Molecular Applications in Zoology

Introduction, sample collection, DNA extraction, PCR and electrophoresis

Zuzana Starostová

Syllabus

1. Introduction, DNA extraction, PCR (20.2.2024, lecturer Zuzana Starostová)

2. Next generation sequencing – introduction, short-read and long-read sequencing methods, assemblies (27.2.2023, lecturer Radka Reifová)

3. How to read and make phylogenetic trees (5.3.2024, lecturer Zuzana Starostová)

4. Use of molecular phylogenetics in zoology (12.3.2024, lecturer Zuzana Starostová)

5. Microsatellites and molecular identification of species, individuals and sex (19.3.2024, lecturer Pavel Munclinger)

6. Molecular phylogeography (26.3.2024, lecturer Pavel Hulva)

7. Population structure and paternity studies (2.4.2024, lecturer Pavel Munclinger)

8. Next generation sequencing – applications: whole genome sequencing, exome sequencing, ddRAD sequencing, metagenomics (9.4.2024, lecturer Radka Reifová)

9. Functional genetic variability: From SNP to selection (16.4.2024, lecturer Michal Vinkler)

10. Gene expression – quantitative PCR, expression microarrays, transcriptome sequencing and analysis, epigenomics (23.4.2024, lecturer Radka Reifová)

11. Cytogenetic methods and their application in zoology (30.4.2024, lecturer František Šťáhlavský)

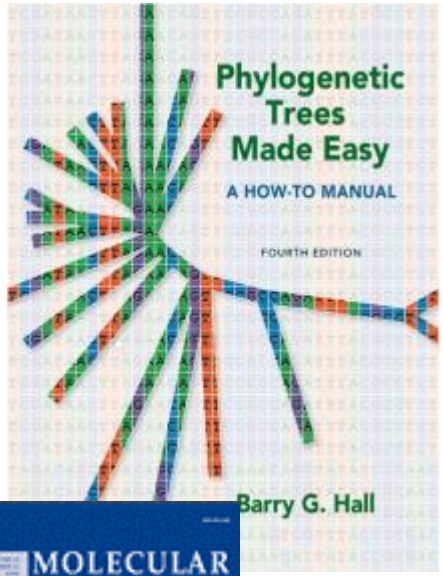
Practicals:
Day 1: database search and basic DNA sequence data handling

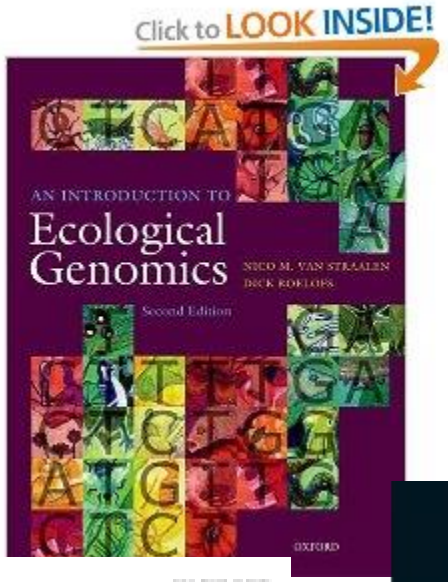
Day 2: lab work – molecular method of sex determination in birds –PCR, gel electrophoresis + cytogenetic methods

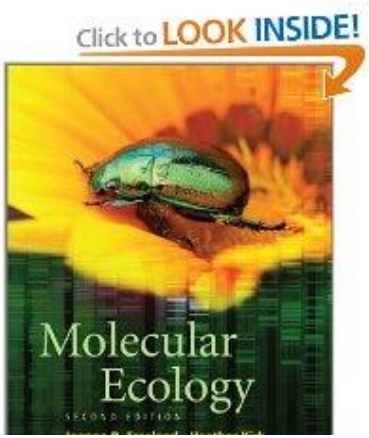
Exam:
written test covering content of the lectures

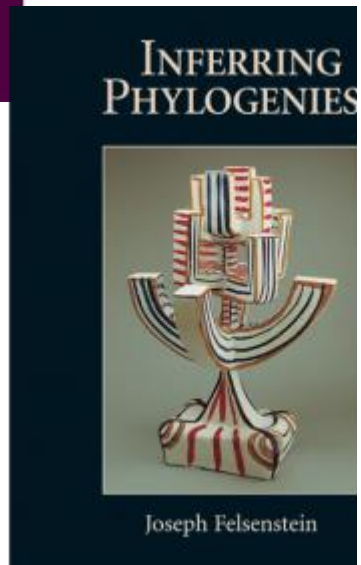
Literature:

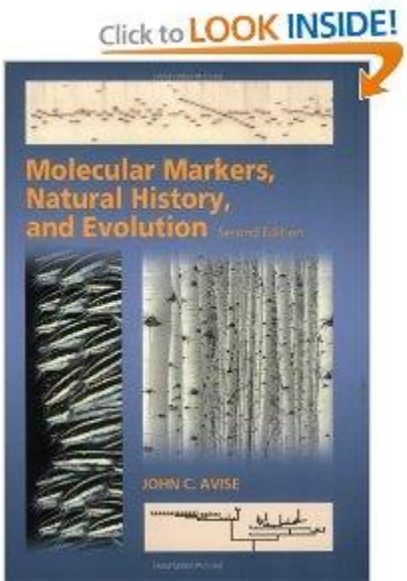
https://zuzanastarostova.webnode.cz/news/molecular-applications-in-zoology/











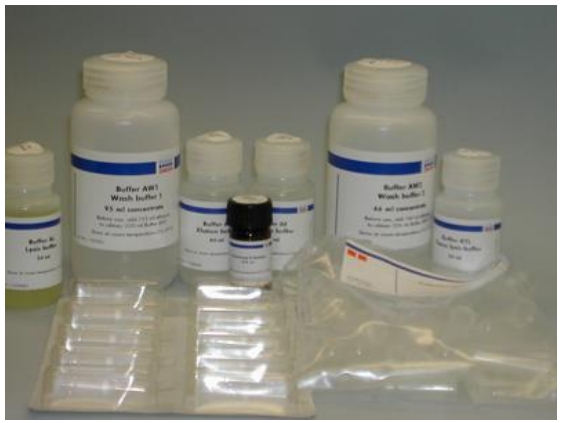
Molecular techniques and zoology

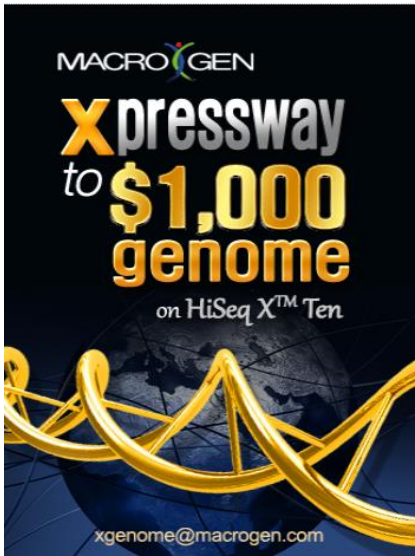
- since 90s molecular techniques more available- automatization, lower costs
- commercial synthesis of oligonucleotides, sequencing services, user friendly kits

→ routine application of molecular techniques into fields like ecology, systematics, ethology, conservation biology…

- new methods – new resources - new questions

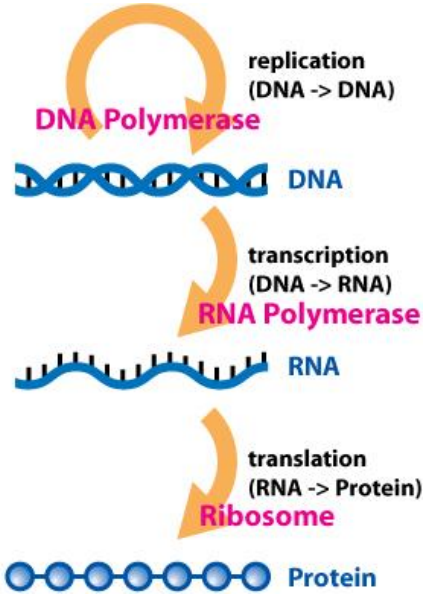
Wikipedia

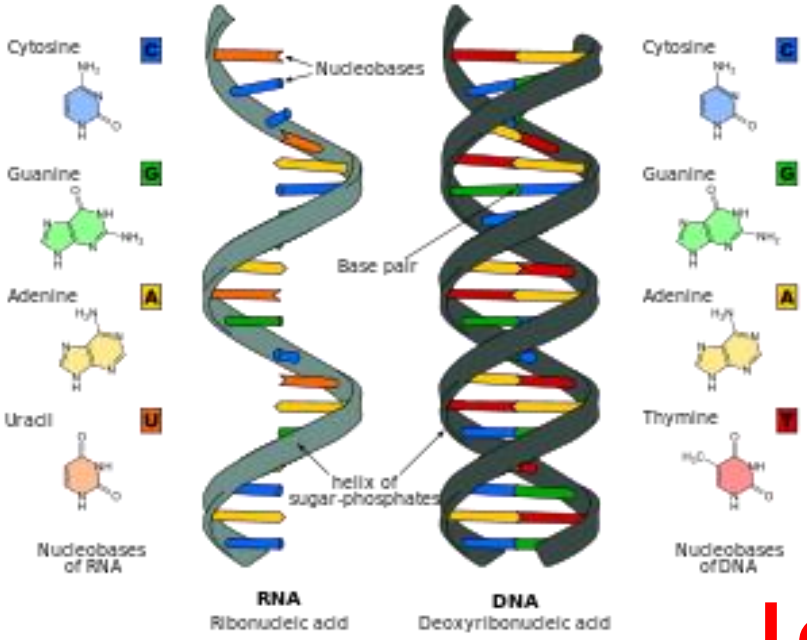




Nucleic acids

- Nucleic acids are macromolecules that store genetic information and enable protein production
- DNA and RNA – molecules composed of strands of nucleotides
- DNA is composed of a phosphate-deoxyribose sugar backbone and the nitrogenous bases adenine (A), guanine (G), cytosine (C), and thymine (T).
- RNA has ribose sugar and the nitrogenous bases A, G, C, and uracil (U).



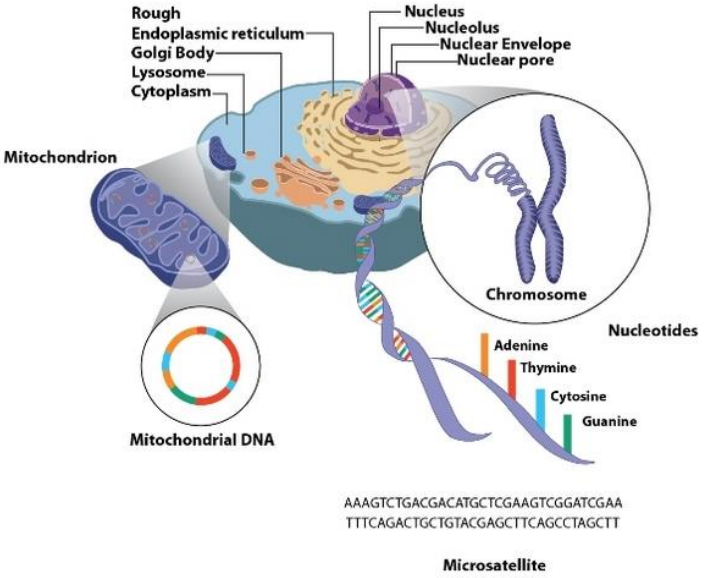


More about RNA in lesson # 9

Let's talk about DNA

DNA

- is carrying the genetic instructions used in the growth, development, functioning, and reproduction of all known living organisms



Structure:

- two polynucleotide strands
- the nitrogenous bases of the two separate polynucleotide strands are bound together, according to base pairing rules (A with T and C with G), with hydrogen bonds to make double-stranded DNA
- the nitrogenous bases - two types:
  pyrimidines are thymine and cytosine;
  purines are adenine and guanine
- two strands of DNA run in opposite directions to each other and are thus **antiparallel**

Sources of DNA for sampling:

- Destructive sampling – tissue (fresh, frozen, dry, preserved in ethanol) – ideal spleen, muscle- e.g. tongue

- Invasive but non-destructive sampling- buccal smears, fin clips, tail tips (rodents), tissue biopsy, blood sample

- Non-invasive sampling (DNA collected without capturing or usually even touching an animal)- feces, hair, feathers, saliva, shed skin, eggshell

  - DNA yield and quality tend to be lower (higher risk of contamination)



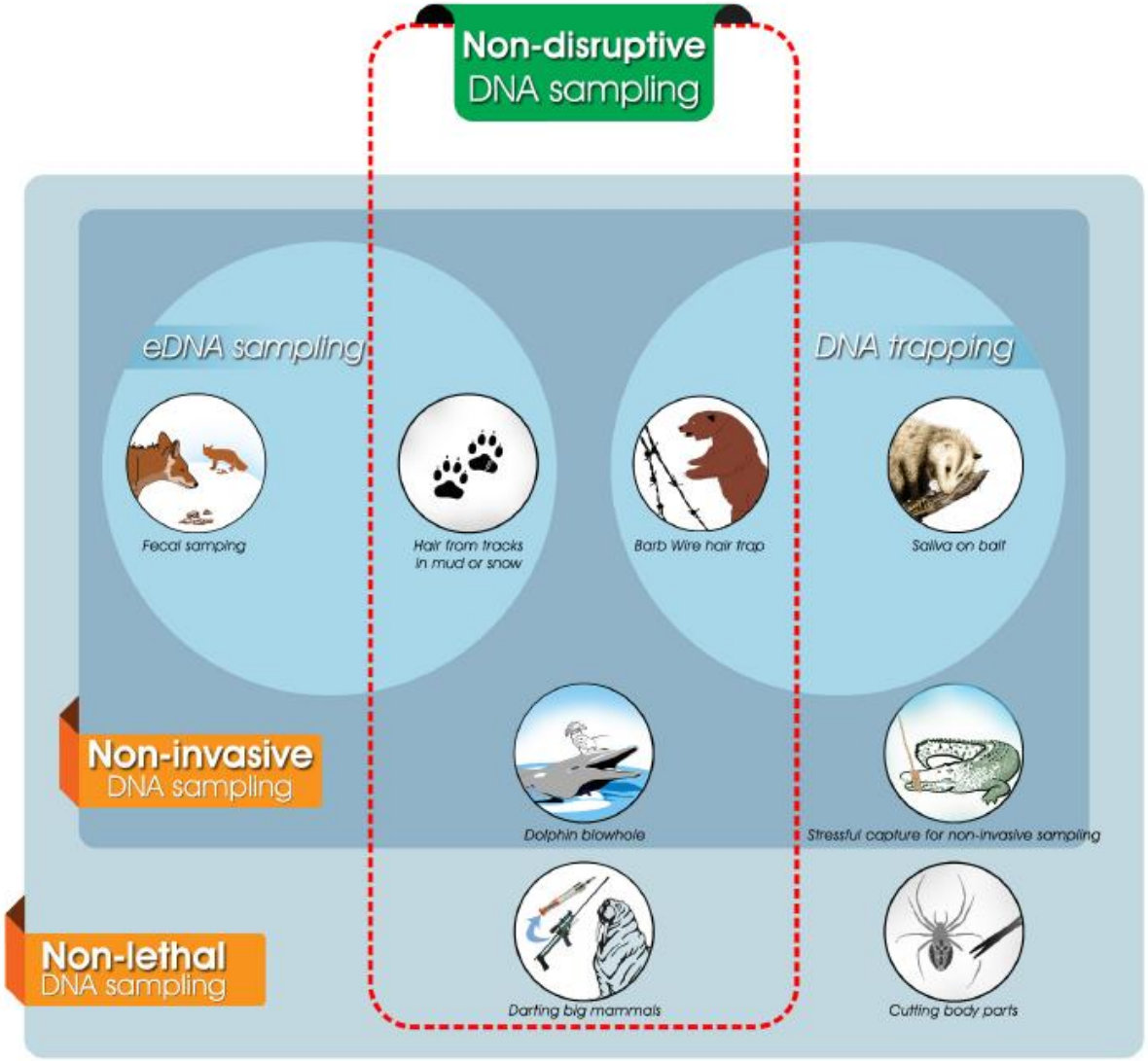



Figure 1. Non-disruptive DNA sampling methods, and their overlaps with non-invasive and non-lethal sampling methods.

Lefort M, Boyer S, Barun A, Emami Khoyi A, Ridden J, Smith VR, Sprague R, Waterhouse BR, Cruickshank RH. (2015) Blood, sweat and tears: non-invasive vs. non-disruptive DNA sampling for experimental biology. PeerJ PrePrints 3:e1580 https://doi.org/10.7287/peerj.preprints.655v3

fur traps

- different for large vs. small species
- effectiveness increased by attractants - cats - *Valeriana officinalis*, deer - anise

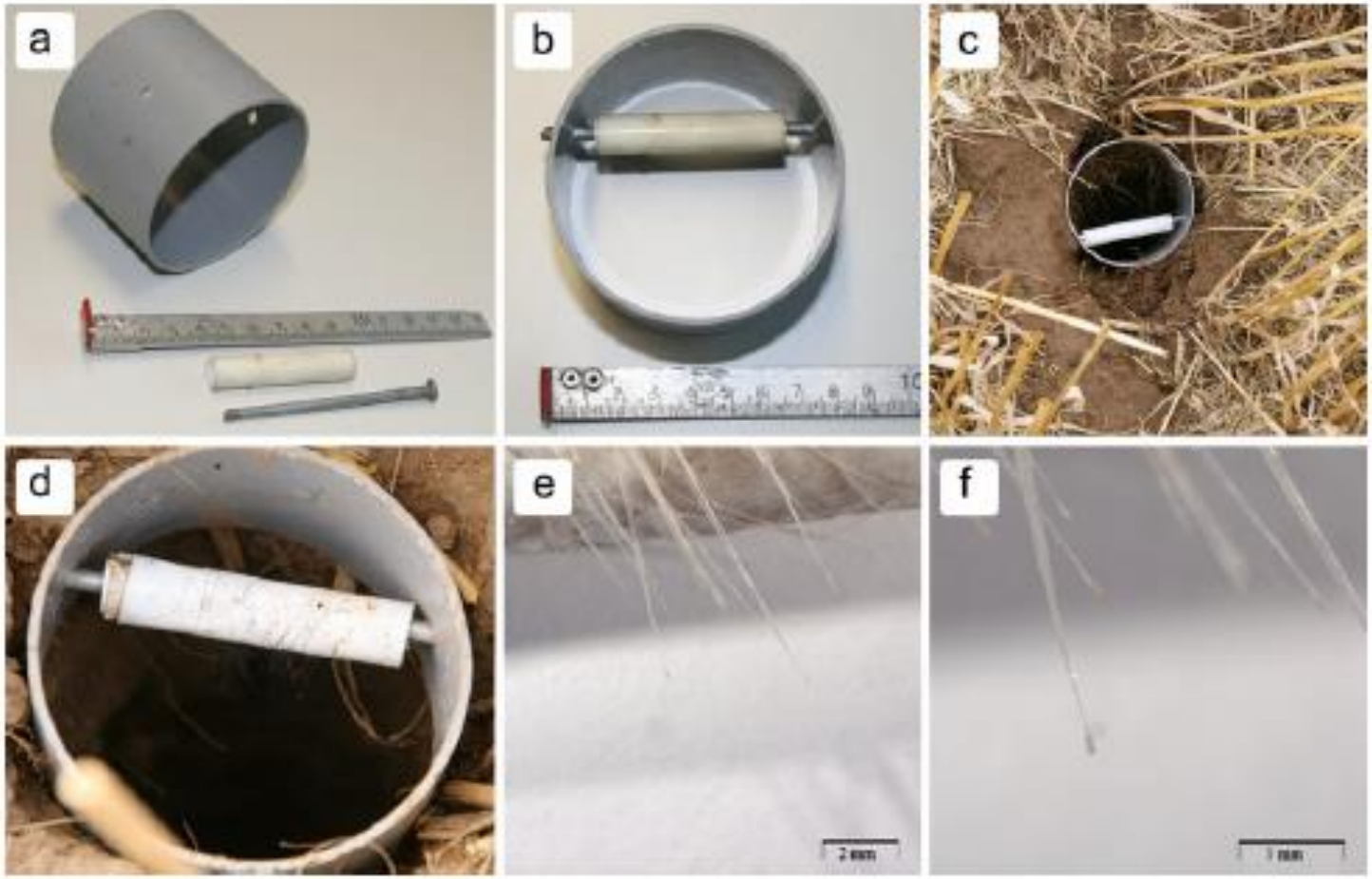


Fig. 1 Materials and application of the optimized hair trap. The trap consists of a PVC pipe with two holes, an iron nail, and a plastic roll (**a**). The plastic roll is wrapped with double-faced adhesive tape and attached inside the pipe by the nail (**b**). Traps are placed in entrances of burrows (**c**), and as an animal passes, hairs are plucked by the rolling tape (**d**). Most of plucked hair contained bulbs with a sufficient amount of follicular tissue (**e** and **f**)

Eur J Wildl Res (2011) 57:991–995
DOI 10.1007/s10344-011-0543-9

TECHNICAL NOTES

An optimized hair trap for non-invasive genetic studies of small cryptic mammals

Tobias Erik Reiners · Jorge A. Encarnação · Volkmar Wolters

eDNA

Environmental DNA or **eDNA** is DNA that is collected from a variety of environmental samples (soil, water, snow, air) rather than directly sampled from an individual organism

cave-dwelling amphibian *Proteus anguinus* inhabits subterranean waters of the north-western Balkan Peninsula. Because only fragments of its habitat are accessible to humans, this endangered salamander's exact distribution has been difficult to establish

SCIENTIFIC REPORTS

OPEN

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Environmental DNA in subterranean biology: range extension and taxonomic implications for *Proteus*

Špela Gorički[1,*], David Stanković[1,2,3,*,†], Aleš Snoj[2], Matjaž Kuntner[4], William R. Jeffery[5], Peter Trontelj[6], Miloš Pavićević[7], Zlatko Grizelj[8], Magdalena Năpăruș-Aljančič[1,9] & Gregor Aljančič[1]

Loch Ness Monster may be a giant eel, say scientists

5 September 2019

eDNAir: proof of concept that animal DNA can be collected from air sampling

Elizabeth L. Clare, Chloe K. Economou, Chris G. Faulkes, James D. Gilbert, Frances Bennett, Rosie Drinkwater and Joanne E. Littlefair

School of Biological and Chemical Sciences, Queen Mary University of London, London, United Kingdom

ABSTRACT

Environmental DNA (eDNA) is one of the fastest developing tools for species biomonitoring and ecological research. However, despite substantial interest from research, commercial and regulatory sectors, it has remained primarily a tool for aquatic systems with a small amount of work in substances such as soil, snow and rain. Here we demonstrate that eDNA can be collected from air and used to identify mammals. Our proof of concept successfully demonstrated that eDNA sampled from air contained mixed templates which reflect the species known to be present within a confined space and that this material can be accessed using existing sampling methods. We anticipate this demonstration will initiate a much larger research programme in terrestrial airDNA sampling and that this may rapidly advance biomonitoring approaches. Lastly, we outline these and potential related applications we expect to benefit from this development.

Subjects Animal Behavior, Biodiversity, Conservation Biology, Ecology, Zoology
Keywords airDNA, eDNA, Biomonitoring, Biodiversity, Terrestrial

More: https://www.the-scientist.com/environmental-dna-can-be-pulled-from-the-air-68645

Clare at al. 2021. eDNAir: proof of concept that animal DNA can be collected from air sampling. PeerJ 9:e11030 https://doi.org/10.7717/peerj.11030

Special challenges:

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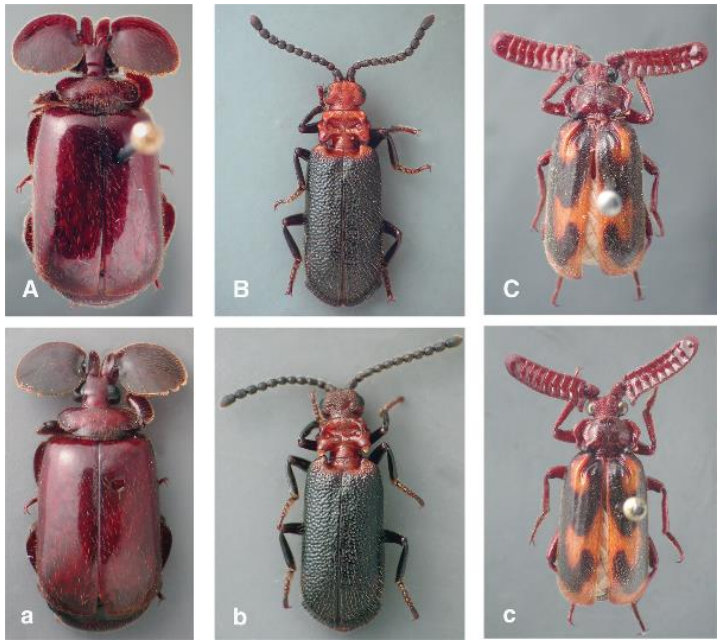
PLoS one

DNA Extraction from Dry Museum Beetles without Conferring External Morphological Damage

M. Thomas P. Gilbert[1,2]*, Wendy Moore[3], Linea Melchior[1,4], Michael Worobey[1]



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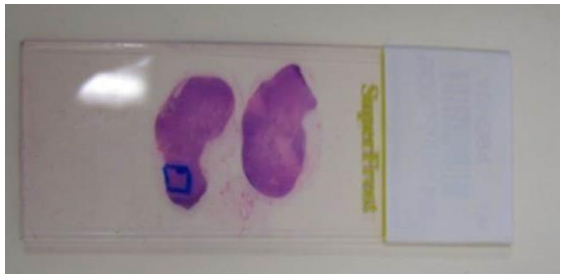
PLoS one

The Isolation of Nucleic Acids from Fixed, Paraffin-Embedded Tissues–Which Methods Are Useful When?

M. Thomas P. Gilbert[1¤]*, Tamara Haselkom[1], Michael Bunce[2], Juan J. Sanchez[3], Sebastian B. Lucas[4], Laurence D. Jewell[5], Eric Van Marck[6], Michael Worobey[1]



Fossils as a source of DNA – ancient DNA

- authentic ancient DNA - up to several hundred thousand years old samples
- bones, artificially or naturally mummified animal remains, remains in permafrost, fossil eggs, paleofaeces
- extremely high risk of contamination- specialized laboratories
- typically short fragments of DNA, but now also whole genomes

  (e.g.: mt genomes of mammoth, moas, whole genome of the Neanderthal)

DNA of extinct birds extracted from ancient eggshell

By Pallab Ghosh
Science correspondent, BBC News

Researchers have found that eggshells of extinct bird species are a rich source of preserved DNA.

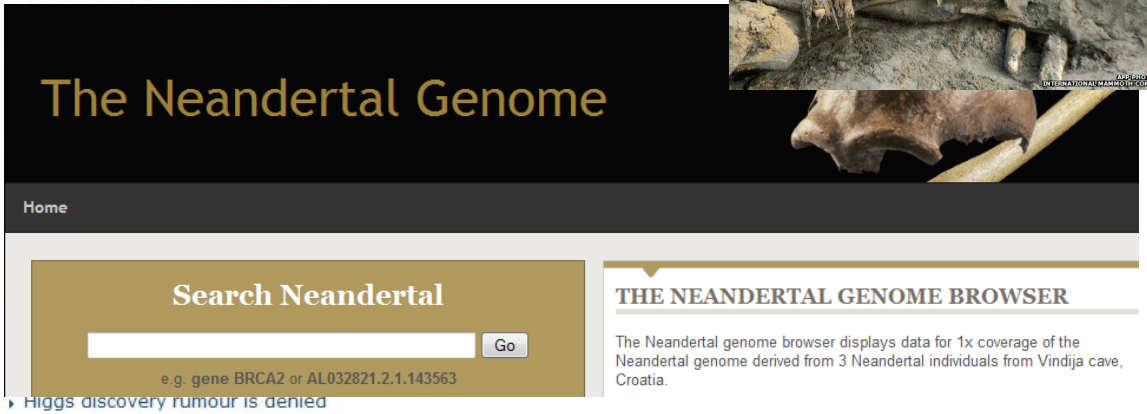
An international team isolated the delicate DNA molecules of species including the massive "elephant birds" of the genus *Aepyorni*.

The Proceedings of the Royal Society B research demonstrated the approach also on emu, ducks and the extinct moa.

The team says that the technique will enable researchers to learn more about ancient birds and why they died out.

Eggs have long been studied but only now is their DNA being isolated

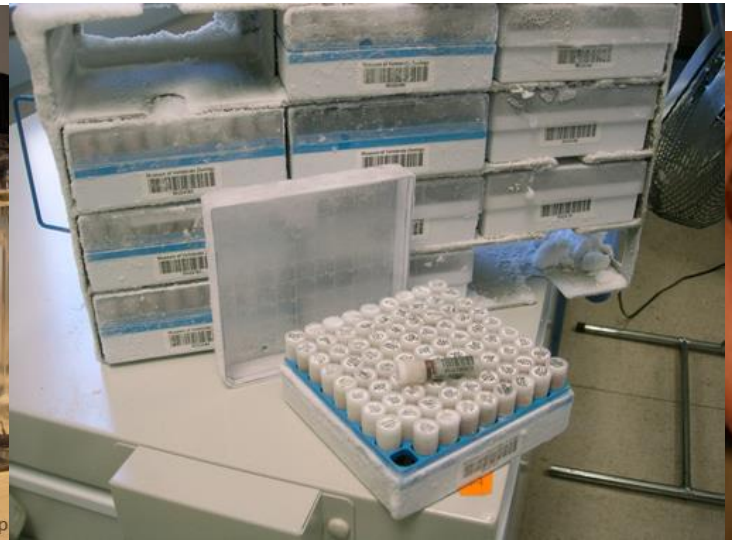
SEE ALSO
- Antique giant bird's egg on sale
  25 Mar 09 | Kent
- Extinct mammoth DNA decoded



- Higgs discovery rumour is denied

Storing samples for DNA extraction:

- frozen (–20°C usually OK, -80°C for long periods)
- dry
- fixed tissue in 96% ethanol (formaldehyde not ideal, but not impossible)
- samples for RNA isolation can be stored in *RNAlater (or* -80°C)



Genetic banks:

GLOBAL REGISTRY OF BIODIVERSITY REPOSITORIES

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Biorepository: Department of Zoology, Charles University in Prague

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Institution Information

DNA extraction

- different methods and technologies are available
- all methods involve **disruption** and **lysis** of the starting material followed by the **removal of proteins** and **other contaminants** and finally **recovery of the DNA**
- removal of proteins - proteinase K
- choice of a method depends on: the required quantity of the DNA, the purity required for downstream applications, and the time and costs

Examples of methods:

- organic extraction – phenol/chloroform
- specialized kits usually using binding of the DNA to a solid-phase support

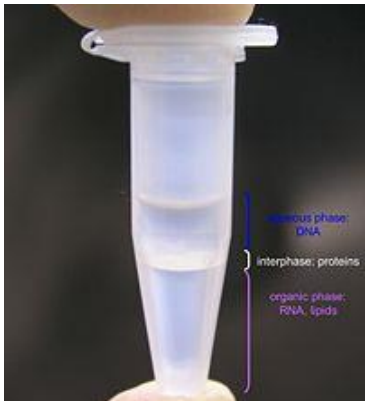
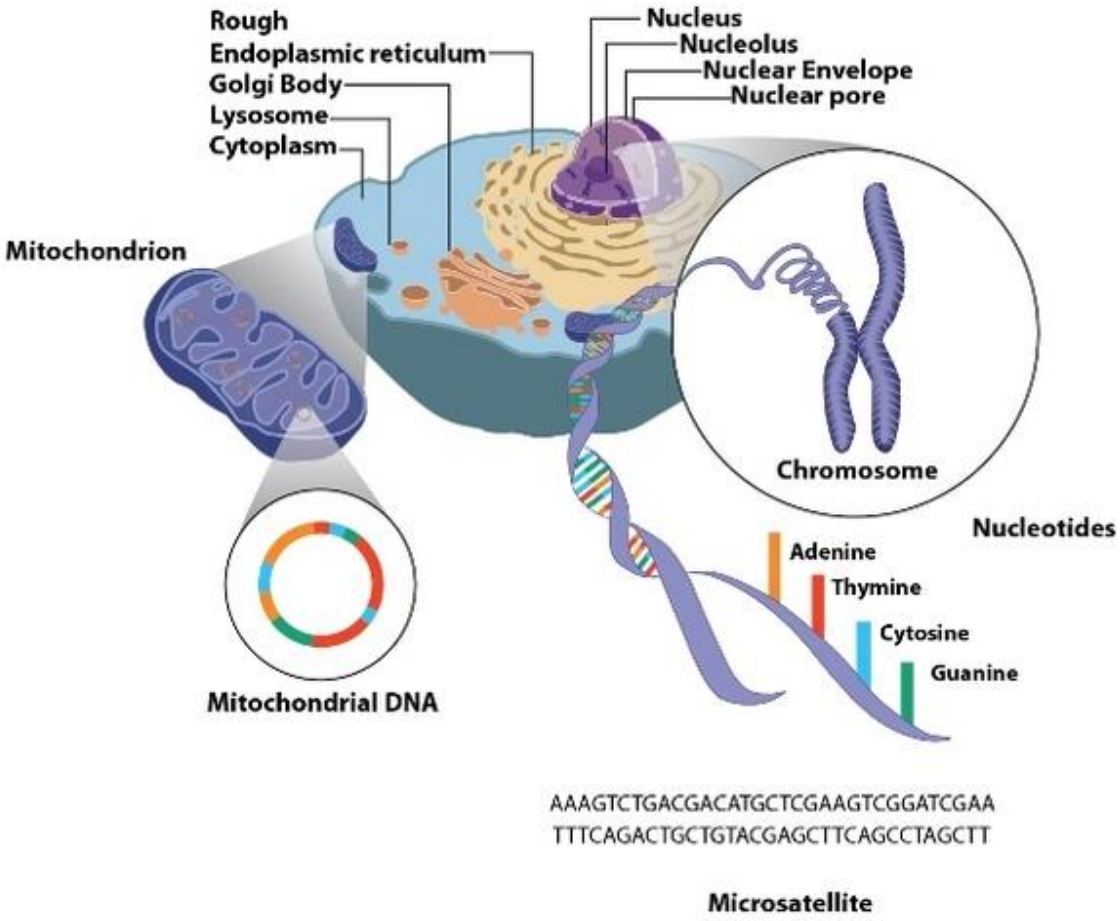


Table 1. Maximum Amounts of Starting Material

Sample	Amount
Animal tissue (see Table 3, page 23)	25 mg (spin-column protocols) 20 mg (DNeasy 96 protocols)
Mammalian blood (see Table 4, page 23)	100 µl
Bird or fish blood (with nucleated erythrocytes)	10 µl
Mouse tail	0.6–1.2 cm
Rat tail	0.6 cm
Cultured cells	5×10^6
Bacteria	2×10^9

Different genes or genomes = different questions



Location of **mtDNA and nuclear DNA in an animal cell**. The mitochondria of an animal cell harbor maternally transmitted mtDNA. Nuclear DNA, which is transmitted by both parents, is found in the nucleus, and contains repeated elements termed microsatellites.

nuclear DNA

-biparental inheritance in diploid sexually reproducing organisms

-nuclear coding genes- lower mutation rate (in mammals mtDNA ca 10x higher mutation rate)

-microsatellite loci - mutate much more rapidly than most other types of sequences

-sex chromosomes - in mammals, the Y chromosome is the paternally inherited - most of it does not undergo recombination - useful marker for retracing male lineages

Different genes or genomes = different questions

mitochondrial DNA

-uniparentally inherited- maternal (but exceptions exist)

-mtgenomes relatively small- between 15 000 and 17 000 bp

-structure, size and arrangement of genes are relatively conserved

- no recombination = offspring will usually have exactly the same mitochondrial genome as its mother

- the mutation rate is relatively high

- non-coding control region (incl. D-loop), evolves rapidly in many taxa

-small effective population size – sensitive to demographic events such as bottlenecks

-available universal primers

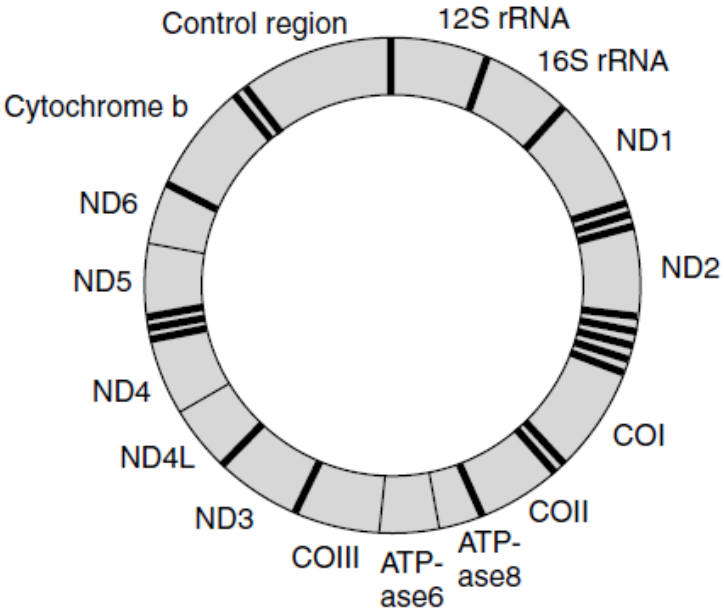
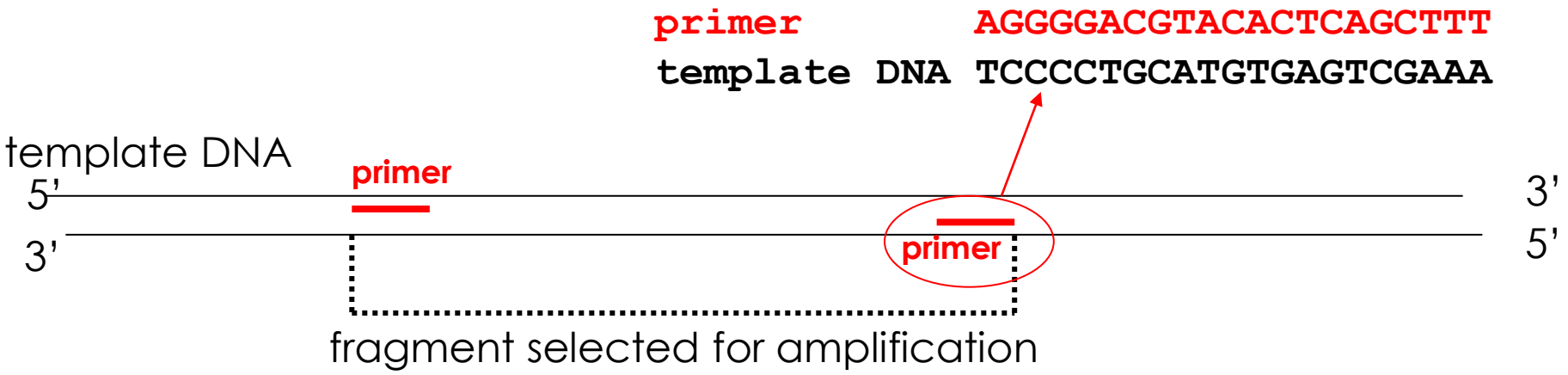


Figure 2.1 Typical gene organization of vertebrate mtDNA. Unlabelled dark bands represent 22 transfer RNAs (tRNAs). Gene abbreviations starting with ND are subunits of NADH dehydrogenase and those starting with CO are subunits of cytochrome *c*.

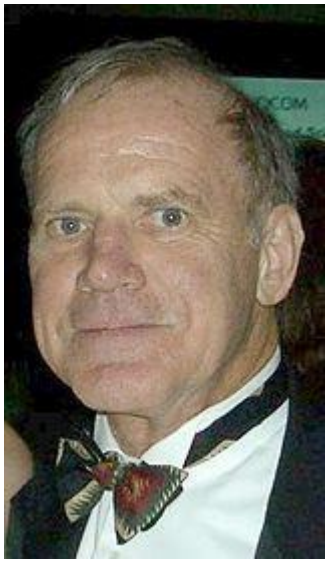
Molecular Ecology, 2nd Edition

Polymerase Chain Reaction = PCR

- method for rapid amplification of DNA sequences – selected fragment of DNA
- amplified fragment of DNA is defined by pair of primers
- Primer - short DNA fragments containing sequences complementary to the target region



Polymerase Chain Reaction = PCR



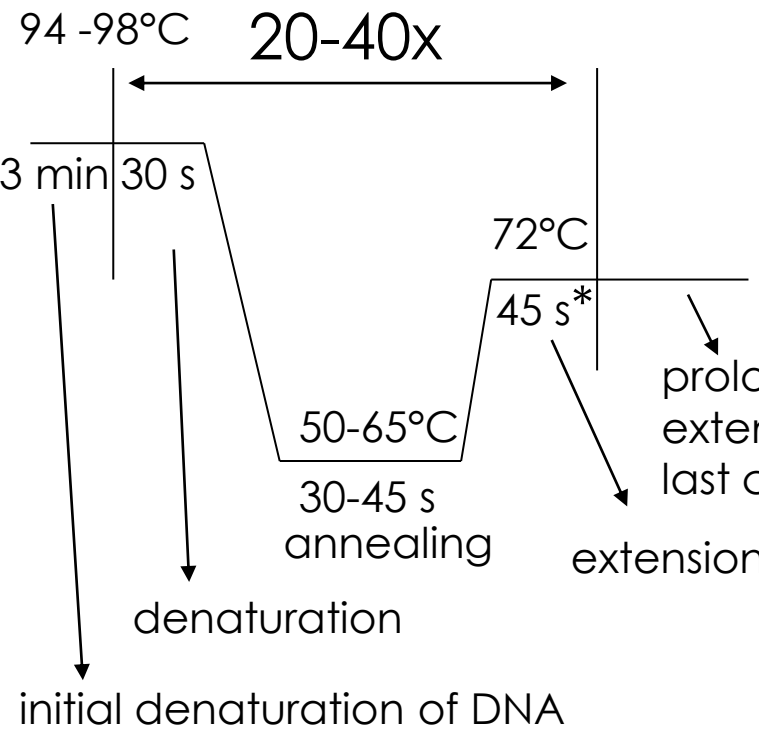
- developed in mid 80s by Kery Mullis

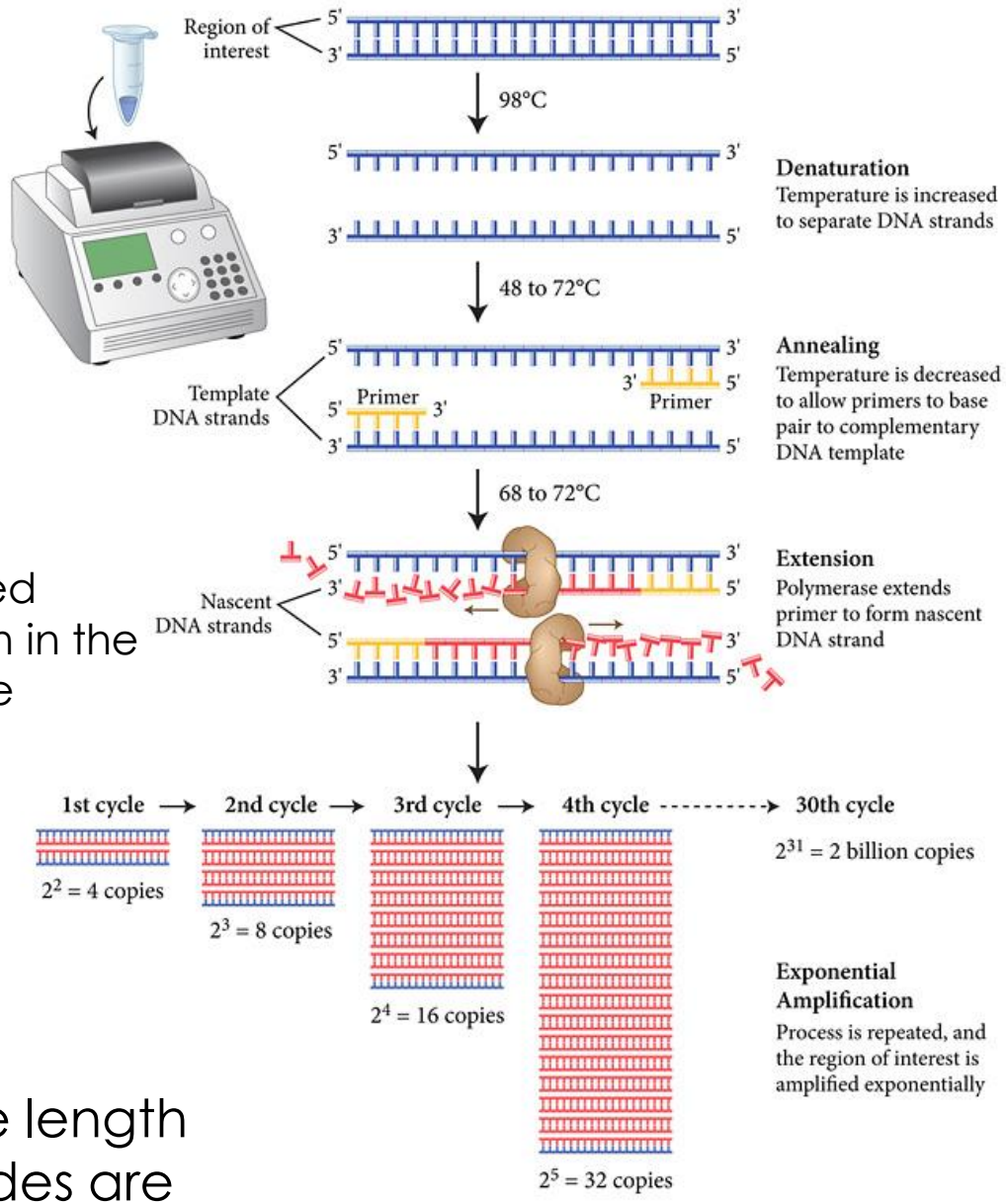
- 1993 – Kery Mullis and Michael Smith awarded the Nobel Prize in Chemistry for their work on PCR

PCR uses repeated cycles of heating and cooling to make many copies of a specific region of DNA

3 steps

1. temperature is raised to near boiling - causing the double-stranded DNA to separate (denature) into single strands
2. temperature is decreased and short DNA sequences (primers) bind (anneal) to complementary matches on the target DNA sequence
3. at a slightly higher temperature the enzyme Taq polymerase binds to the primed sequences and adds nucleotides to extend the second strand





* Extension time depends on the length of amplified fragment- nucleotides are incorporated in the rate cca 35 nucleotides per second

source: New England Biolabs

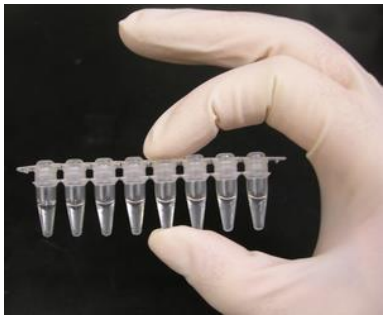
Polymerase Chain Reaction = PCR

PCR requires:

- two specific oligonucleotide primers
- thermostable DNA polymerase - *Taq* polymerase
- dNTP's
- template DNA
- $MgCl_2$
- buffer (supplied with the polymerase)
- water

PCR is performed in the thermal cycler (also known as a thermocycler or PCR machine)

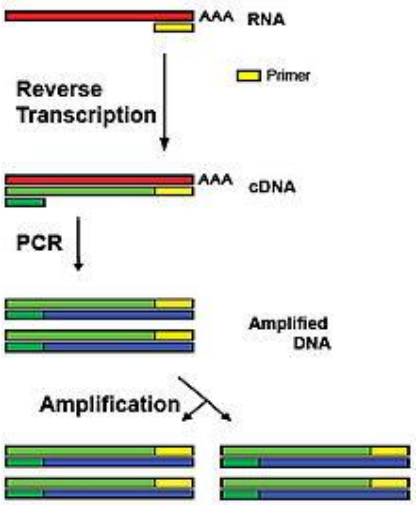
PCR usually prepared in small volumes – 20-100 µl



PCR primers

- short oligonucleotides 17-30bp long
- sequences complementary to the target region
- no repetitive sequences
- no sequence complementarity within or between primers – secondary structures, primer dimer
- annealing temperature dependent upon primer sequence (~ 50% GC content)
- software for primer design (web tools)

RT-PCR (reverse transcription PCR) and Real-Time PCR

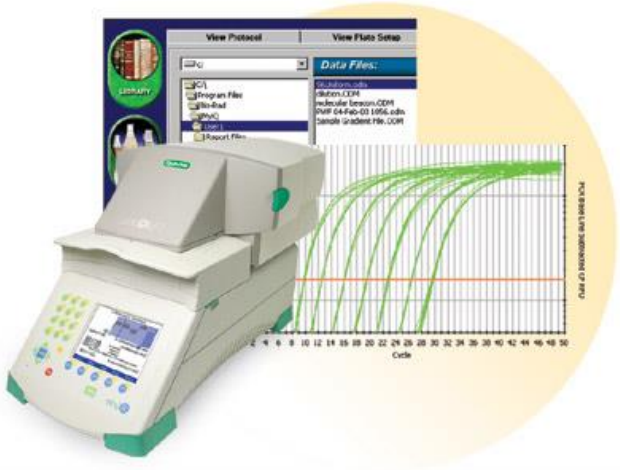


RT-PCR – method to detect RNA expression

- modification of PCR, RNA is template
- qualitatively detect gene expression through creation of complementary DNA (cDNA) transcripts from RNA

Real-Time PCR (also qPCR)

- quantitatively measure the amplification of DNA using fluorescent probes
- specially modified cycler



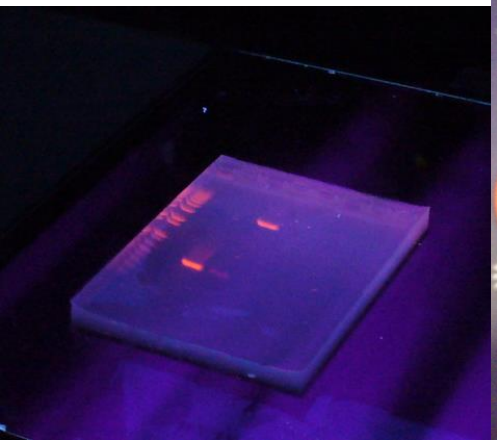
Gel electrophoresis

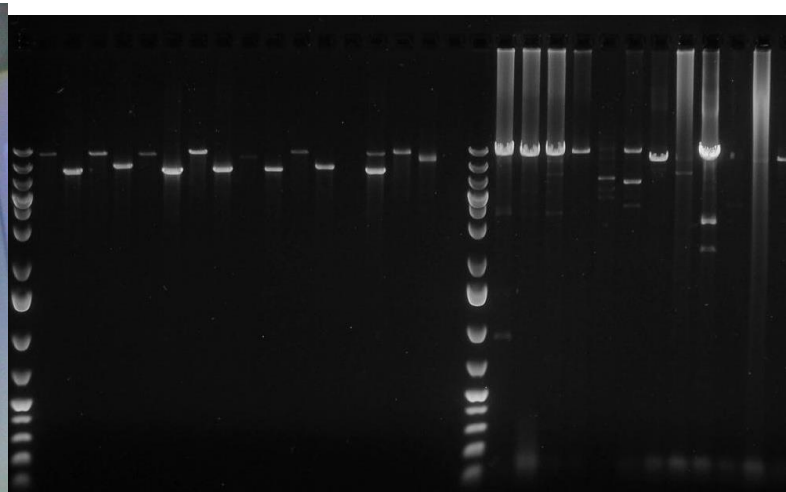
method for separation and analysis of macromolecules (DNA, RNA, proteins) based on their size and charge

electrophoresis = transport by electricity

-**DNA** is negatively charged – fragments of different length are separated by applying an electric field - they move through a gel (usually agarose gel, for more precise separation polyacrylamide gel)

-shorter molecules move faster and migrate farther than longer ones





Gel electrophoresis

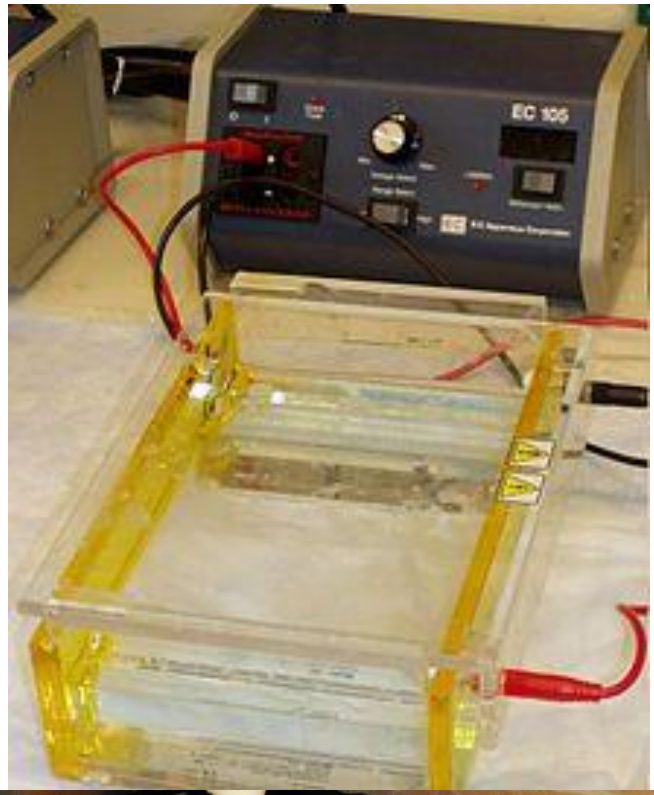
- electric field consists of a negative charge at one end which pushes the molecules through the gel, and a positive charge at the other end that pulls the DNA

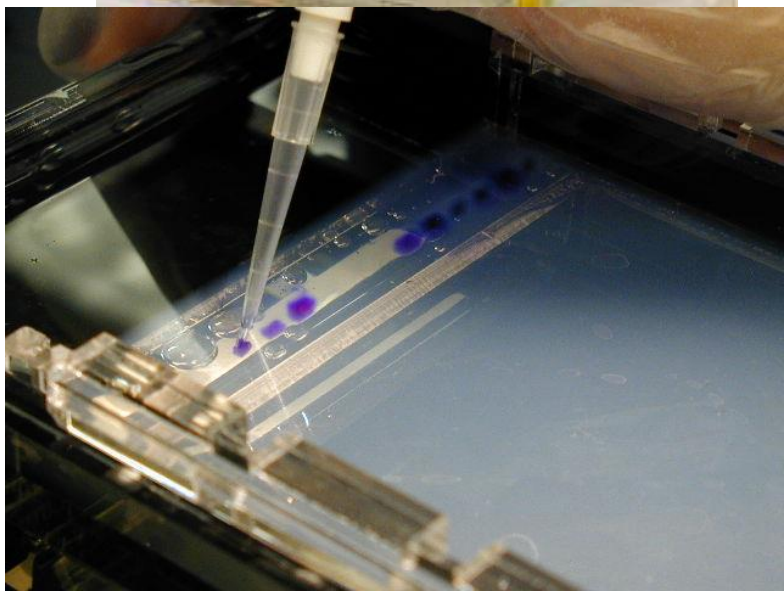
- molecules being sorted are dispensed into a well in the gel material

- gel is placed in an electrophoresis chamber, which is then connected to a power source

- when the electric current is applied fragments molecules move

-the different sized molecules form distinct bands on the gel





Gel electrophoresis

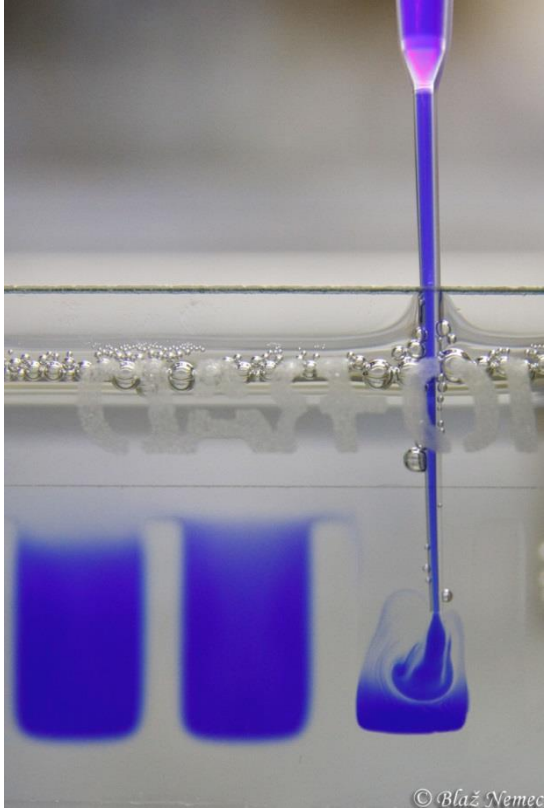
- electrophoresis chamber is filled with buffers
  - provide ions that carry a current and maintain stable pH

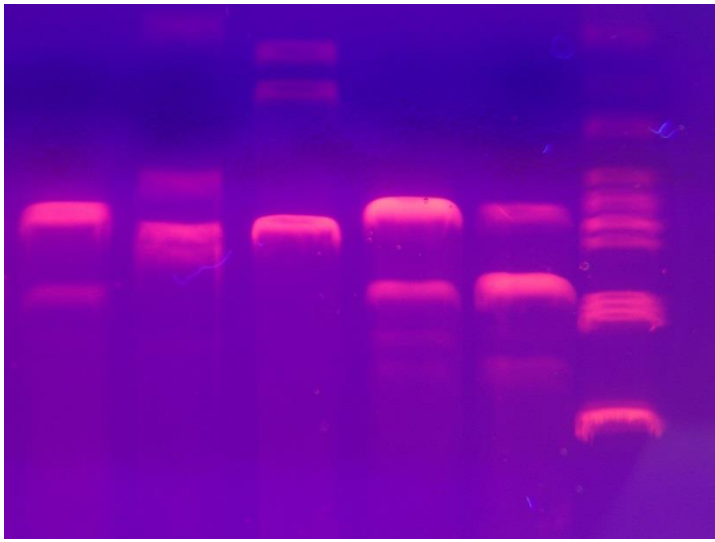
- most common buffers for nucleic acids Tris/Acetate/EDTA (TAE) or Tris/Borate/EDTA (TBE) buffers

- samples are mixed with dye for monitoring

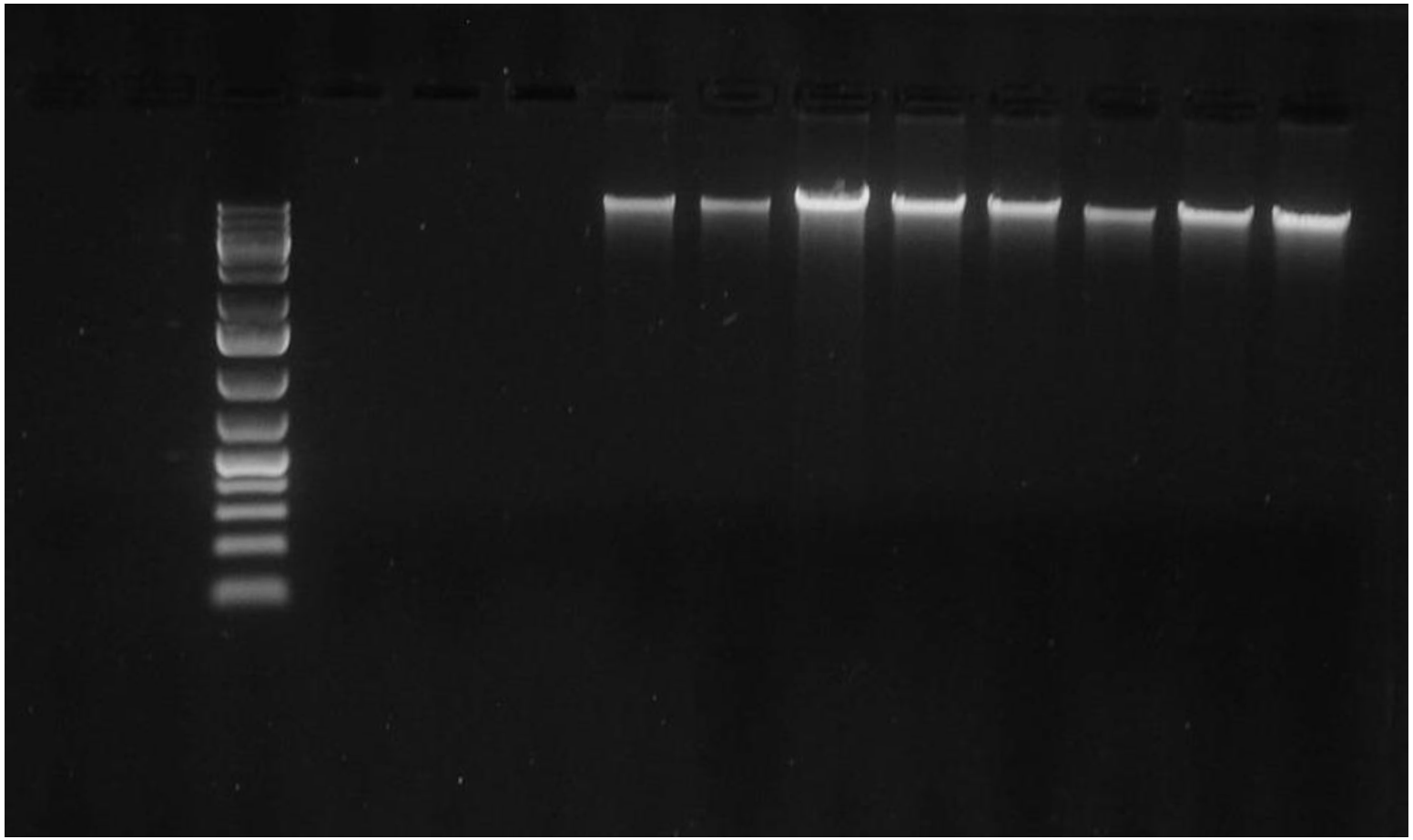
-to visualize DNA fragments - ethidium bromide bind to the DNA - visible under UV (SYBR Green also used)

-size standard (ladder) for estimating size of fragments



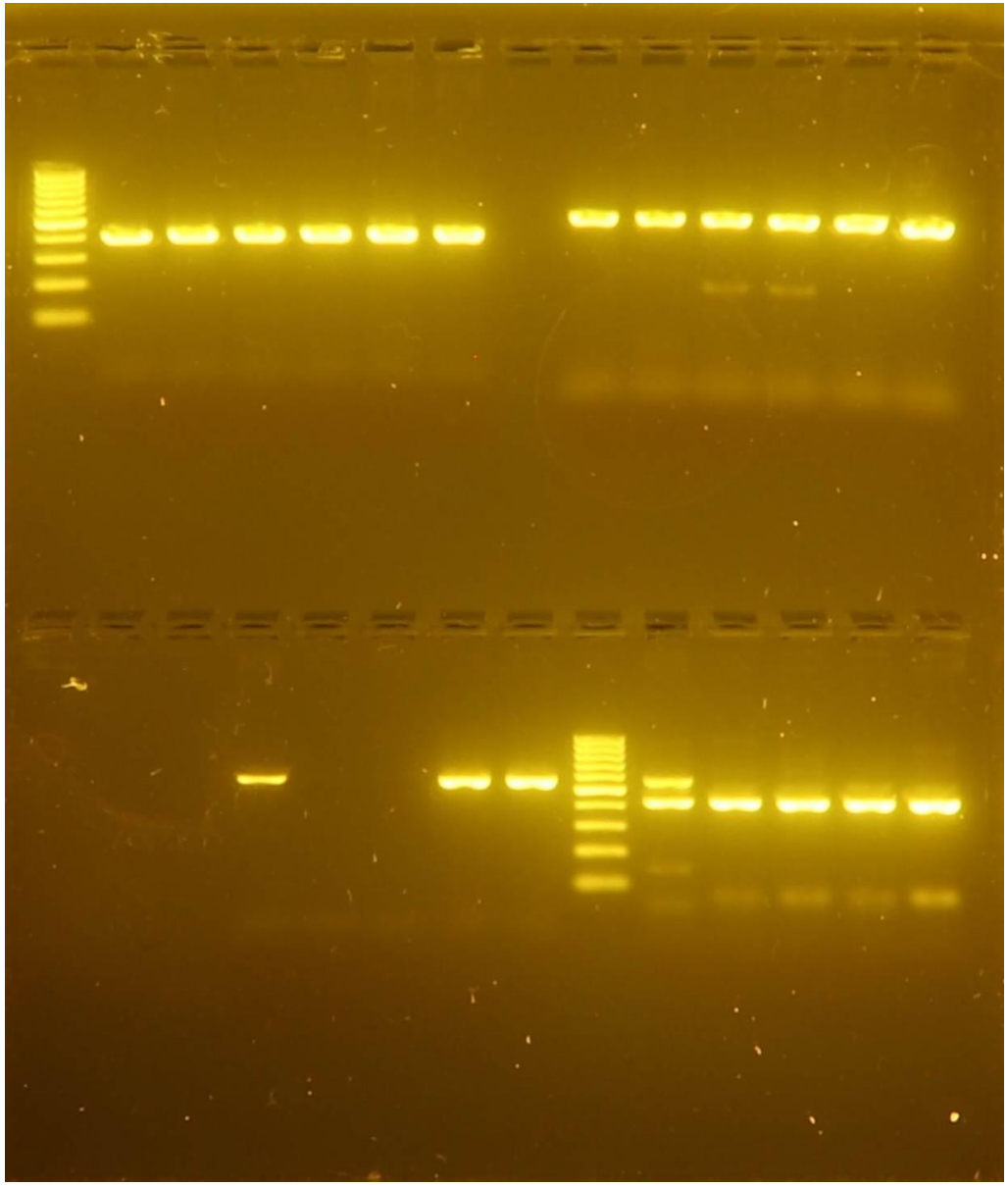


ELFO of total genomic DNA

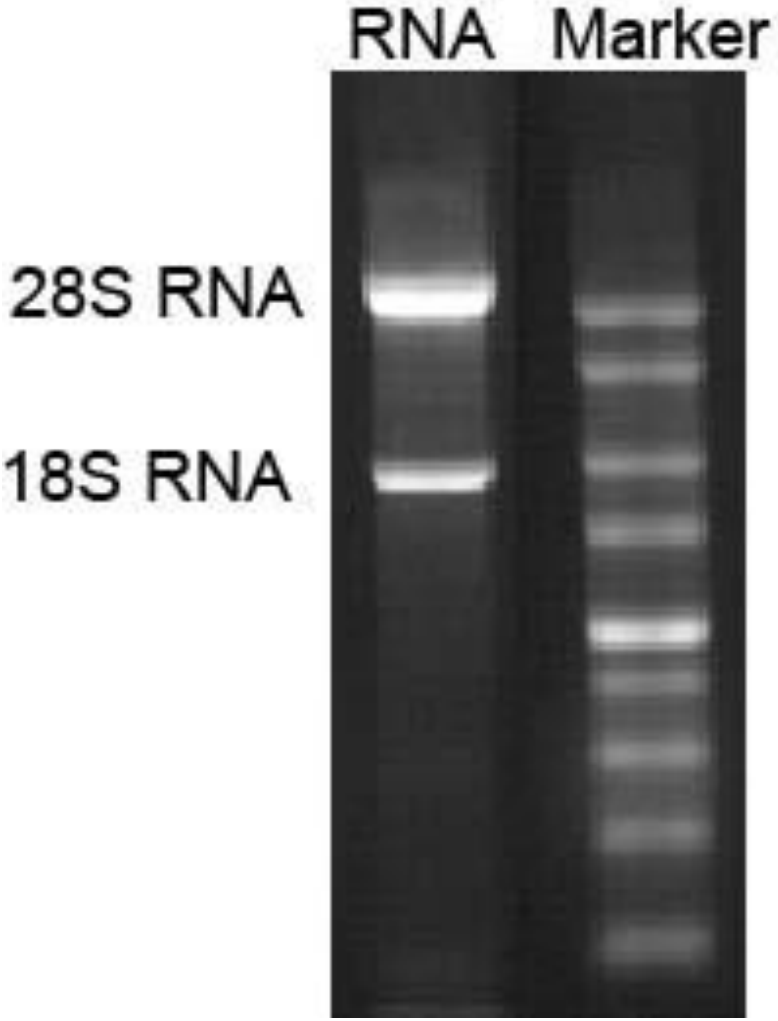


ELFO after PCR

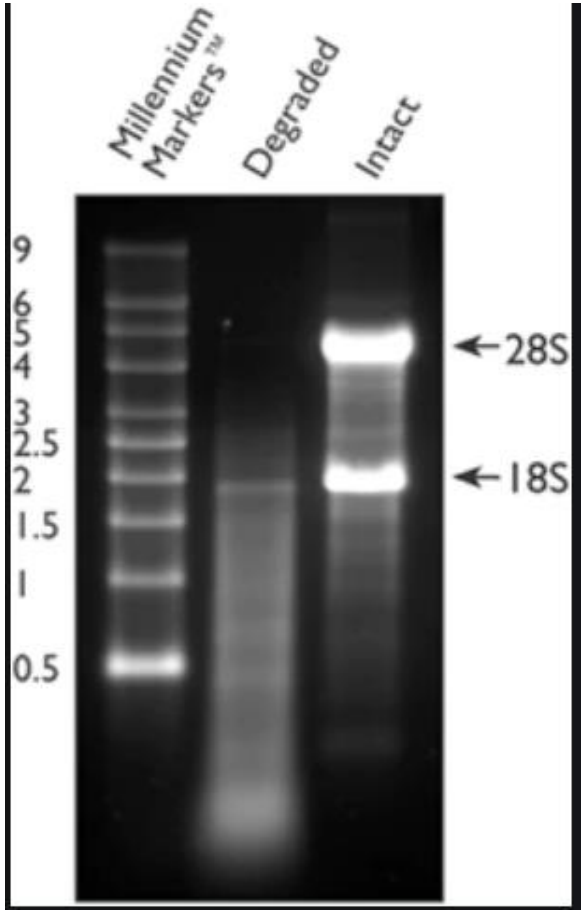
Size standard (ladder) →



RNA gel electrophoresis



www. labguide.cz

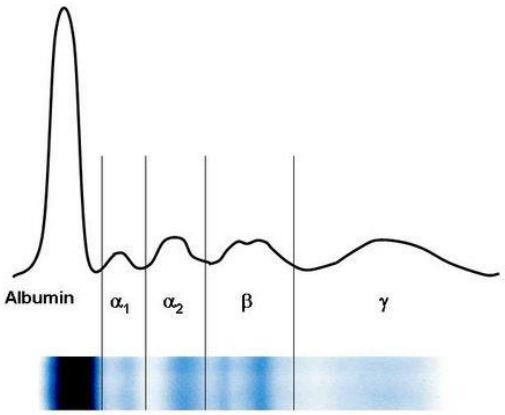


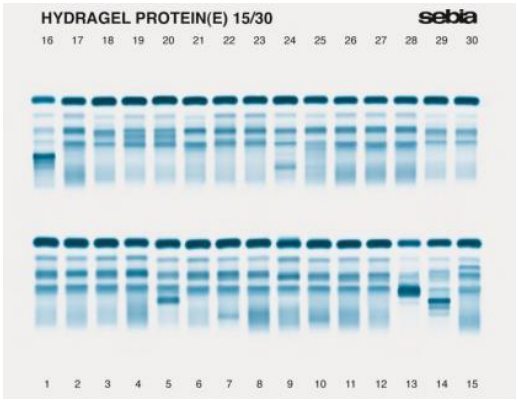
www.thermofisher.com

Protein electrophoresis:

at pH 8-9 proteins- negatively charged = they migrate through the gel while exposed to an electric current (proteins with a negative charge will migrate towards the positively charged anode)

common use: medicine – e.g. electrophoresis of blood serum – information about type of inflammation – acute/chronic, disease diagnostics …
In case of blood serum – separation into five major fractions by size and electrical charge: serum albumin, alpha-1 globulins, alpha-2 globulins, beta 1 and 2 globulins, and gamma globulins





Characteristic Patterns of Acute-Reaction Proteins Found on Serum Protein Electrophoresis and Associated Conditions or Disorders

Increased albumin
Dehydration

Decreased albumin
Chronic cachectic or wasting diseases
Chronic infections
Hemorrhage, burns, or protein-losing enteropathies
Impaired liver function resulting from decreased synthesis of albumin
Malnutrition
Nephrotic syndrome
Pregnancy

Increased alpha$_1$ globulins
Pregnancy

Decreased alpha$_1$ globulins
Alpha$_1$-antitrypsin deficiency

Increased alpha$_2$ globulins
Adrenal insufficiency
Adrenocorticosteroid therapy
Advanced diabetes mellitus
Nephrotic syndrome

Decreased alpha$_2$ globulins
Malnutrition
Megaloblastic anemia
Protein-losing enteropathies
Severe liver disease
Wilson's disease

Increased beta$_1$ or beta$_2$ globulins
Biliary cirrhosis
Carcinoma (sometimes)
Cushing's disease
Diabetes mellitus (some cases)
Hypothyroidism
Iron deficiency anemia
Malignant hypertension
Nephrosis
Polyarteritis nodosa
Obstructive jaundice
Third-trimester pregnancy

Decreased beta$_1$ or beta$_2$ globulins
Protein malnutrition

Increased gamma globulins
Amyloidosis
Chronic infections (granulomatous diseases)
Chronic lymphocytic leukemia
Cirrhosis
Hodgkin's disease
Malignant lymphoma
Multiple myeloma
Rheumatoid and collagen diseases (connective tissue disorders)
Waldenström's macroglobulinemia

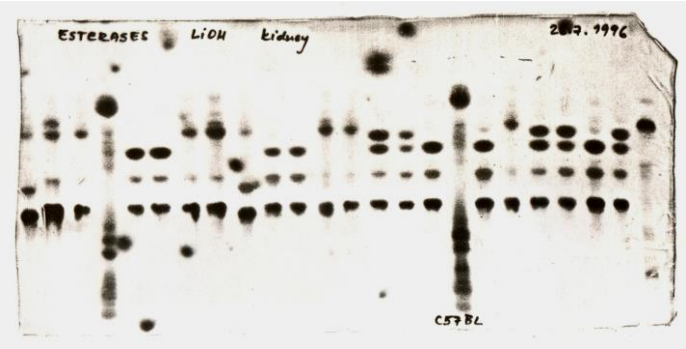
Decreased gamma globulins
Agammaglobulinemia
Hypogammaglobulinemia

Protein electrophoresis and zoology:

method: variation in the electrophoretic mobilities of proteins can be used as a measure of nucleotide sequence variation in nuclear DNA

- genetic variability within population, cryptic species, phylogeny reconstruction

samples: tissues, blood, saliva, urine



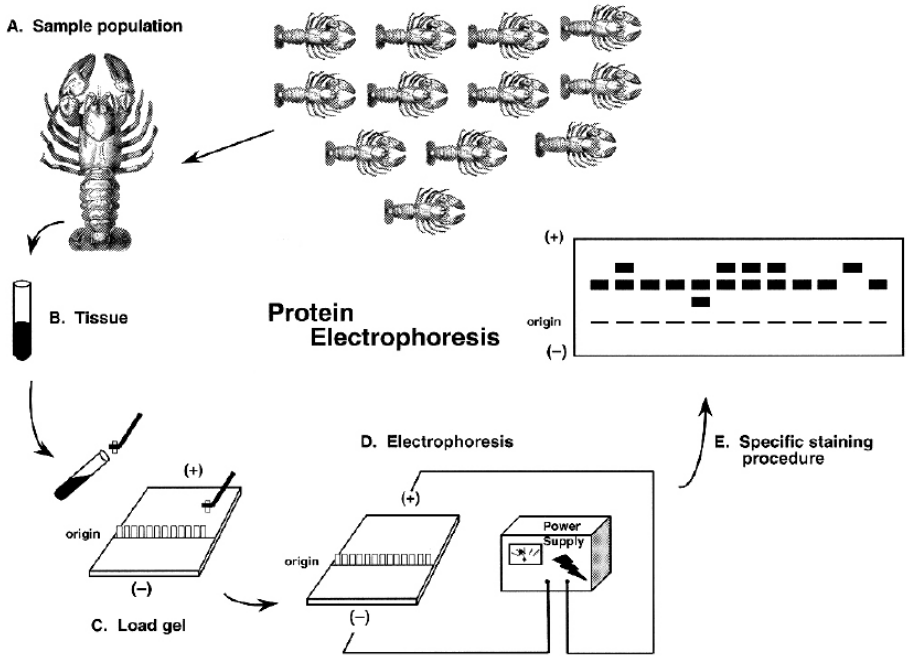


Fig. 8.1 Graphic depicting the steps involved in the protein electrophoresis method. A Drawing samples from a natural population. B Dissecting a tissue sample from an individual to make a tissue extract. C Loading the extract onto a starch gel using filter paper wicks. D Running a cur[r]ent through the gel to separate the proteins by charge and size. E Adding a specific staining recipe to the sliced gel to visualise specific protein products (see also Plate 29).

Examples of the use of PCR + ELFO methods:

- DNA sequencing – Sanger sequencing – next lecture

- molecular sex determination – more on March 19

- SSCP

PROTOCOL

Single-strand conformation polymorphism (SSCP) for the analysis of genetic variation

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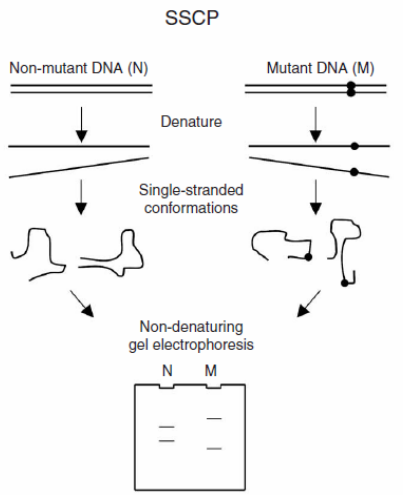


Figure 1 | The principle of PCR-based SSCP analysis. A point mutation (represented by a dot on a DNA strand) leads to the formation of different single-strand conformations of the mutant DNA (M) compared with the non-mutant molecule (N), resulting in differential mobilities in a non-denaturing gel matrix.

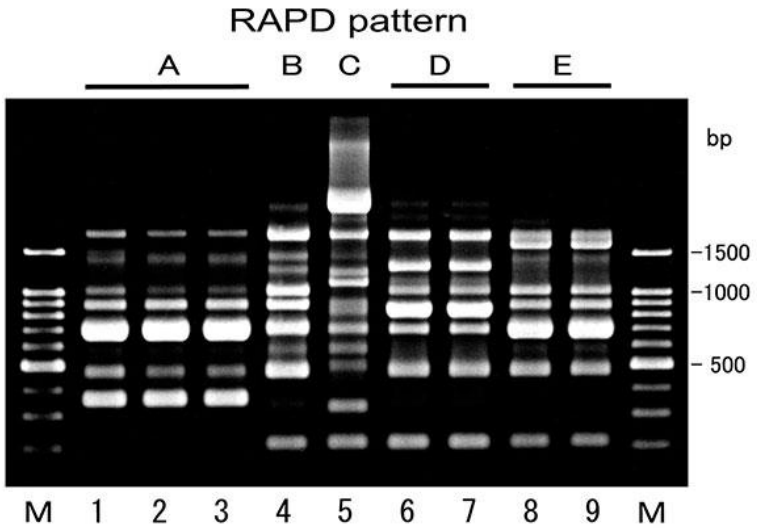
- after denaturation, single-stranded DNA undergoes 3-dimensional folding = unique conformational state based on its DNA sequence
- difference in " shape" between two single-stranded DNA strands with different sequences can cause them to migrate differently through an electrophoresis gel (number of nucleotides still the same)
use: intraspecific studies: e.g. genotyping to detect homozygous/heterozygous individuals; virology- virus variants

RAPD = randomly amplified polymorphic DNA

- does not require any specific knowledge of the DNA sequence of the target organism
- markers are DNA fragments from PCR amplification of random segments of genomic DNA with **single primer** of **arbitrary nucleotide sequence** (primers 8-12 nucleotides)
- PCR reaction with one primer, if primers bind to the DNA in reasonable proximity – fragment is amplified, primers bind to the genomic DNA on many positions = fragments of different length – **species specific pattern**

x

- method sensitive to lab conditions, problems with reproducibility



Use: RAPD is an inexpensive yet powerful typing method for many bacterial species.

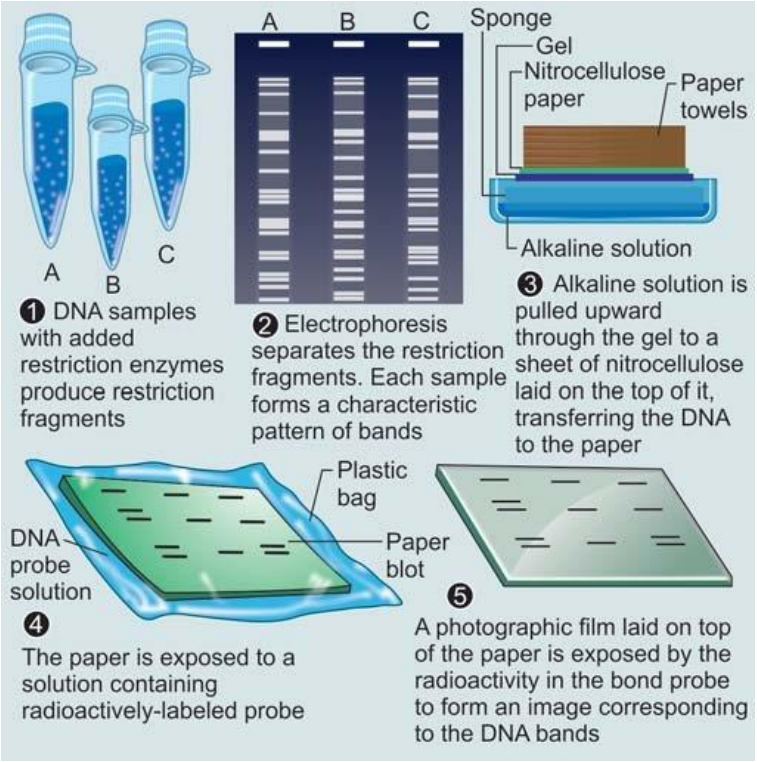
Figure. Random amplified polymorphic DNA (RAPD) patterns of CTX-M-2 β-lactamase-producing *Escherichia coli* isolated from cattle. Lanes M, 100-bp DNA ladder; lanes 1–9, strains GS528, GS542, GS547, GS553, GS554, GS721, GS733, GS631, and GS671, respectively. Five RAPD patterns, A to E, were produced with RAPD analysis primer 4 (Amersham Pharmacia Biotech, Piscataway, NJ). Shiraki et al. 2004

RFLP – Restriction Fragment Length Polymorphism

restriction enzyme sites - specific sequences of nucleotides (4-8 base pairs in length), which are recognized by restriction enzymes

Method: DNA sample is digested into fragments by one or more restriction enzymes, and the resulting restriction fragments are then separated by gel electrophoresis according to their size, after that transferred to a membrane via the Southern blot procedure. Hybridization of the membrane to a labeled DNA probe then determines the length of the fragments which are complementary to the probe

Use: formerly a tool in genome mapping and genetic disease analysis, basis for early methods of genetic fingerprinting - useful in the identification of samples retrieved from crime scenes, in the determination of paternity, and in the characterization of genetic diversity …



AFLP = amplified fragment length polymorphism

- PCR-based technique that uses selective amplification of a subset of digested DNA fragments to generate and compare unique fingerprints for genomes of interest
- does not require prior information regarding the targeted genome, high reproducibility and sensitivity for detecting polymorphism at the level of DNA sequence

Use: to assess genetic diversity within species or among closely related species, to infer population-level phylogenies and biogeographic patterns, to generate genetic maps
- template can be also cDNA – then useful for studying differences in gene expression and searching for target genes