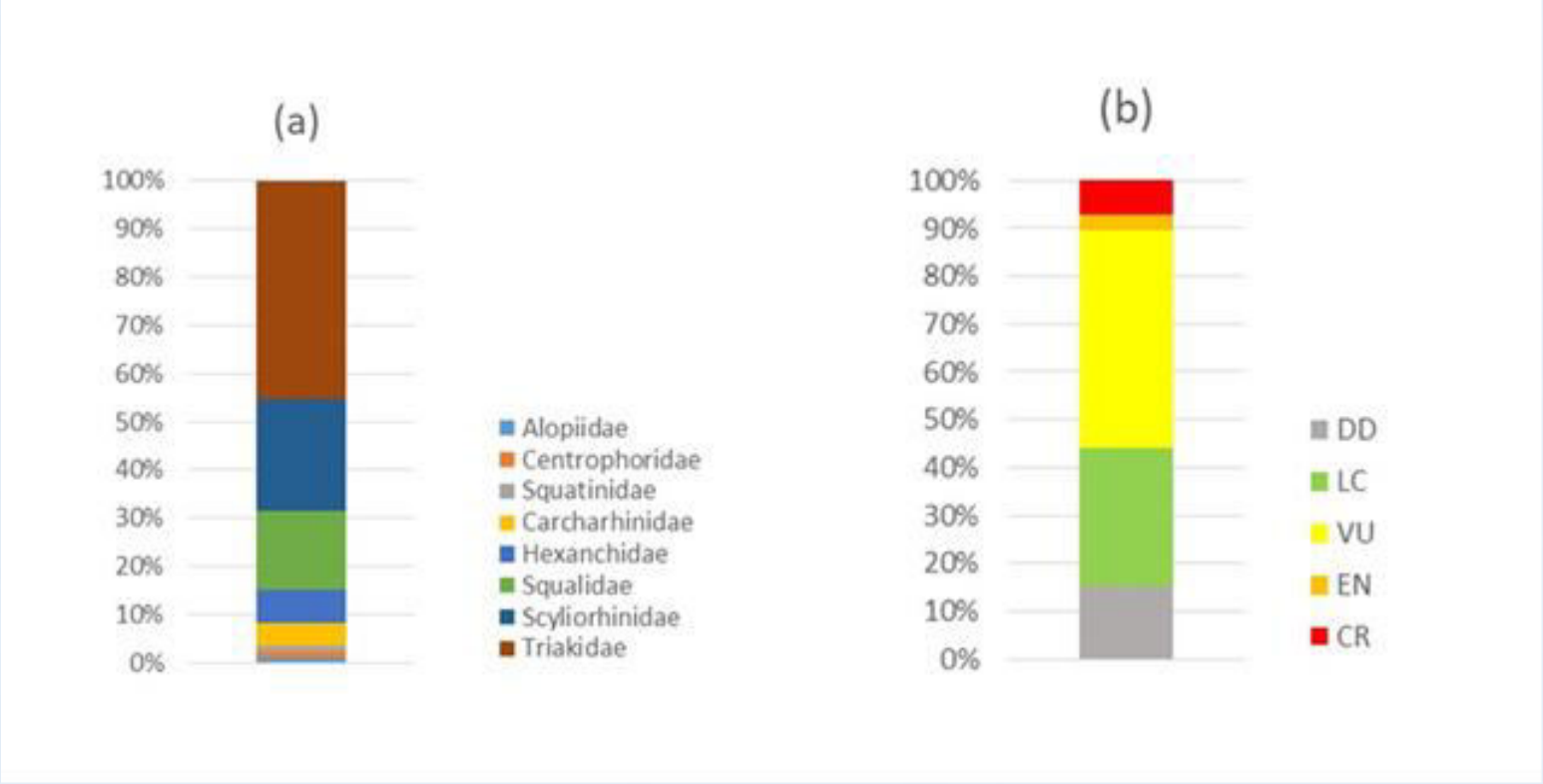
Fraud detection

- Detection of fraudulent commercialisation of seafood products has been extensively reported, mainly by substituting species with others of a different price or identity
- According to the Greek Food & Safety legislation requirements only the *Mustelus spp*. products should be sold as “galeos”
- Only half out of the 87 specimens analyzed were classified as *Mustelus spp*., according to the two methodologies
- The rest of the samples were classified to different Elasmobranchii species
- Marine products labelled as “galeos” have higher commercial value (15-18 euros/kg), in contrast to other Elasmobranchii species with the common name “sapounas” (mainly *Hexanhcus griceus* and rarely *Cetorhinus maximus*), with much lower value (5 euros/kg)

Species composition (i.e., number of individuals) as genetically identified from the "galeos" shark meat samples. *Mustelus spp: M. mustelus, M. asterias* and *M. punctulatus*; Other species: *S. canicula, S. blainville* and *Centrophorus sp.*



Composition of the identified species by family (a) and conservation status for the Mediterranean (b) according to Dulvy et al. (2016)



8. Next Generation Sequencing analysis (NGS)

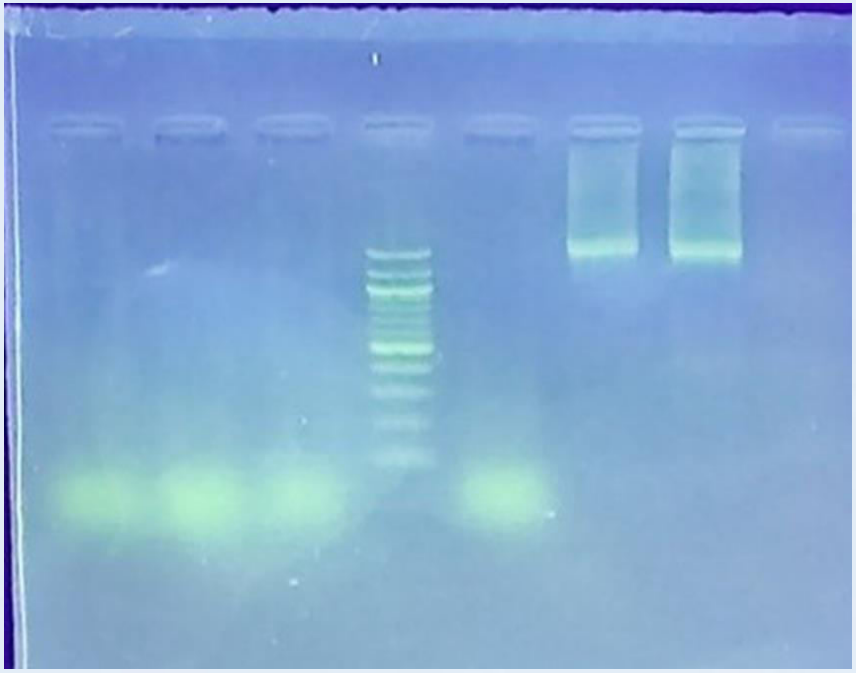
Next Generation Sequencing

- Next-generation sequencing (NGS), also known as high-throughput sequencing, is the catch-all term used to describe a number of different modern sequencing technologies including:

- Illumina (Solexa) sequencing
- Roche 454 sequencing
- Ion torrent: Proton / PGM sequencing
- SOLiD sequencing
- Oxford nanopore technology

- These recent technologies allow us to sequence DNA and RNA much more quickly and cheaply than the previously used Sanger sequencing, and as such have revolutionized the study of genomics and molecular biology

Procedure – Oxford Nanope



- PCR reaction – 16S rRNA = 1650bp
- Each sample has a **unique barcode** (PCR adapter) which is attached in the universal primer
- This adapter discriminates the samples during the sequencing procedure
- After PCR we add all the samples in one eppendorf tube

Oxford Nanopore Protocol

- ***Step 1. Library construction***

  0.7 µl PCR + 0.3 µl sequencing **adaptor** (common) + 4 µl H_2O => 5 µl

- ***Step 2. Fuel preparation***

  175.5 µl Flush Buffer (FLB) + 4.5 µl Flush Tether (FLT) => 180 µl *fuel*

- ***Step 3. Flow cell fuel loading***

  To load the fuel into the flow cell, insert the tip of the pipette into the port

- ***Step 4. Add loading beads into the library***

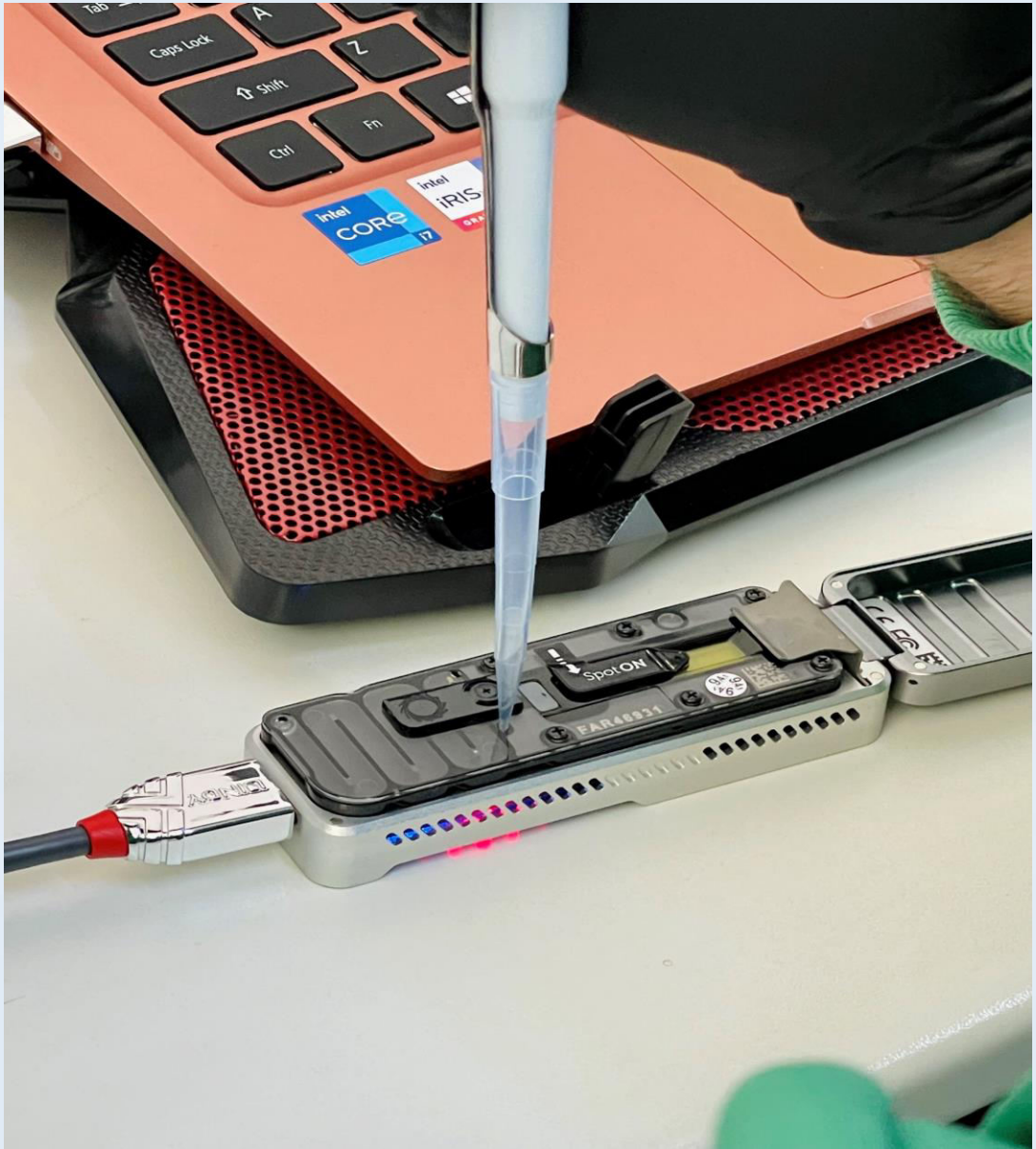
  5 µl of the DNA library, 10 µl of the loading beads, 15 µl of the Sequencing buffer.

- ***Step 5. Flow cell library loading***

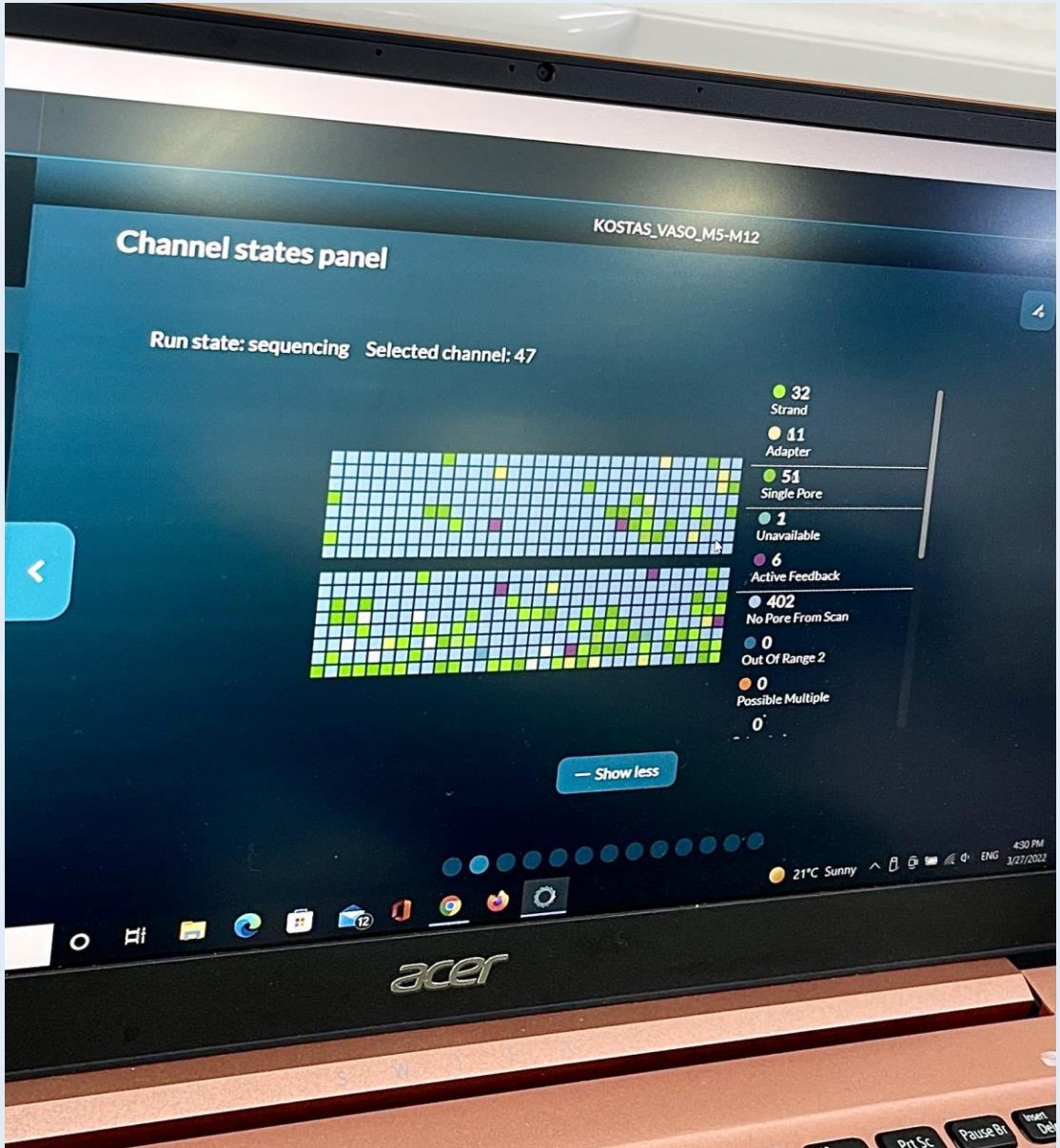
  Load the library constructed into the flow cell.

MiNION device

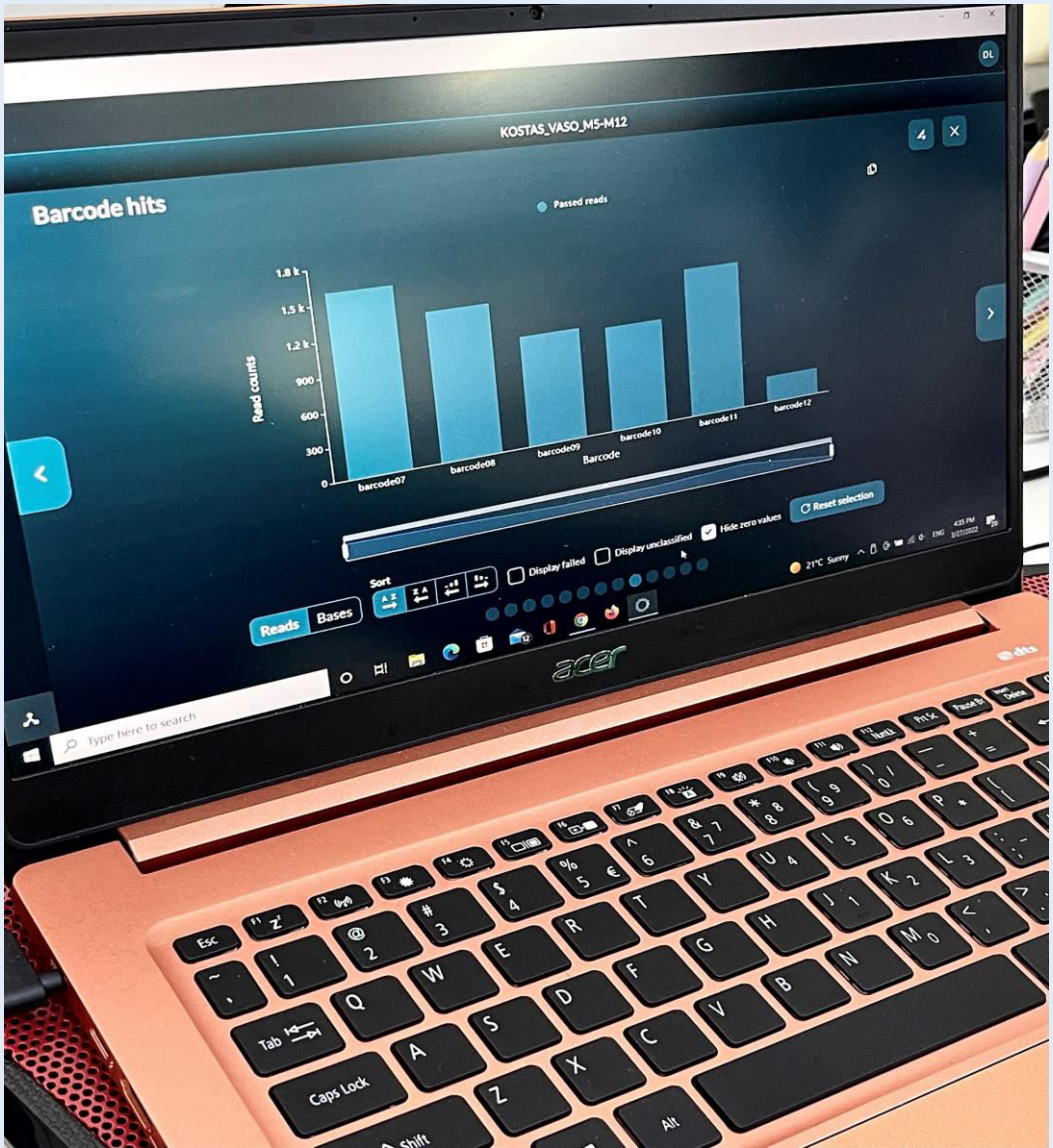
MiNION device



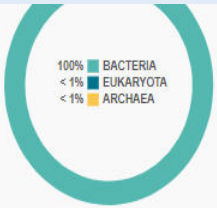
MiNION device



MiNION device



Results



READS ANALYSED
169,640

READS CLASSIFIED
168,424

READS UNCLASSIFIED
1,216

Taxa at Rank: Family

Filter...

Taxon	Cumulative Reads
Rhodobacteraceae	28,637
Mycoplasmataceae	22,905
Flavobacteriaceae	15,232
Spirochaetaceae	6,846
Campylobacteraceae	5,185
Clostridiaceae	**2,999**
Erysipelotrichaceae	2,786
Aerococcaceae	2,443
Bacillaceae	2,192
Akkermansiaceae	2,134
Microbulbiferaceae	1,763
Peptostreptococcaceae	1,705

« 1 2 3 4 5 ... 31 »

Reads per Barcode ID

ID	Read Count	
barcode01	65,409	EXCLUDE
barcode02	104,231	EXCLUDE

EXCLUDE ALL

Reads per Exit Status

Unclassified	1,216
Workflow successful	168,424

BACK

FAMILY Clostridiaceae

RESULTS | TAXONOMY TREE

READS
2,999

NCBI TAXONOMY ID
31979

Source: Wikipedia

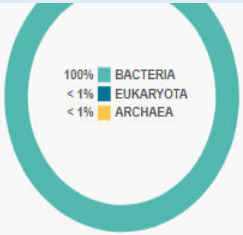
LINEAGE
SUPERKINGDOM Bacteria
CLADE Terrabacteria group
PHYLUM Firmicutes
CLASS Clostridia
ORDER Clostridiales
FAMILY Clostridiaceae

Of the 2999 reads, 90 were classified as **Clostridiaceae** and the rest to one of the **59 child taxa**:

Taxon	Reads	Rank
Clostridium	508	genus
Clostridium fermenticellae	354	species
Clostridium septicum	236	species
Clostridium butyricum	230	species
Clostridium perfringens	183	species
Sarcina sp. JB2	162	species
Alkaliphilus metalliredigens QYMF	147	strain
Alkaliphilus oremlandii OhILAs	115	strain
Clostridium chauvoei	114	species
Clostridium sp. CT4	113	species

« 1 2 3 4 5 6 »

Results



READS ANALYSED
169,640

READS CLASSIFIED
168,424

READS UNCLASSIFIED
1,216

Taxa at Rank: Genus

Filter...

Taxon	Cumulative Reads
Mycoplasma	18,083
Polaribacter	7,290
Sulfitobacter	4,054
Erysipelothrix	2,760
Poseidonibacter	2,720
Ruegeria	2,452
Aerococcus	2,434
Parasedimentitalea	2,390
Clostridium	2,318
Akkermansia	2,134
Sediminispirochaeta	1,819
Microbulbifer	1,763

« 1 2 3 4 5 ... 101 »

Reads per Barcode ID

ID	Read Count	
		EXCLUDE ALL
barcode01	65,409	EXCLUDE
barcode02	104,231	EXCLUDE

BACK

GENUS Mycoplasma

RESULTS | TAXONOMY TREE

READS
18,083

NCBI TAXONOMY ID
2093

Source: Wikipedia

LINEAGE
SUPERKINGDOM Bacteria
CLADE Terrabacteria group
PHYLUM Tenericutes
CLASS Mollicutes
ORDER Mycoplasmatales
FAMILY Mycoplasmataceae
GENUS Mycoplasma

Of the 18083 reads, 1698 were classified as **Mycoplasma** and the rest to one of the 50 child taxa:

Taxon	Reads	Rank
Mycoplasma penetrans HF-2	9,808	strain
Mycoplasma iowae	3,292	species
Mycoplasma mobile 163K	686	strain
Mycoplasma hyorhinis MCLD	441	strain
Mycoplasma pneumoniae	286	species
Mycoplasma capricolum subsp. capripneumoniae	262	subspecies
Mycoplasma bovoculi M165/69	234	strain
Mycoplasma hyorhinis	204	species
Mycoplasma sp. Phocoena C-264-GEN	183	species
Mycoplasma sp. Mirounga ES2806-NAS	160	species

« 1 2 3 4 5 »

Next Generation Sequencing

- The four main advantages of NGS over classical Sanger sequencing are speed, cost, sample size and accuracy
- NGS is significantly cheaper, quicker, needs significantly less DNA, and is more accurate and reliable than Sanger sequencing
- Only one read can be taken at a time in Sanger sequencing, whereas NGS is massively parallel, allowing 300Gb of DNA to be read on a single run on a single chip.
- The reduced time, manpower and reagents in NGS mean that the costs are much lower.

Thank you…