LECTURE I

Biochemical Methods of Characterization in Cellular (Patho)Physiology

Διατμηματικό Πρόγραμμα
Μεταπτυχιακών Σπουδών
στη Βιοανόργανη
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LABORATORY OF INORGANIC CHEMISTRY
AND ADVANCED MATERIALS
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Outline of the presentation

- **Electrophoresis**
- **Electrophoretic Mobility Shift Assay (EMSA)**
- **Polymerase Chain Reaction (PCR)**
- **Western Blot Analysis**
- **Confocal Microscopy**
- **Applications**

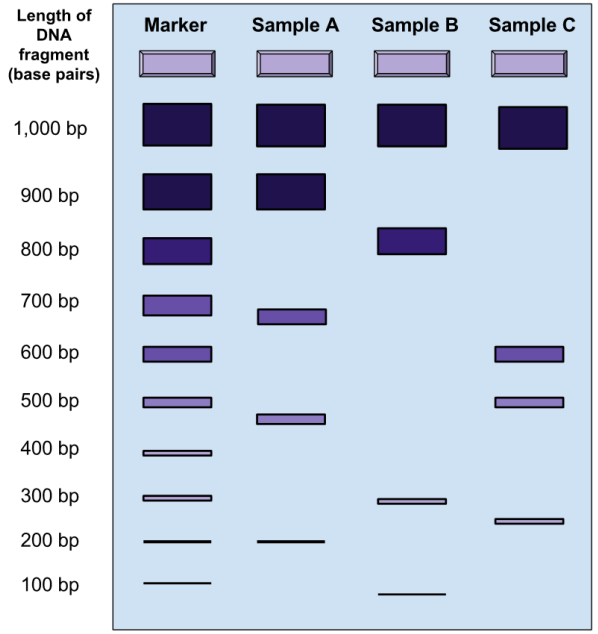
Electrophoresis

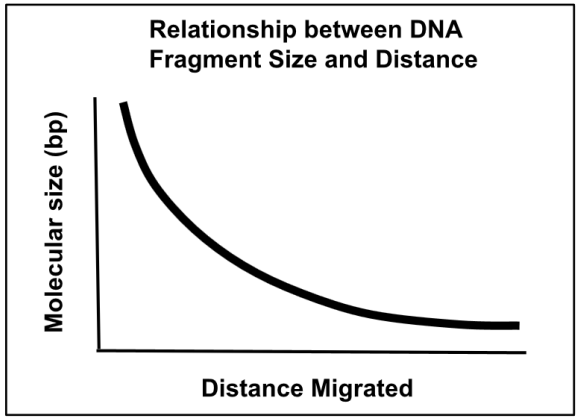
- Gel electrophoresis is a method through which molecules can be separated and analyzed.
- Such molecules include DNA, RNA, and proteins, as well as fragments of molecules thereof.
- The molecules are separated according to
    a) size and
    b) electric charge.
- The molecules can subsequently be compared to other molecules based on size and charge. which is especially useful if the size and charge of specific molecules

Electrophoresis

Gel electrophoresis got its name from the fact that the

a) substrate on which the method is developed is a gel
b) Electricity is used to mobilize charge molecules through the gel and thus separate them under the influence of a field





Electrophoresis

Charge

Gel electrophoresis works by taking advantage of the electric charge of molecules under investigation. Molecules have an electric charge. As a result, they move under the influence of an electric field.

- Separation of positively charged molecules is called **cataphoresis**
- Separation of negatively charged molecules is called **anaphoresis**

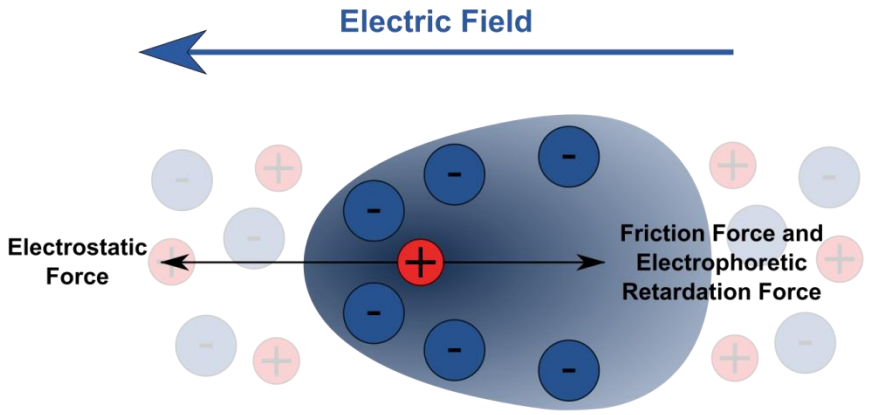
Size

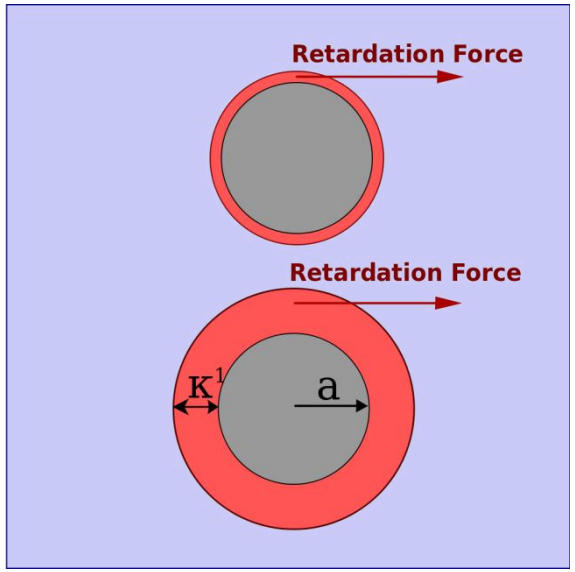
The gel (e.g. agarose) is a matrix. It can be thought of as a sieve, containing small pores. As a result, smaller molecules move through the gel faster than larger molecules. This means that smaller charged molecules move further toward the positive electrode than larger molecules. It is because of this property that molecules can be compared to each other through gel electrophoresis.

Electrophoresis -Applications

Gel electrophoresis has a variety of applications.

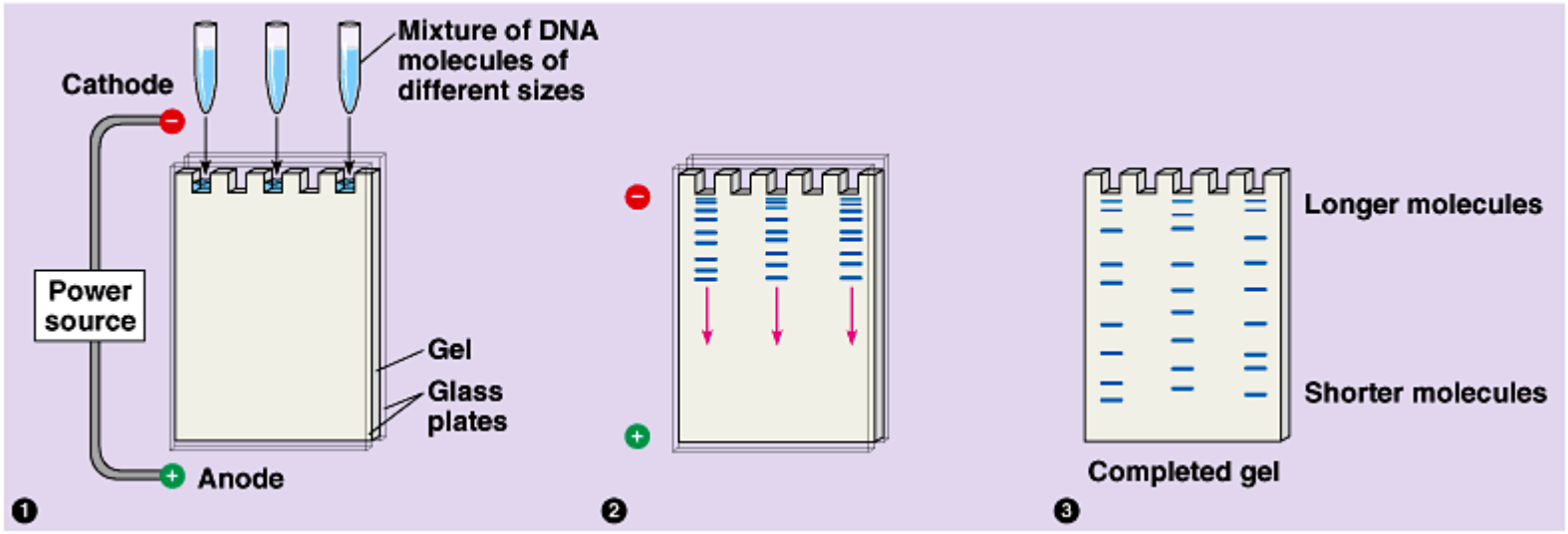
- **Forensics.** It can be used in forensic testing, to see if the DNA from a sample found at a crime scene matches the DNA of a suspect.
- **Paternity.** It can also be used in paternity testing, by comparing the DNA of both the child and the potential father. If the potential father is the real father they will share much of the same DNA.
- **Disease.** It can be used for diagnosis of disease (or prognosis) based on biomarker proteins or genes known to undergo structural modification as a result of oxidative stress or mutations.
- T**ampering** of food products with additive or foreign substances (protein identification)
- **Study** of binding interactions of proteins with DNA (Foot-printing)
- **General research.** Identification of new species and comparison with known molecules at the protein or DNA level. A good example of Genealogy in the hierarchy of species (from bacteria to eukaryotes).

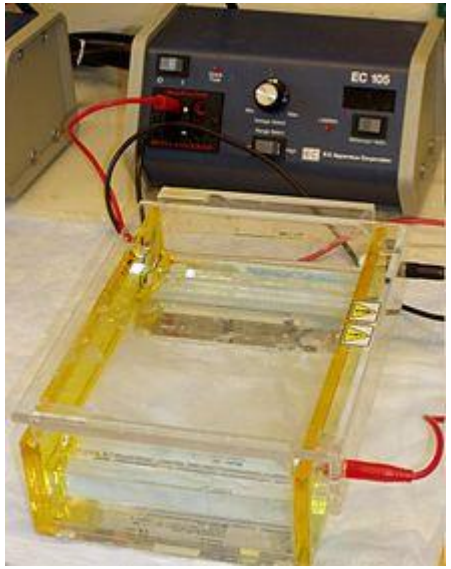


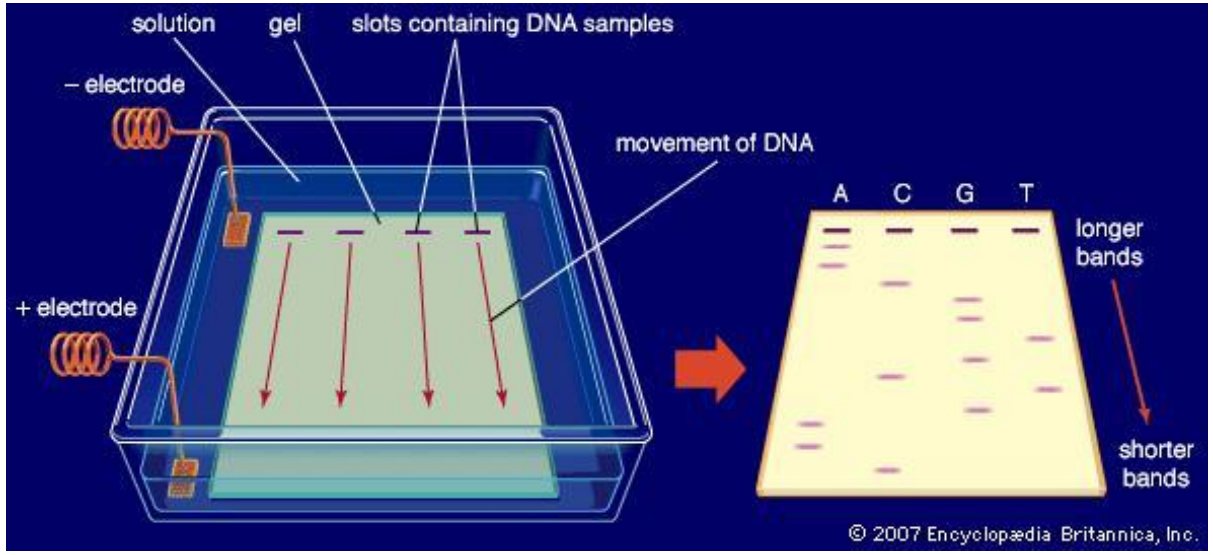


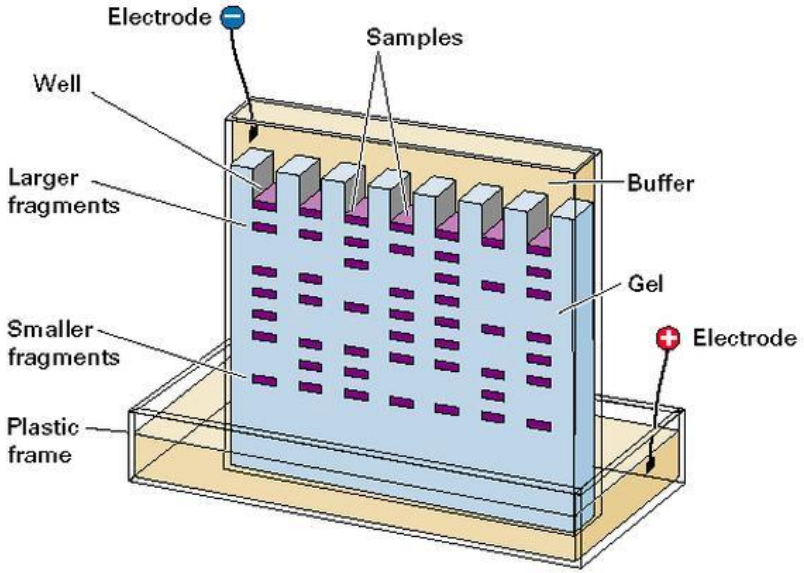
Electrophoresis rides on the fact that:
The electric surface charge of molecules is influenced by an external electric field through electrostatic Coulomb forces. According to the double layer theory, surface charges are screened through a diffuse layer bearing an absolute charge equal to but of opposite sign to the surface charge. Therefore, there are two forces exerted on the particles, one of opposite direction to the other. The second force is exerted on the diffuse layer at same distance from the particle surface and partly transferred to the surface through viscous stress (electrophoretic retardation force).
Under steady state conditions, **the drift velocity v** is proportional to the electric field. Therefore, the **electrophoretic mobility μ** is defined as $\mu = v/E$.

SDS-PAGE electrophoresis

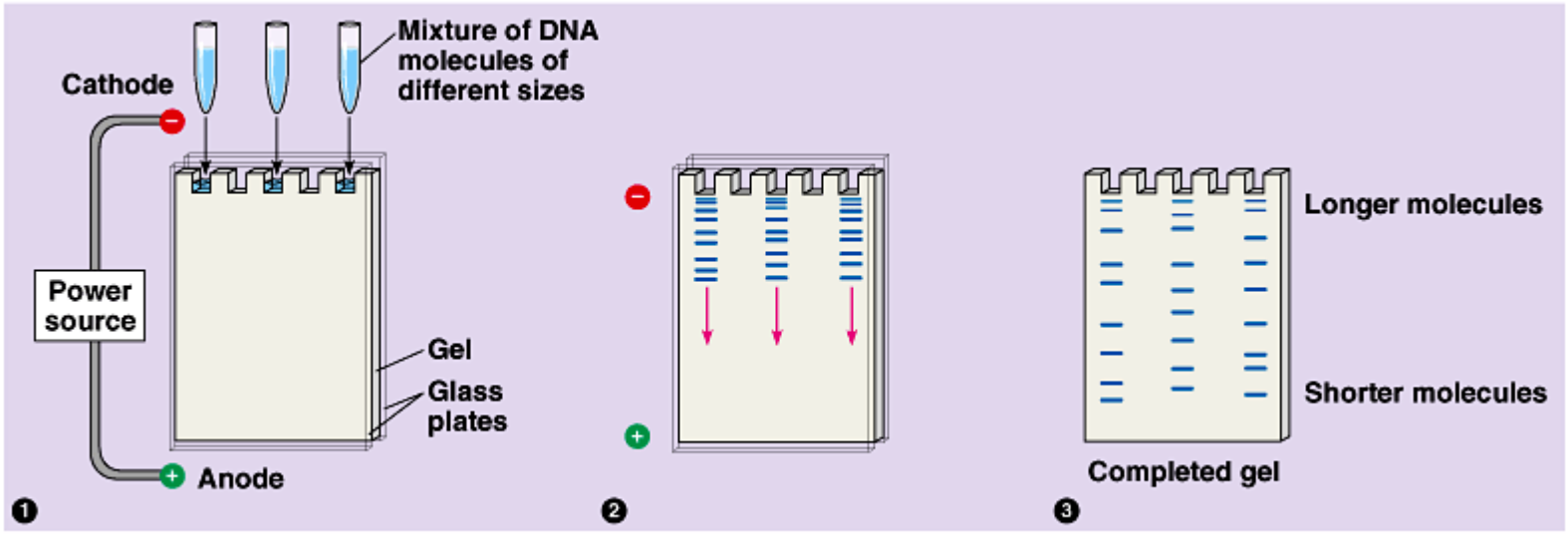


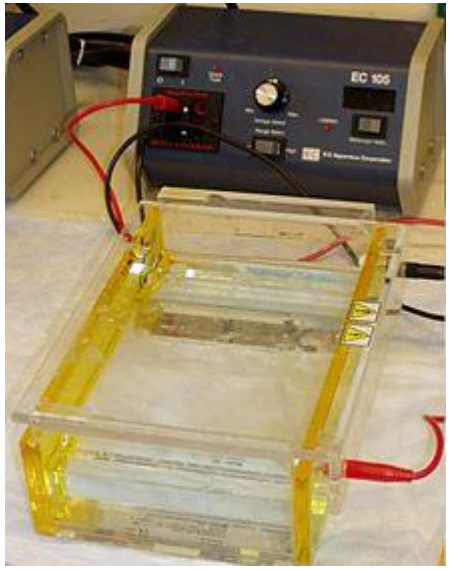


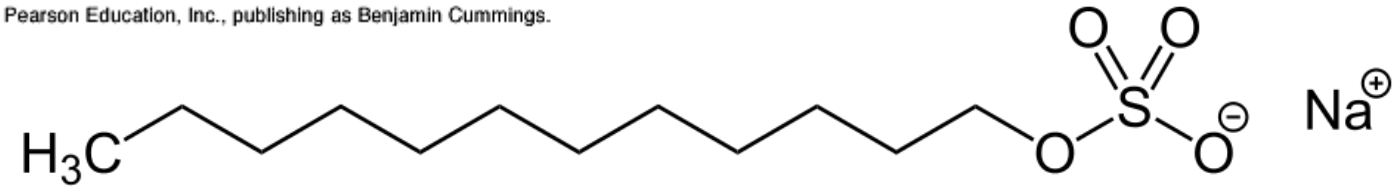


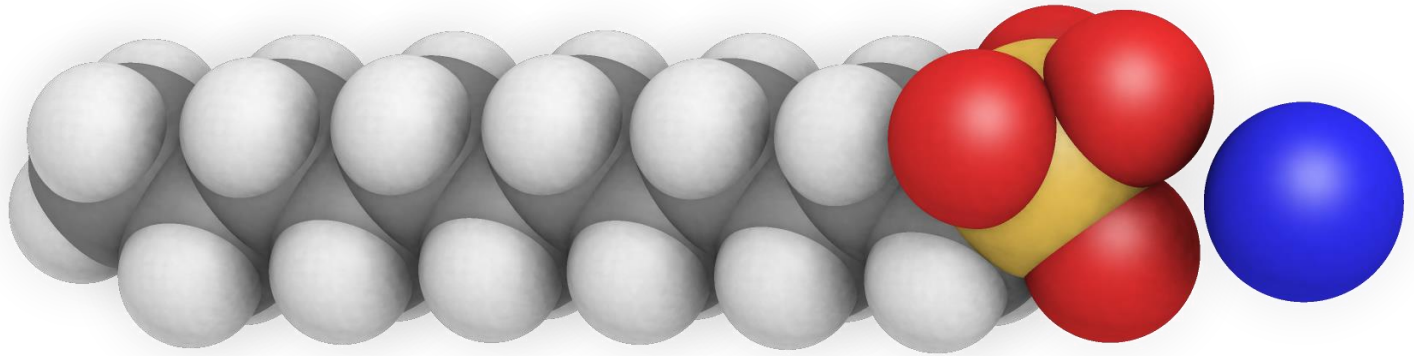


SDS-PAGE electrophoresis



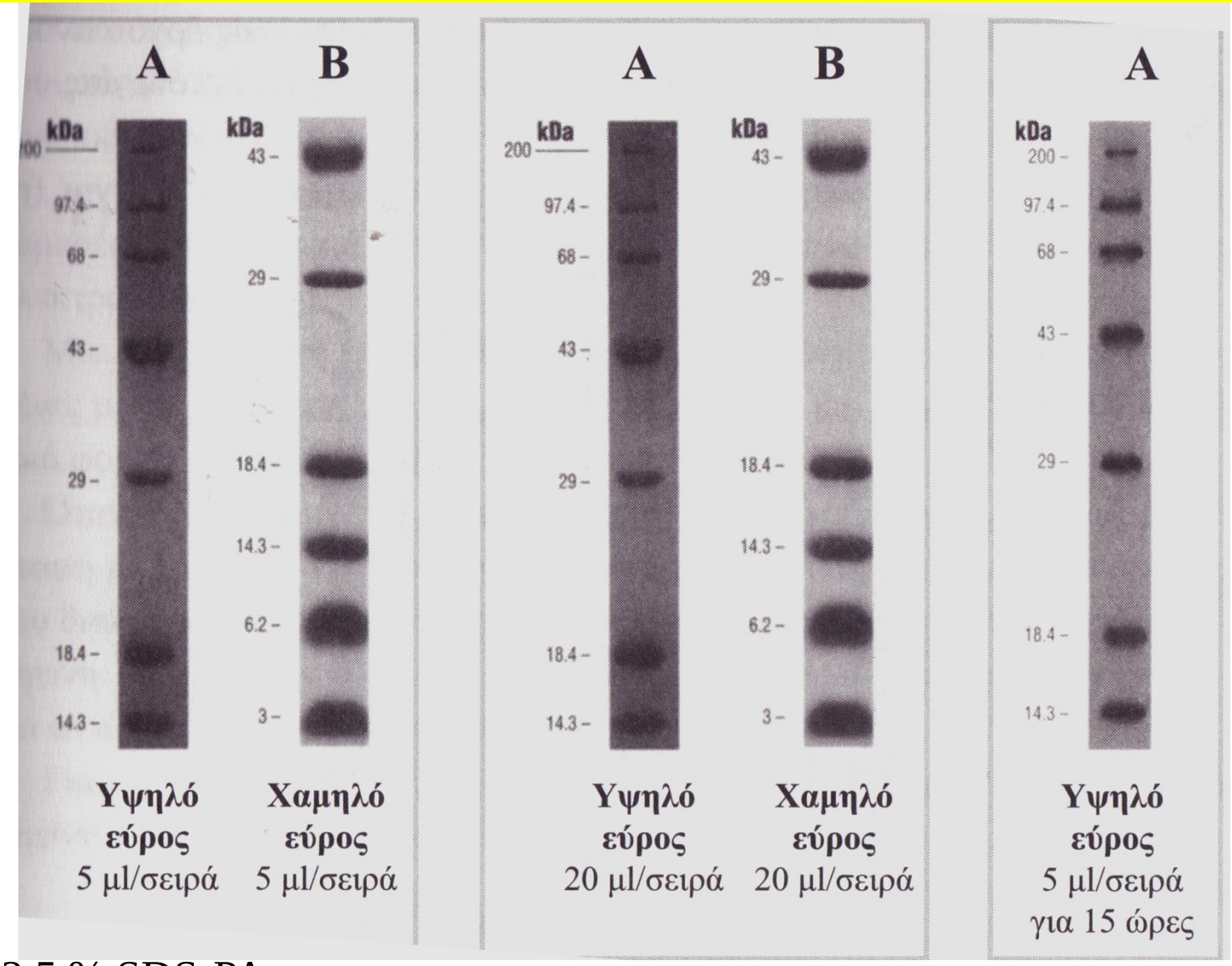






- **Sodium Dodecyl Sulfate**

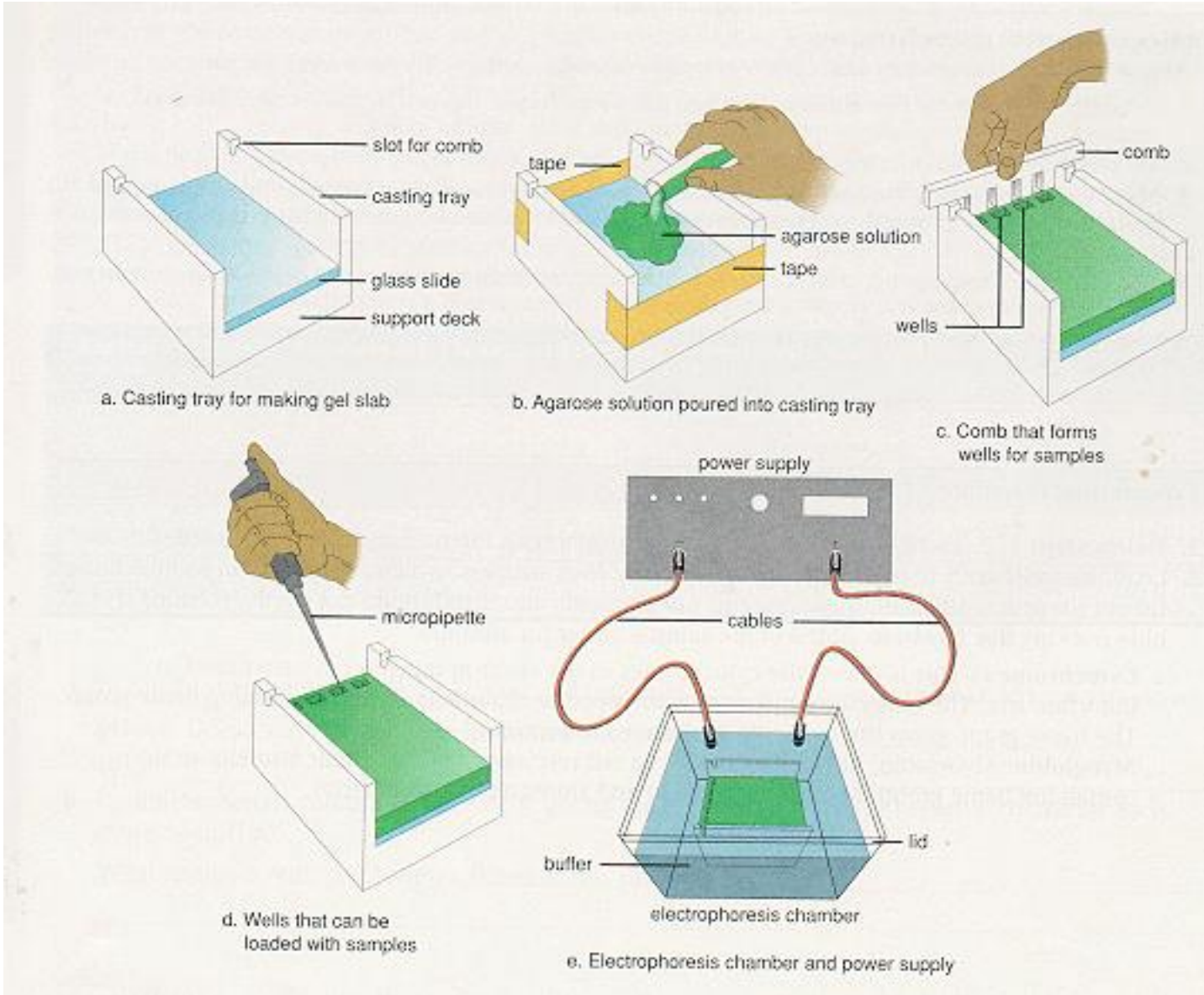
Protein separation



A. Electrophoresis in 12.5 % SDS-PA.

B. Electrophoresis in 15 % SDS-PA. Staining is done with Coomassie Blue

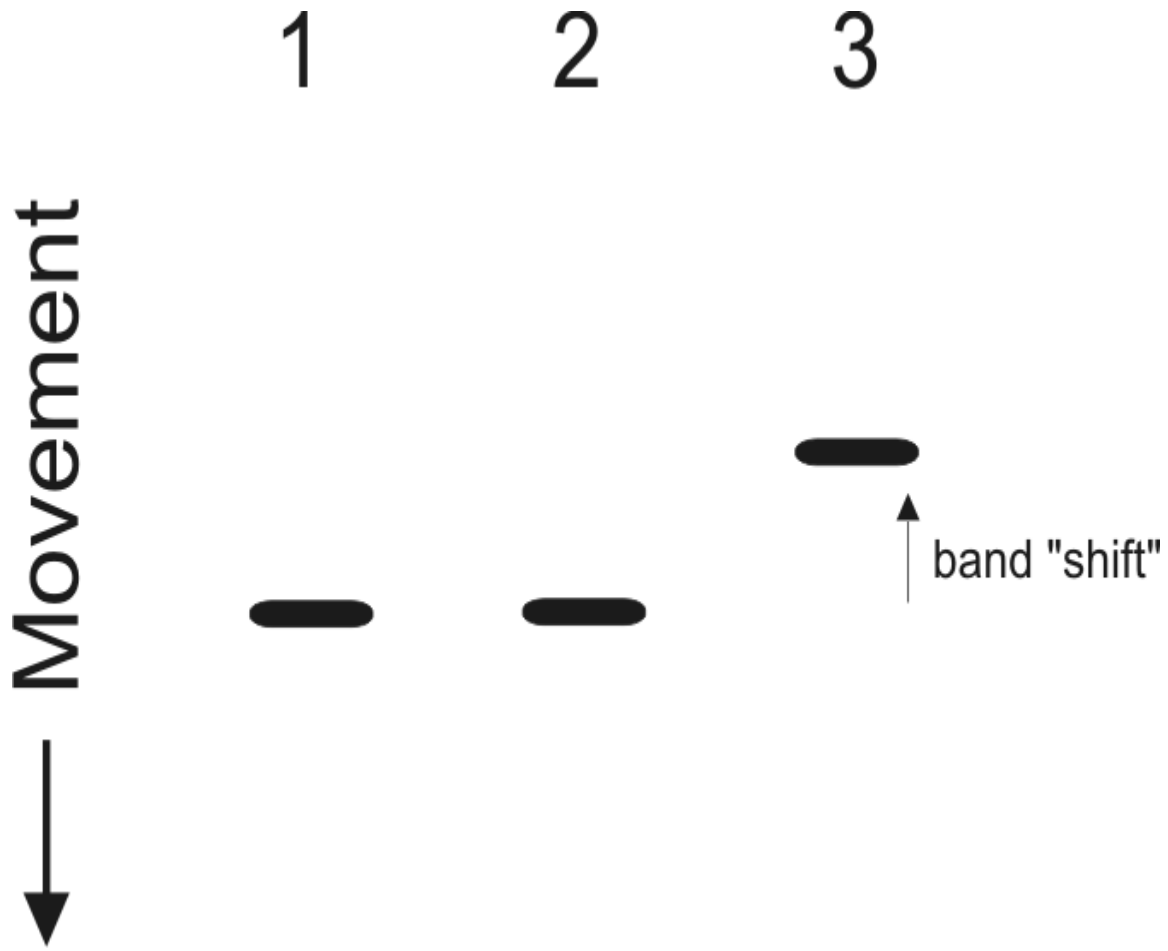
DNA ELECTROPHORESIS



Electrophoretic Mobility Shift Assay (EMSA)

- An **Electrophoretic Mobility Shift Assay** (**EMSA**) or **gel shift assay**, **gel mobility shift assay**, **band shift assay**, or **gel retardation assay**.
- It is an electrophoretic technique which is used to study protein-DNA interactions.
- The technique can determine if a protein or mixture of proteins is capable of binding a specific DNA (or RNA) sequence. Occasionally, it can indicate whether or not more than one protein species are bound to a specific DNA sequence.
- The assays are run in vitro and often in parallel with DNA foot-printing, primer extension, especially so when transcription, replication, repair and processing of DNA or RNA is the target.

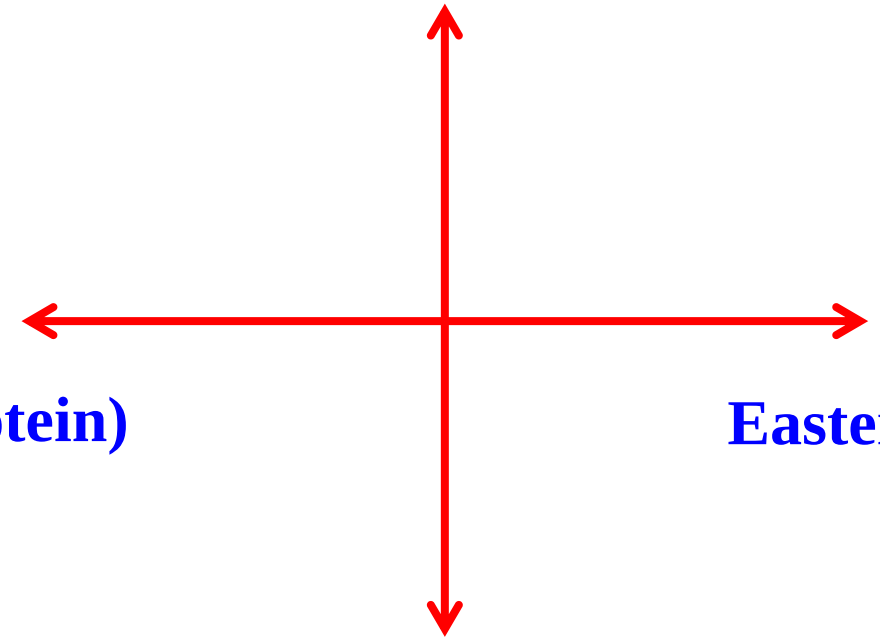
Electrophoretic Mobility Shift Assay (EMSA)



- **Lane 1** is a negative control, and contains only DNA.
- **Lane 2** contains protein as well as a DNA fragment that, based on its sequence, does not interact.
- **Lane 3** contains protein and a DNA fragment that does react. the resulting complex is larger, heavier, and slower-moving. The pattern shown in lane 3 is the one that would result if all the DNA were bound and no dissociation of complex occurred during electrophoresis. When these conditions are not met a second band might be seen in lane 3 reflecting the presence of free DNA or the dissociation of the DNA-protein complex.

Blotting

Northern Blotting (RNA)



Western Blotting (Protein)

Eastern Blotting (Glycosylation)

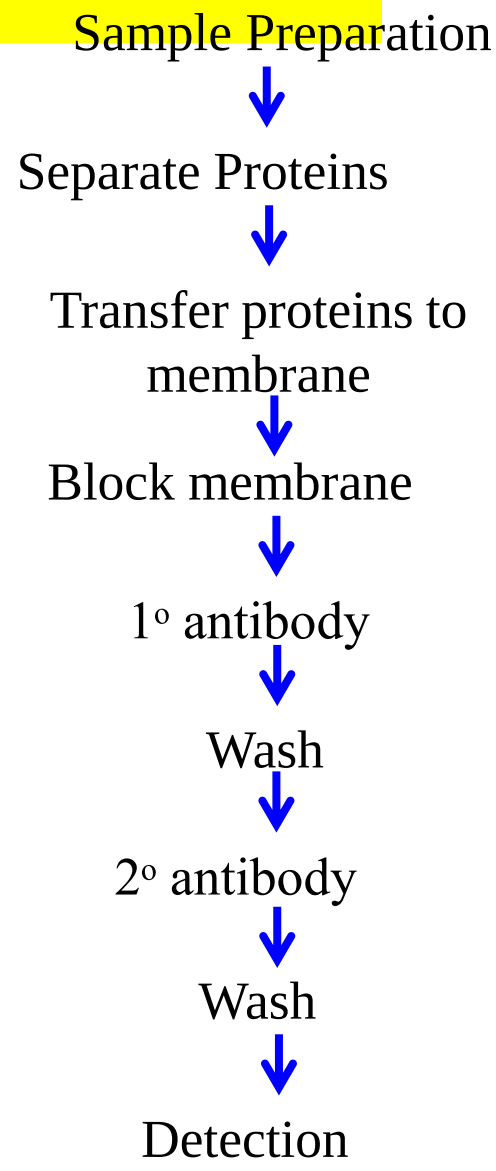
Southern Blotting (DNA)

Introduction

- Proteins are separated by SDS-PAGE and then a specific antibody is used to detect the protein of interest.

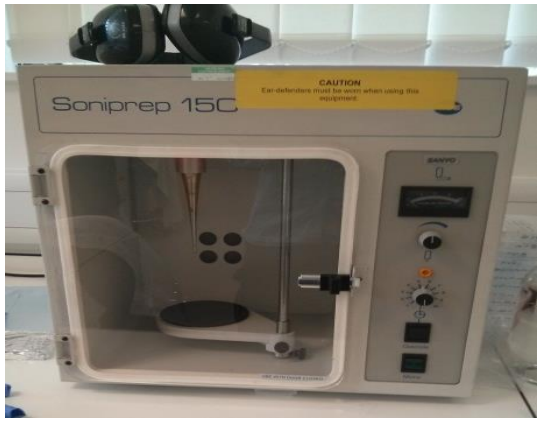
- Questions relevant to potential applications:
  - Is my protein expressed?
  - Do the levels change in response to treatment?
  - Detection of **Post Translation Modifications**
  - Has my protein expression/purification/immuno-precipitation worked properly?

- Other ways of detecting proteins
  - **ELISA (Enzyme-Linked Immunosorbent Assay**) is a biochemical method of detection of an antibody or antigen in a sample.
  - Mass spectrometry
  - Protein Array. Protein microarray (or protein chip) is a high-throughput method used to track interactions and activities of proteins, determine their function.



Sample Preparation and Quantification

- Lysis depends on tissue!
  - Culture Cells -> sonicate
  - Tissue samples -> homogenize
- Centrifuge to remove debris
- Keep cold and use protease inhibitors and phosphatase inhibitors!



Sample Preparation

↓

Separate Proteins

↓

Transfer proteins to membrane

↓

Block membrane

↓

1° antibody

↓

Wash

↓

2° antibody

↓

Wash

↓

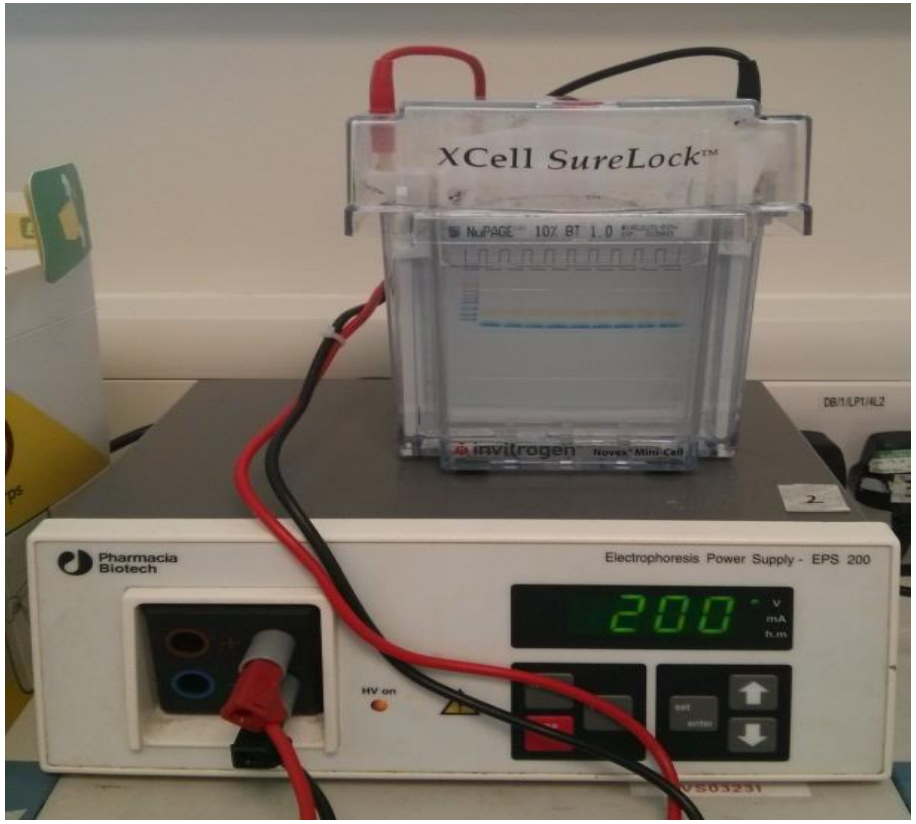
Detection

Sample Preparation and Quantification

- If you want to compare samples, you need to load the same amount of protein in each sample.
- Accurate quantification important
- **Bradford** assay
- **BCA** and **Lowry** assays. Less variable, but more sensitive to pH, detergents, EDTA etc. in lysis buffer
- Determination through measurement of absorbance at 280 nm. Accurate, but sensitive to DNA contamination.

SDS-PAGE

- In order to see proteins, we need to separate them out
- SDS-PAGE electrophoresis. Separation of proteins in sample according to molecular weight.
- Proteins are denatured before SDS-PAGE.
- Pick gel, buffer, running conditions appropriate for size of protein.
- 2D-PAGE is often required.



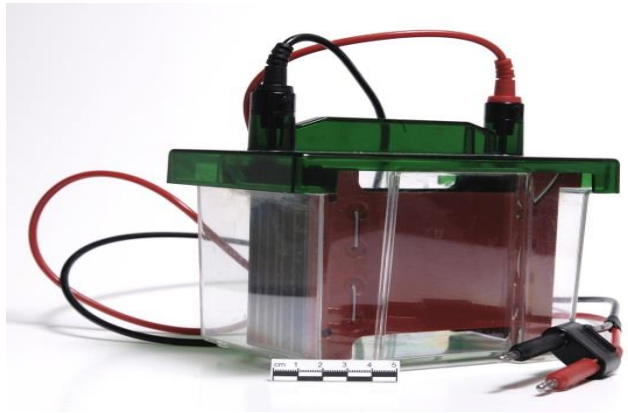
Transfer

Transfer separated proteins onto a membrane, which can then be probed with antibodies to detect the protein of interest.

Membrane can be Nitrocellulose or PVDF

Types of transfer:

Wet



Best for proteins >100kDa

Semi-dry

Quick

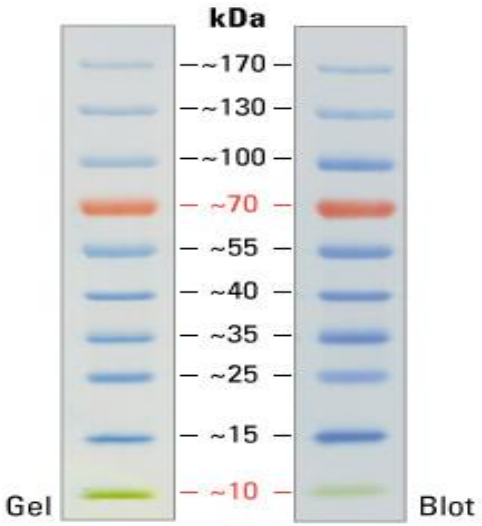
Dry

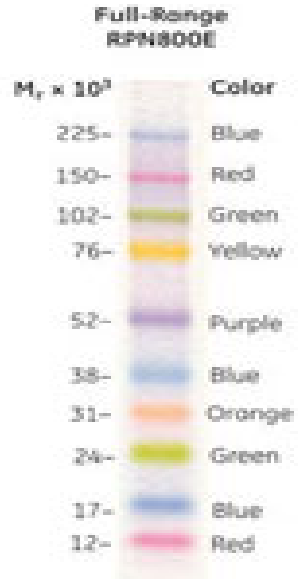
Even quicker

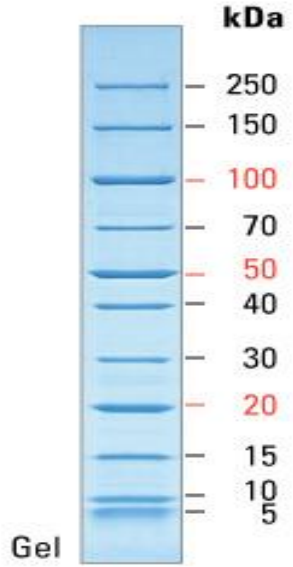
Transfer

Protein transfer to the membrane

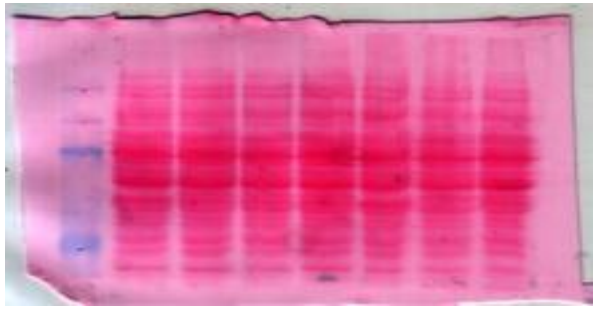
Pre-stained ladder







Ponceau staining (reversible)



Blocking

Fill up the space on the membrane to prevent non-specific antibody binding

Recommended to block for >1 hour

Milk

Strong blocking agent
Less signal
Not-recommended for phospho-proteins
Cheap!

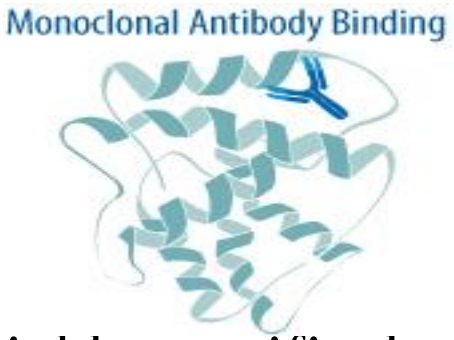
BSA

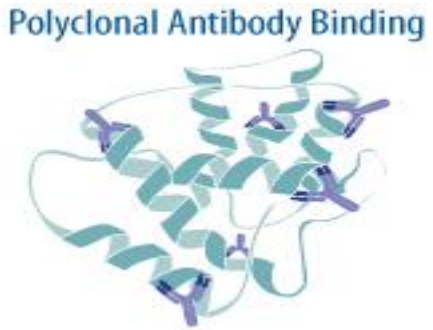
High signal
High background!

Diluted in the same Buffer used for washing (PBST/TBST)

Primary Antibodies

- Antibody specific to your protein of interest
- Monoclonal vs. Polyclonal antibodies

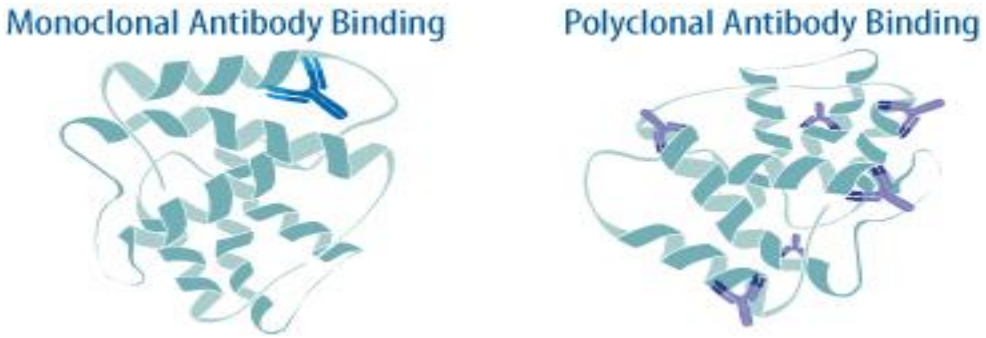


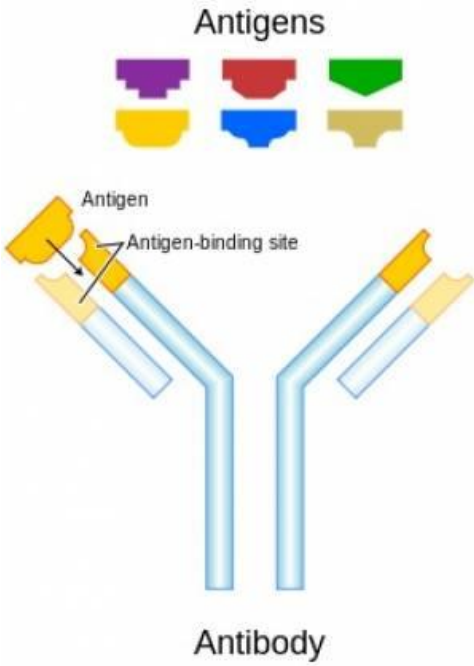


- Monoclonal – one antigen sequence – highly specific, low background, but low signal
  - Commercial monoclonal ~£200 for 100 µl
  - Custom monoclonal >£2000
- Polyclonal – multiple antigen sequences – more background, high signal
  - Custom monoclonal £600-800
- Directly conjugated Horse Radish Peroxidase – fewer steps, lower signal
- Amount of antibody – determined empirically (1/1000 -1/5000)
- Incubate in wash buffer, or blocking buffer for 1-2 hours at RT or overnight at 4^{o}C

Primary Antibodies

- Antibody specific to your protein of interest
- Monoclonal vs. Polyclonal antibodies





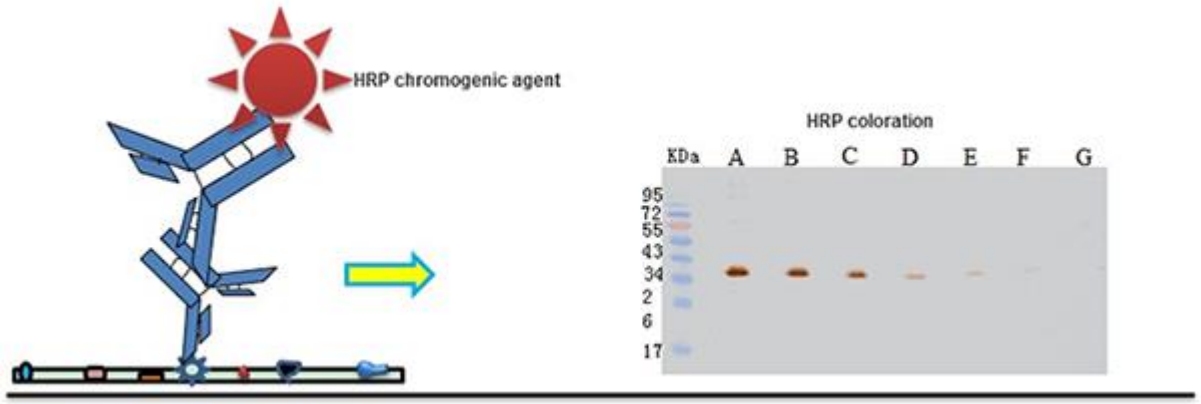
- Primary antibodies bind directly to the protein of interest, but unless they are directly conjugated to a dye or an enzyme, a secondary antibody is needed for detection.
- Conjugated secondary antibodies are used to detect the primary antibody.
- Secondary antibodies are commercially available in a variety of forms, including monoclonal, polyclonal, F(ab')2 fragments, affinity purified, and pre-adsorbed. Several factors influence the choice of secondary antibody (e.g. species specificity of the primary antibody, the class of the primary antibody, the detection method and any special factors on the technique or sample that would require a higher specificity secondary antibody).

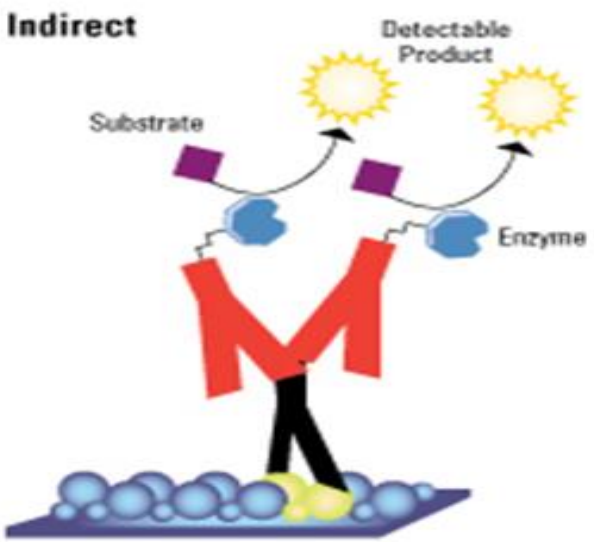
Washing

- Wash off unbound antibody
- Tris-Buffered Saline or Phosphate-Buffered Saline (PBS) buffer
- Tween-20 or Triton-X-100 (or other detergents beginning with T)
- In most cases, it doesn’t matter which agents you use. In the case of phospho-proteins, however, TBST may be better.

Secondary Antibody

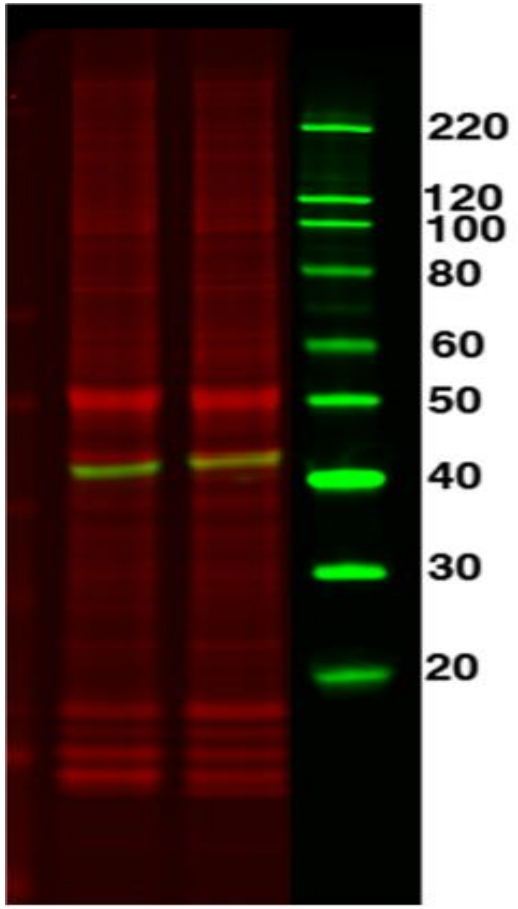
- Antibody against IgG of primary antibody
- Conjugated to a reporter – Horse radish Peroxidase RP, Alexa 488, etc.
- Dilution in range of 1/10000 – 1/100000 in blocking buffer
- Incubate for ~2 hours at RT.





Detection

- Colorimetric – less sensitive
- Radioactive label
- Fluorescently labelled secondary antibody – highly quantitative
- Chemiluminescent – HRP or AP (Alkaline phosphatase) labelled secondary antibody - very sensitive!



Horse Radish Peroxidase

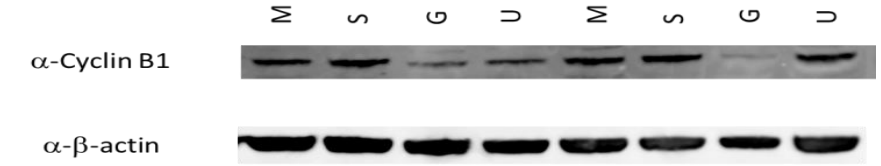
Detection

Chemiluminescent detection

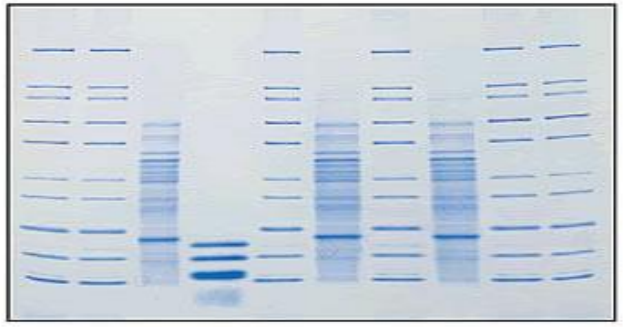
- Mix 2 solutions together -> pipette onto membrane
- Wait 5 minutes
- Image

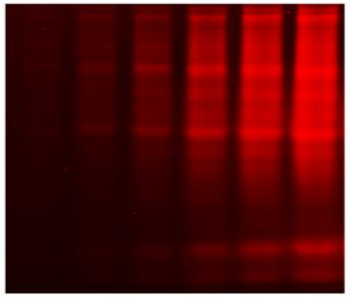
Loading Controls

- Difference between samples? – is it real?
- Normalise to either a loading control or total protein stain.
- Possible loading controls – tubulin, β-actin, GAPDH, other housekeeping protein.



Total protein – colloidal Coomassie blue, fluorescent stains.





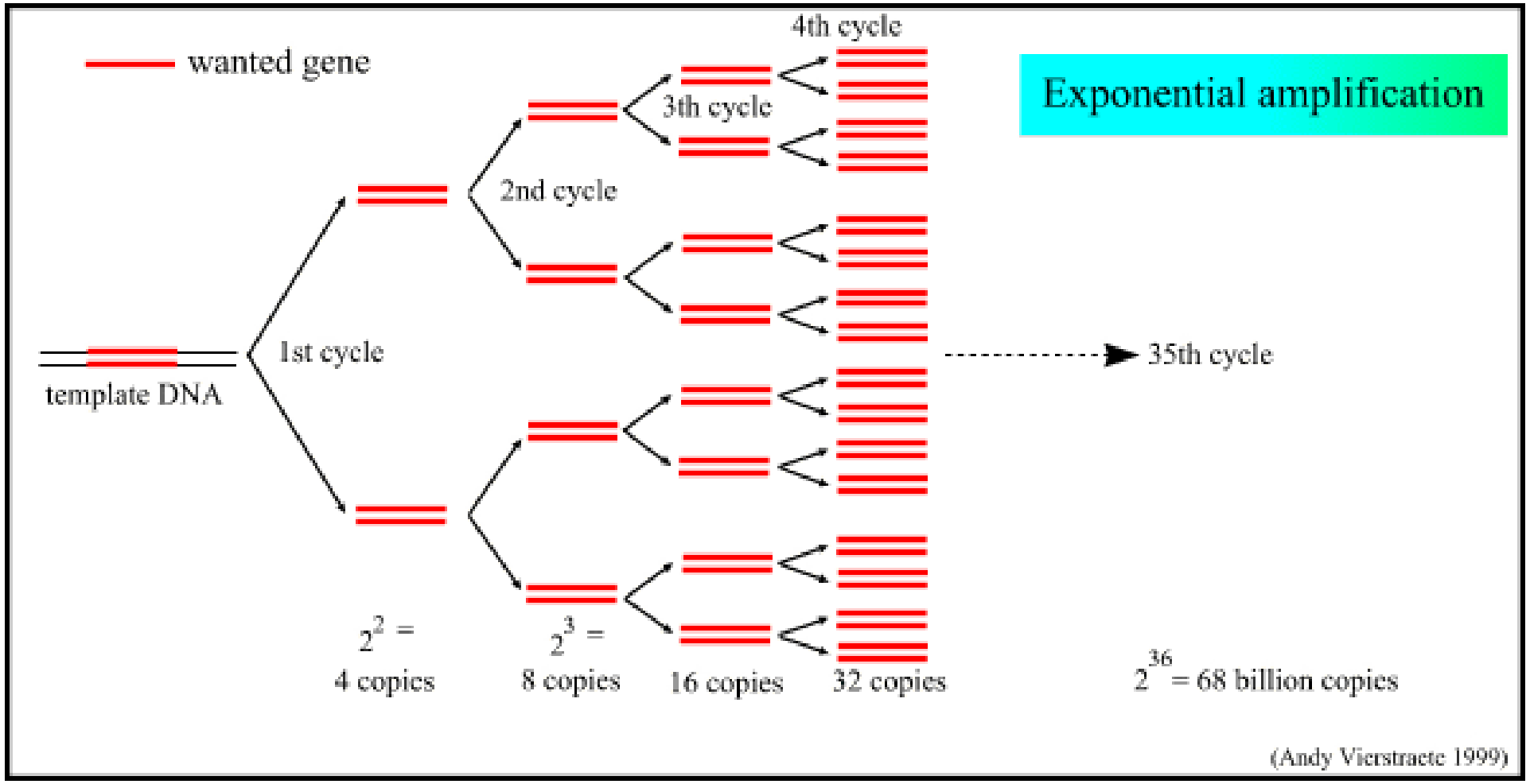
Quantification

- ImageQuant software allows quantification of blot results.
- Bear in mind, signal is not linear!

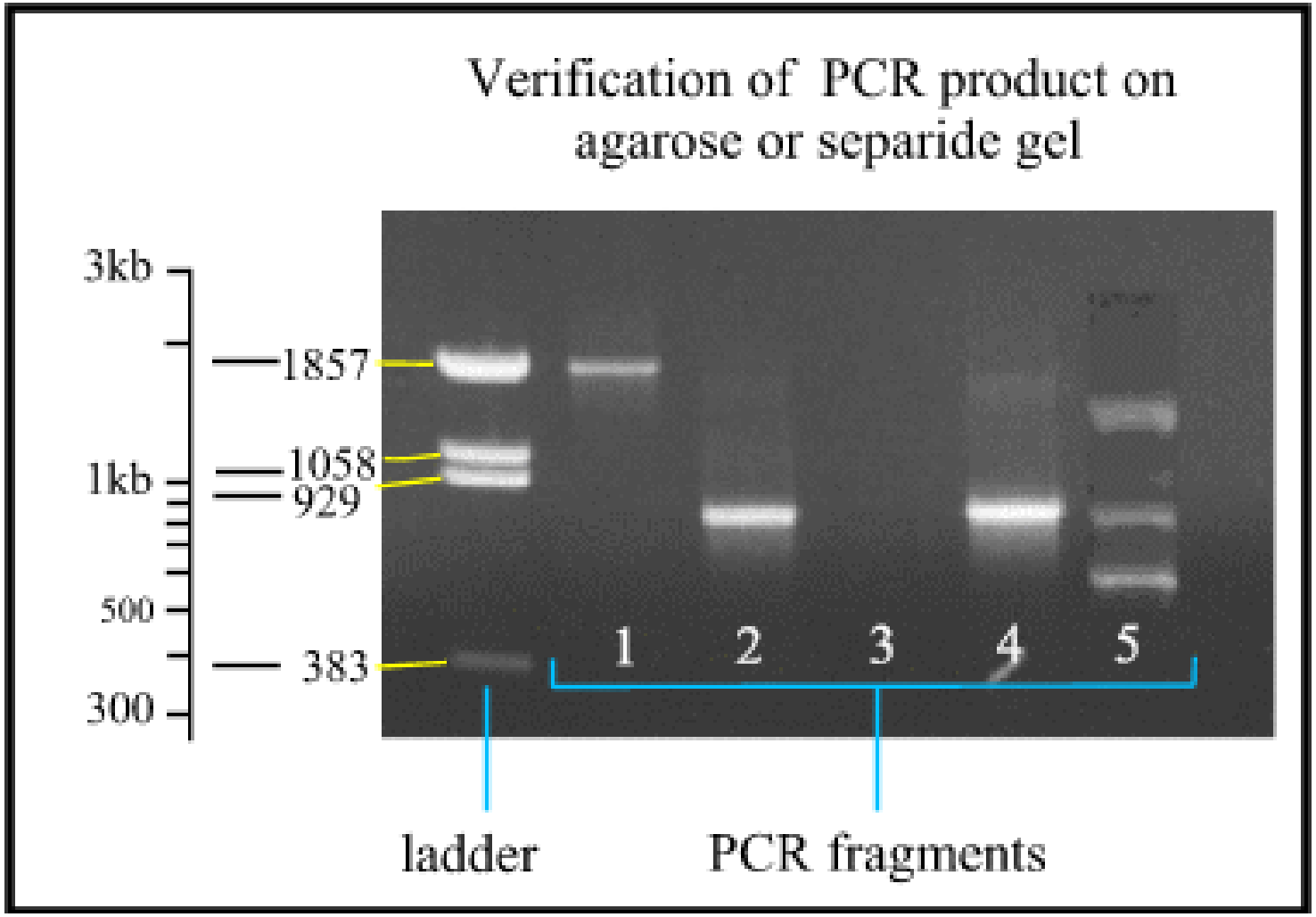
Polymerase Chain Reaction (PCR)

- The technique allows the synthesis of large quantities of DNA from a prototype molecule.
- A small quantity of a body fluid (saliva, sweat, sperm, etc.) or cell(s) is sufficient to allow proliferative augmentation of DNA in quantities warranting credible analysis
- Applications in forensic sciences and generally forensics are ostensible.

POLYMERASE CHAIN REACTION (PCR)



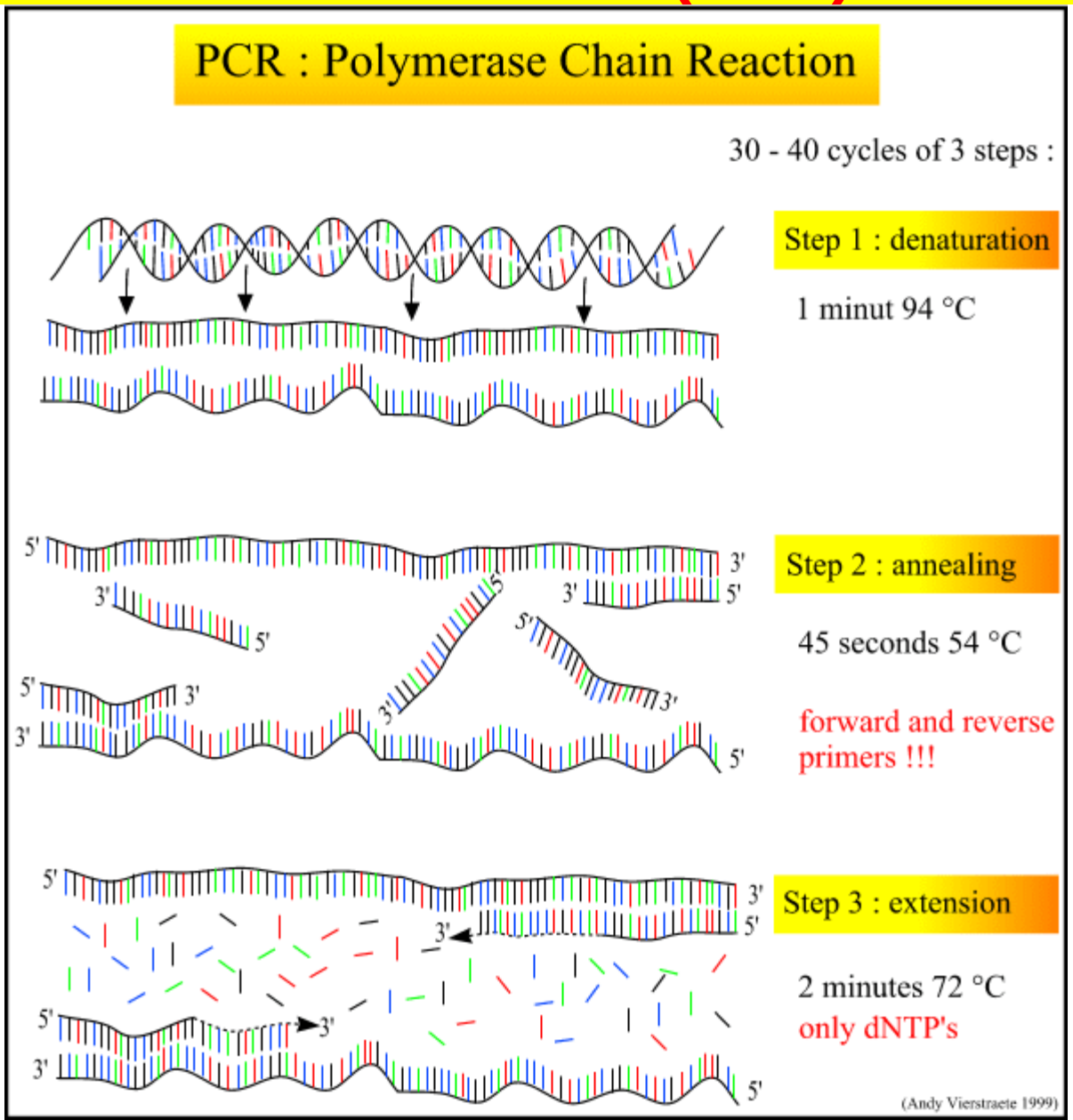
CONFIRMATION OF POLYMERASE CHAIN REACTION (PCR)

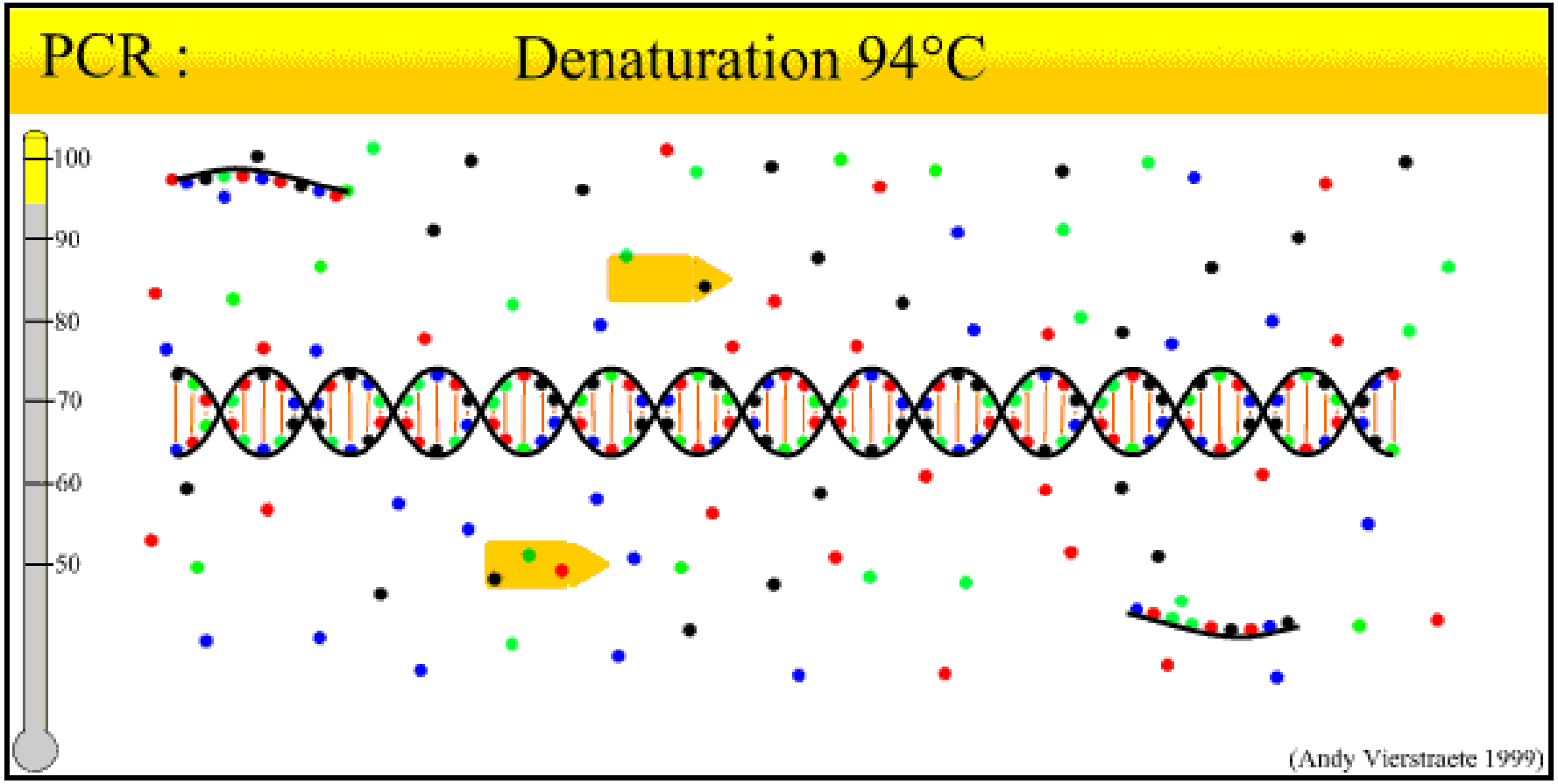


The process

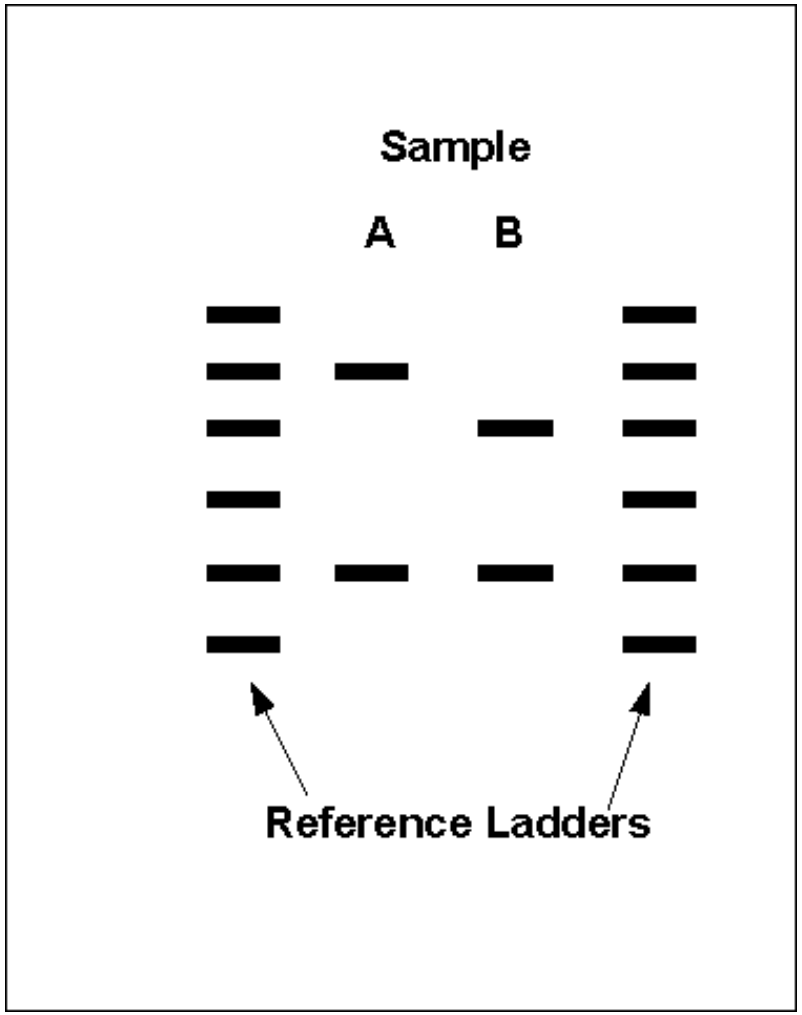
- A small quantity of DNA is required.
- Based on nucleotide sequences of the investigated DNA terminals, **primers** are synthesized, which are complementary to both sequences.
- The use of **thermostable DNA polymerase** (Taq polymerase) leads to under controlled conditions to the synthesis of new DNA clones identical to the regions between the two ends, where the primers are bound.
- Specifically, DNA, **Taq polymerase** and the primers are placed in a vial. The mixture is heated to 94 °C for 1 min and denatures DNA.
- The temperature is lowered to 55 ° C for 30 sec to allow the primers to bind to the complementary ends of DNA.
- The temperature rises to 72 ° C for 1 min, allowing **Taq polymerase** to synthesize two new clones of DNA, each complementary to the original clone.
- The temperature rises to 94 °C for 1 min and denatures the newly synthesized DNA. The cycle is repeated and every time the newly synthesized clones serve as templates for the synthesis of new clone(s).
- The synthesis of millions of copies of starting DNA is achieved within a few hours and 30-35 cycles.
- The advantage of the PCR method is that a single DNA copy from a single cell is sufficient to generate a new sample for further analysis. The disadvantage is that multiple DNA copying could cover a region of 5000 bp. To the contrary, **Restriction Fragment Length Polymorphism (RFLP) analysis** has no limits**.**
- For DNA profiling PCR could be used.
- For RFLP the use of a specific **VNTR** is required, VNTR regions are very large for use in PCR. To deal with this problem, short length regions of contiguous repetitions (contain repeating units of 5-7 nucleotides) (**Short Tandem Repeats**) are employed. The method is called **PCR-STR** and is implemented in combination with RFLP analysis.
- The power of hybridization of nucleic acids through probes of specific regions allows fast and credible technique to recognize the unique characteristics of a person, from which samples are available.

THE PROCESS OF POLYMERASE CHAIN REACTION (PCR)




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

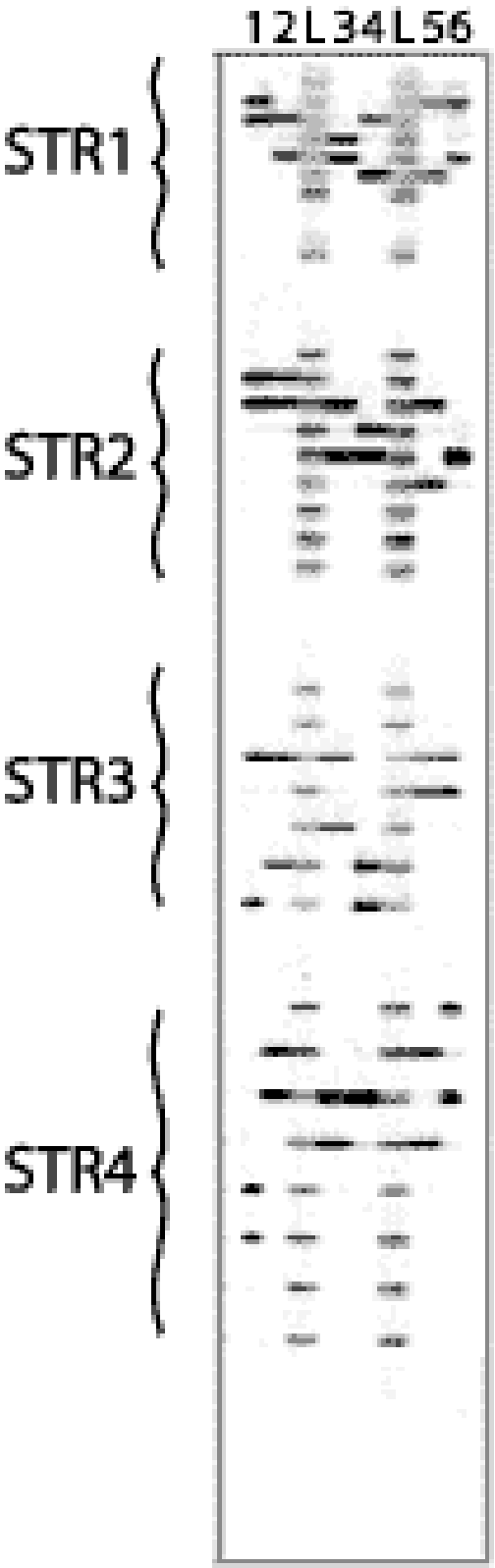
THE PROCESS OF POLYMERASE CHAIN REACTION (PCR-STR)



In this case:

- **Sample A** contains two bands in positions corresponding to two (i.e. CATGCATG) and 5 tetranucleotide repetitions.
  The common terminology, in this case is dubbed type 2,5.

- **Sample B** could be ascribed to type 2,4.
- For a person, each locus has two alleles and both of them could be different (heterozygotes) or the same (homozygotes).

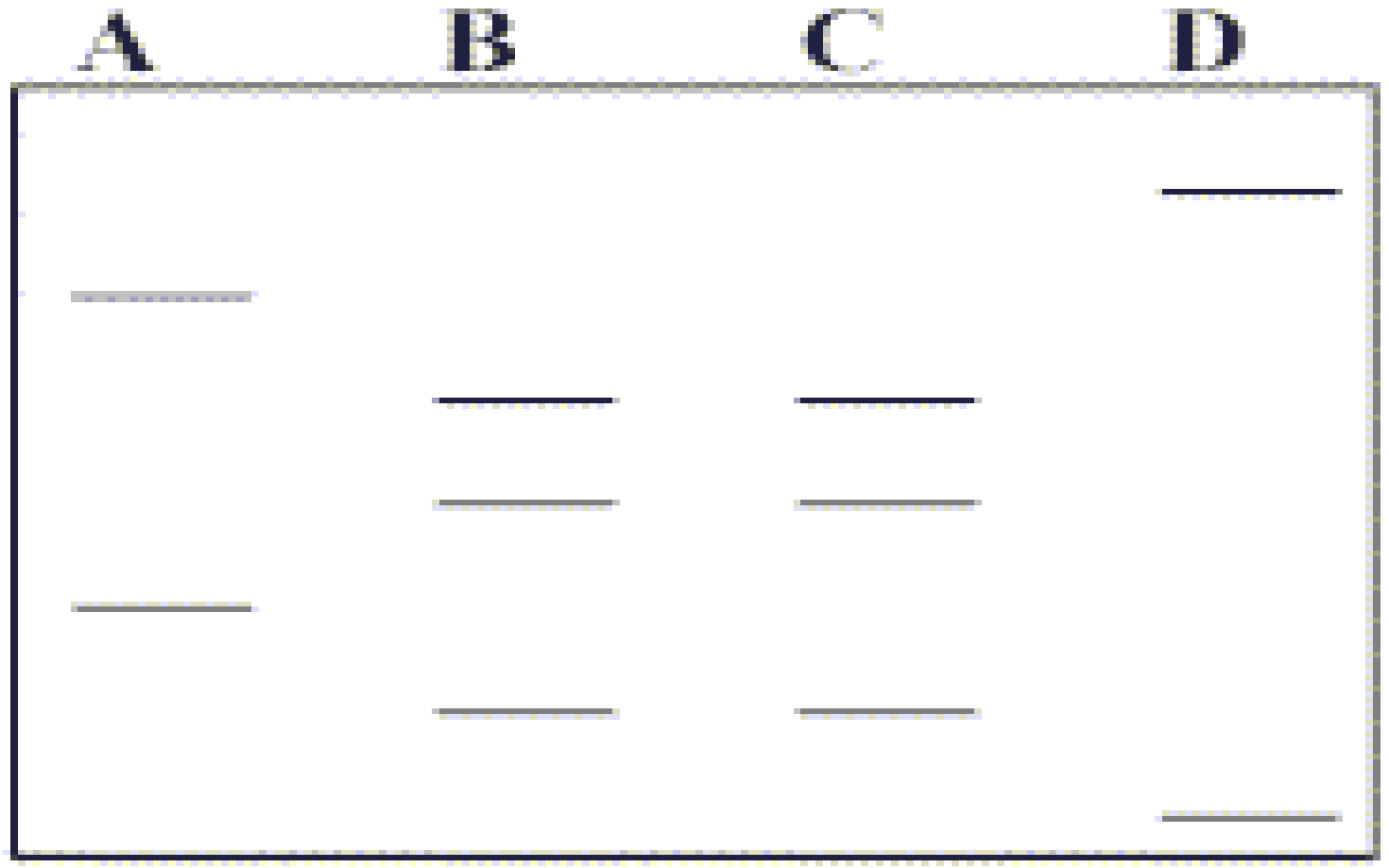


1 is mother
2 is father
3 to 6 are children
L is a scale to indicate DNA fragment size

CASE OF FORENSIC PATERNITY

FORENSICS CASE

A = Victim
B = Evidence
C = Guilty suspect
D = Innocent suspect



CONFOCAL MICROSCOPY

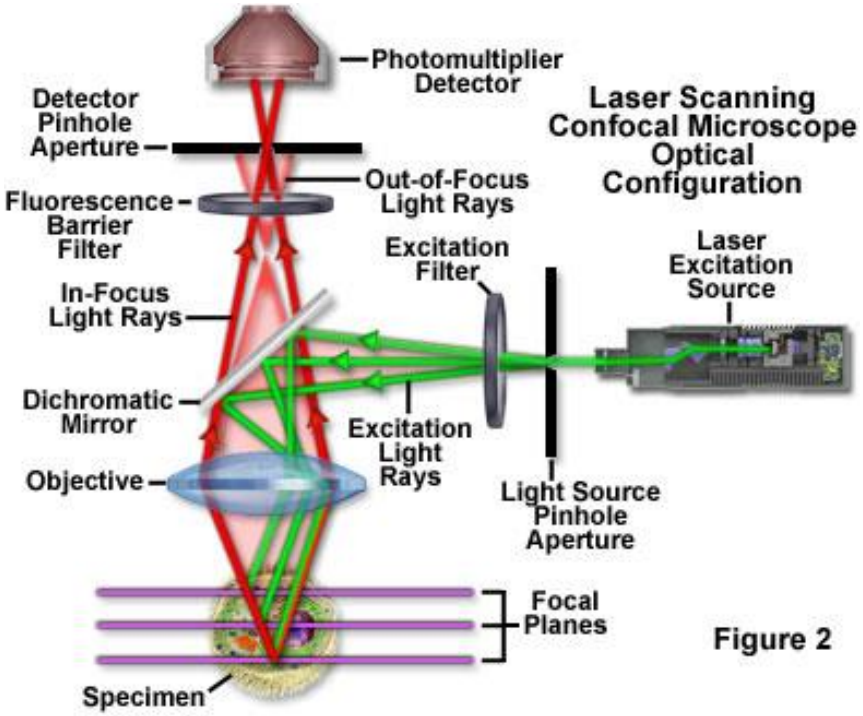
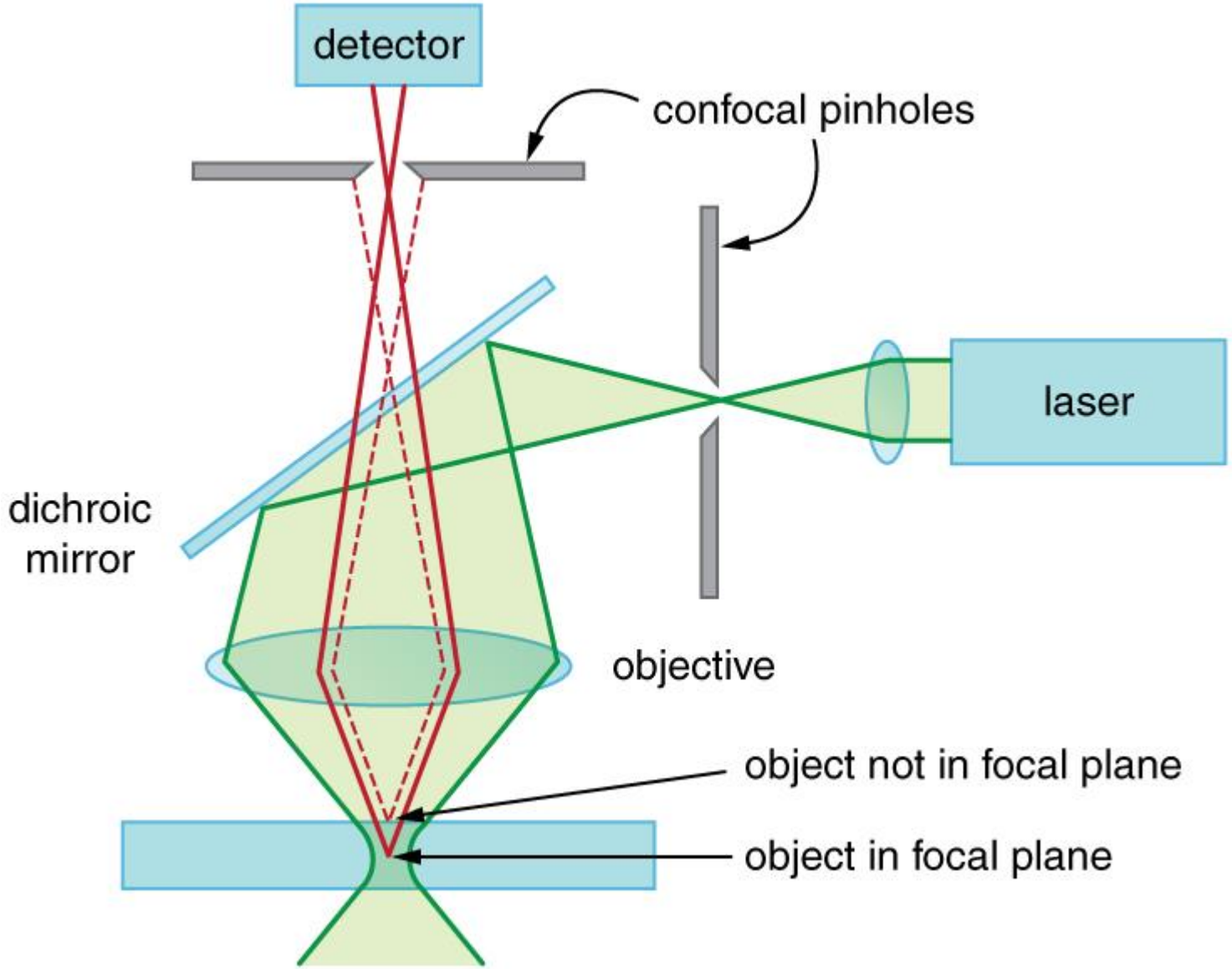
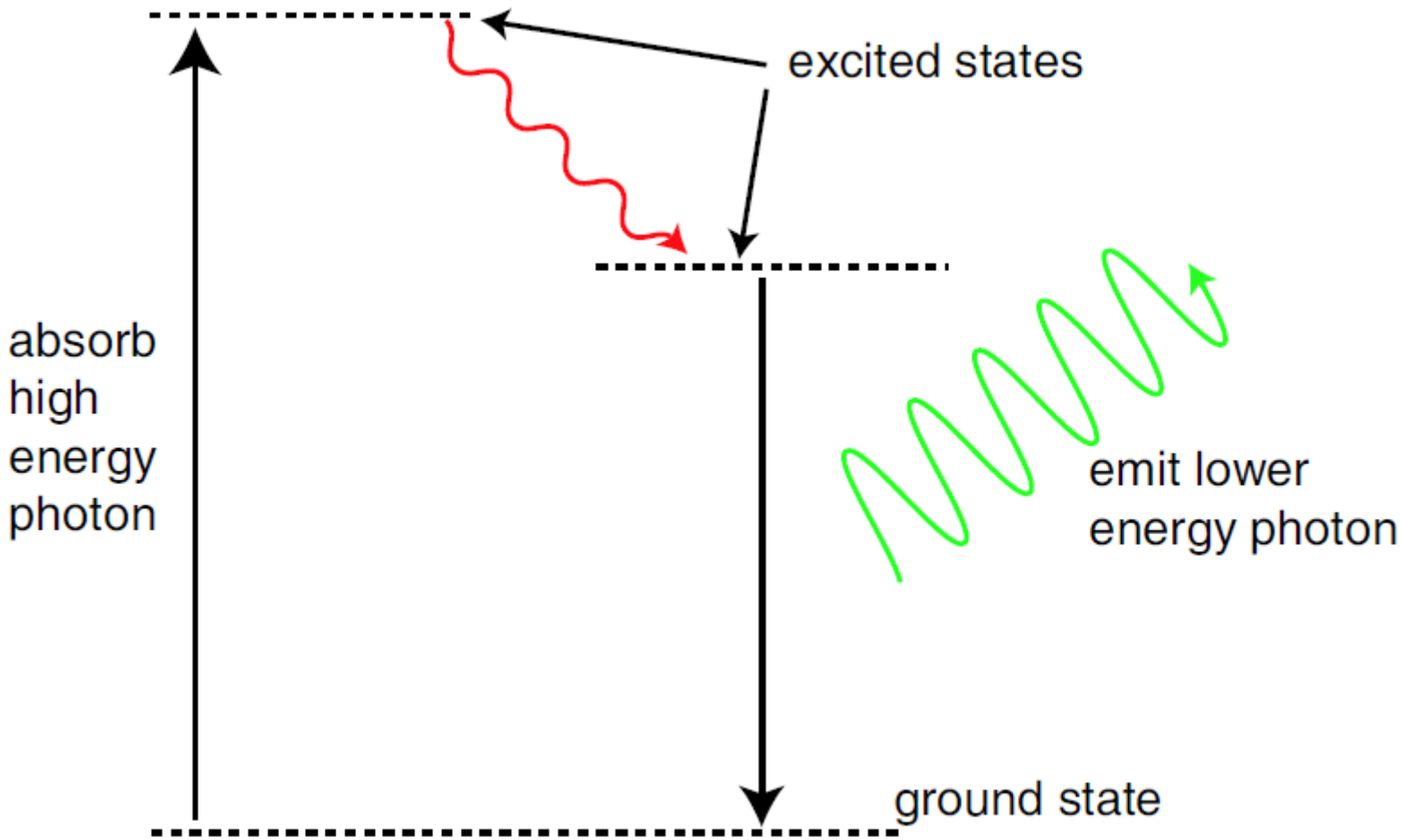
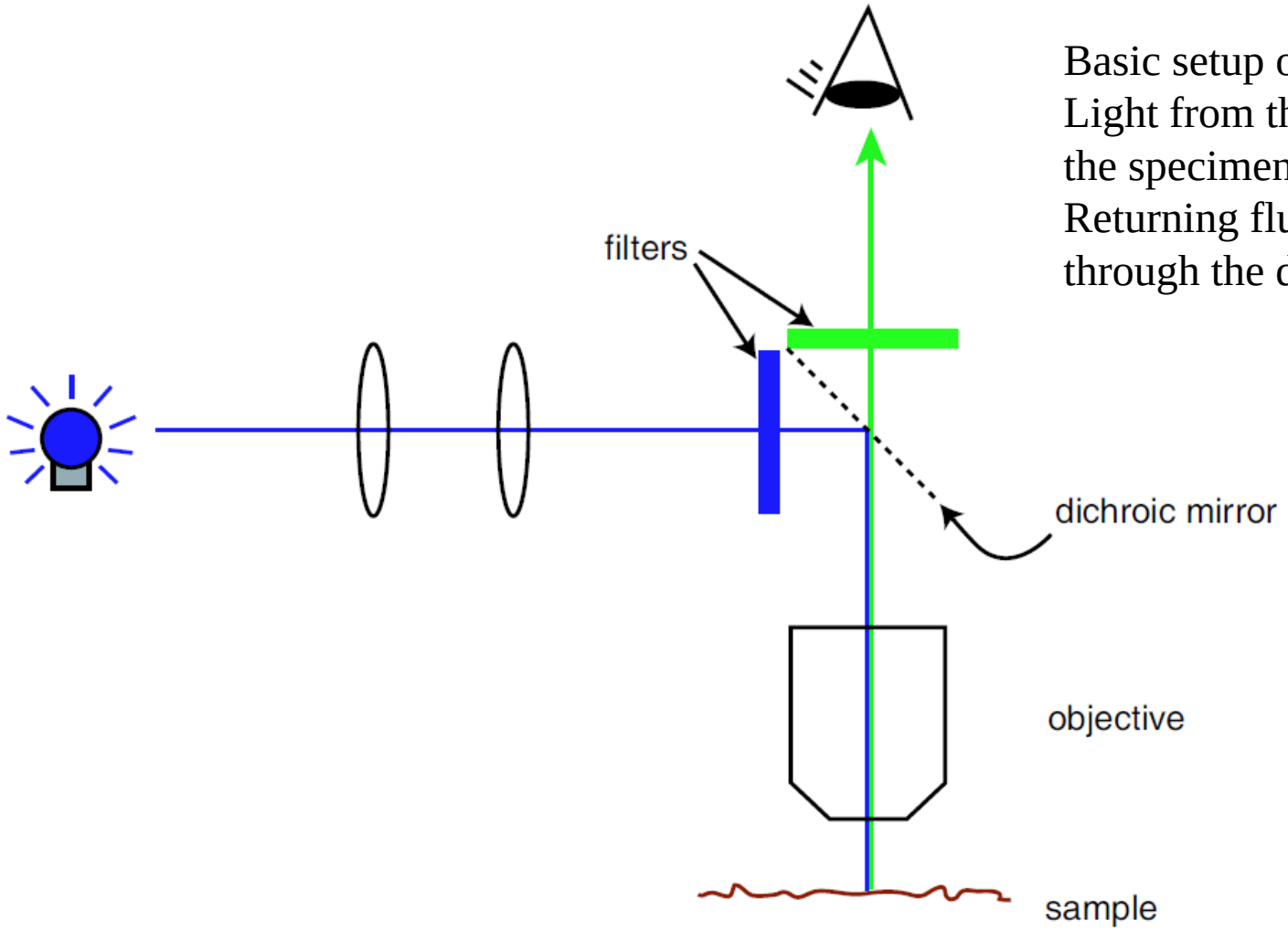


Figure 2

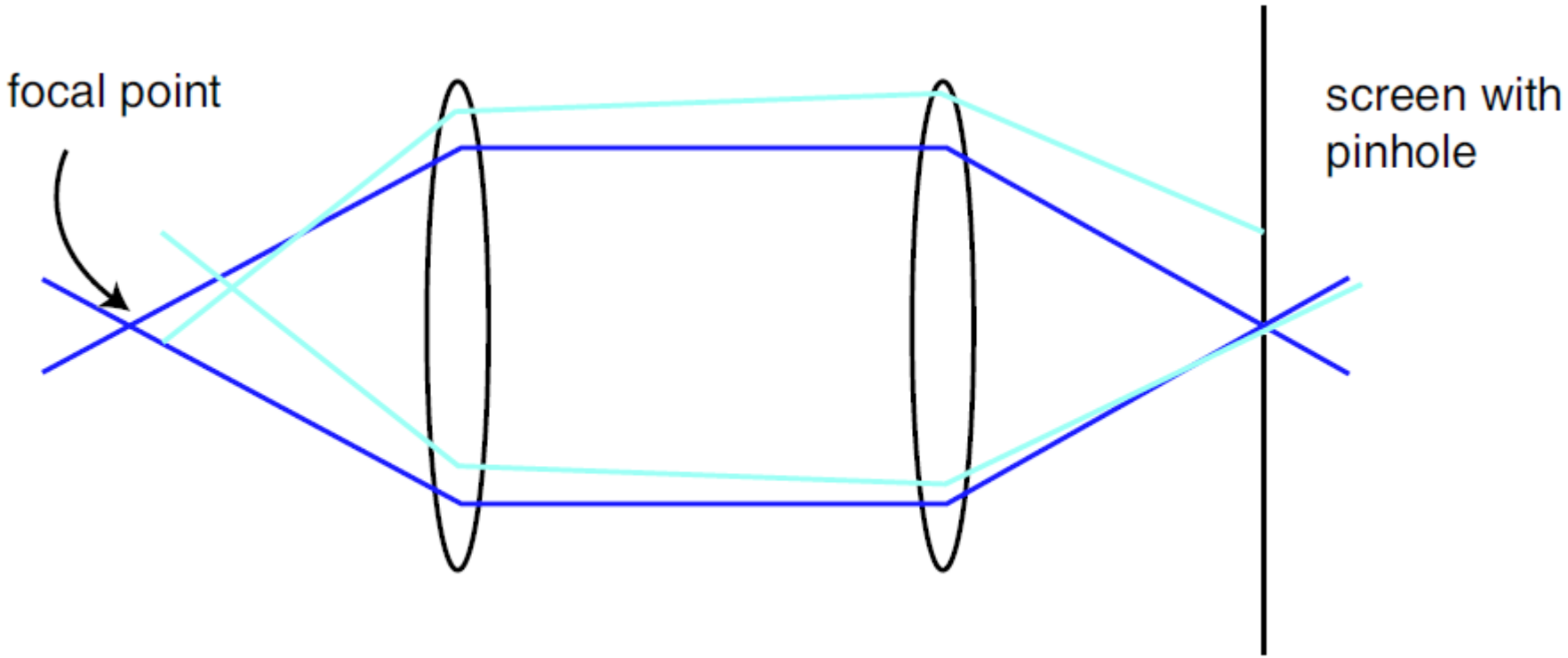



excited states
absorb
high
energy
photon
emit lower
energy photon
ground state

Fluorescence Microscopy

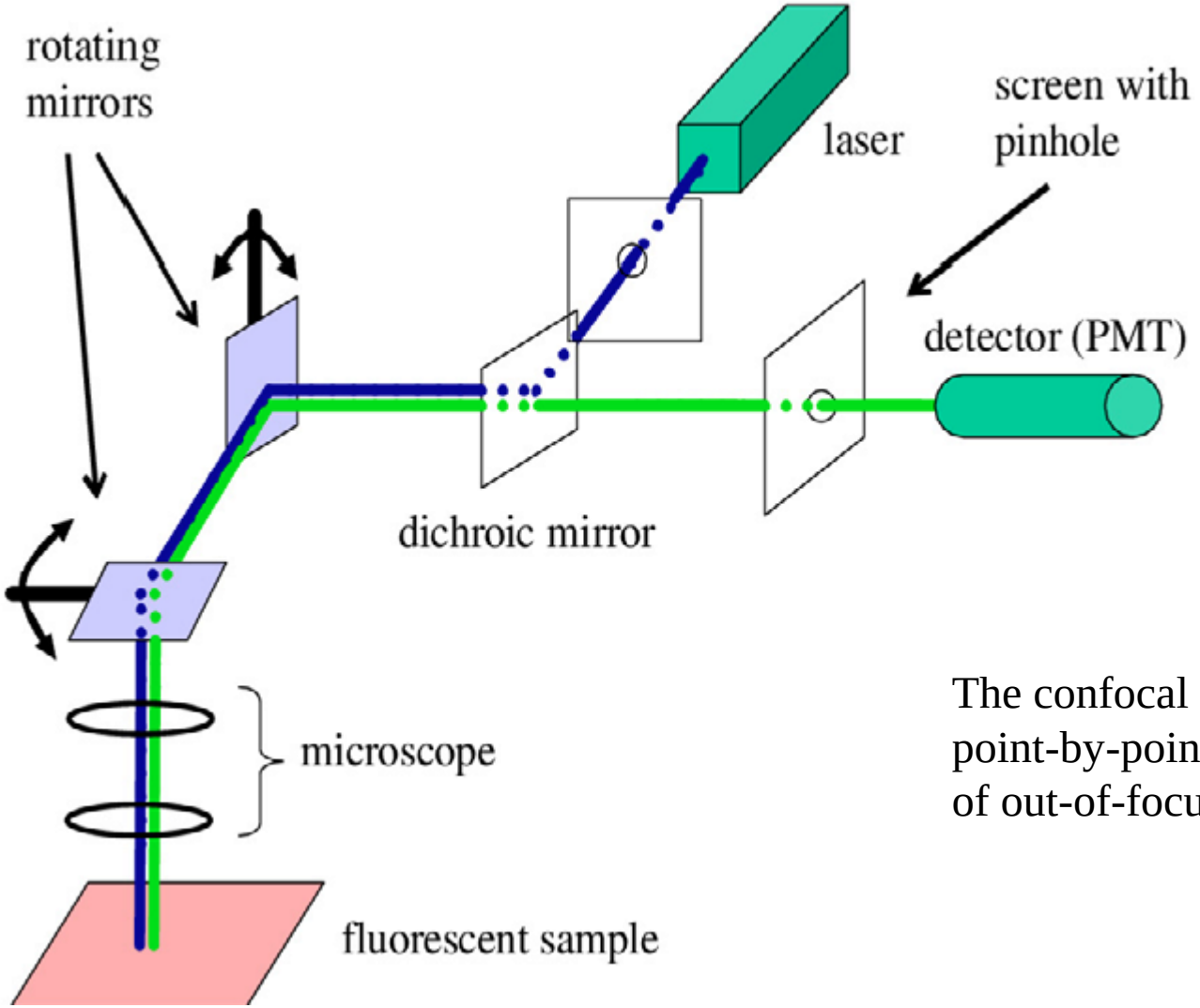


Basic setup of a fluorescence microscope.
Light from the source is reflected off the dichroic mirror toward the specimen.
Returning fluorescence of a longer wavelength is allowed to pass through the dichroic mirror to the eyepiece.



- Rejection of light not incident from the focal plane.
- All light from the focal point that reaches the screen is allowed through.
- Light away from the focal point is mostly rejected.

Confocal Microscopy



The confocal microscope incorporates the ideas of point-by-point illumination of the specimen and rejection of out-of-focus light.

Advantages in Confocal Microscopy

- At any particular point in time only one point of the sample is observed.
- A computer reconstructs the 2D image plane one pixel at a time.
- A 3D reconstruction of the sample can be performed by combining a series of such slices at different depths.

Advantages

1. The 3D capability is one of the main advantages in confocal microscopy.
2. The pinhole filters out background fluorescence that would normally prevent clear imaging of a high volume fraction sample. It is precisely this ‘sectioning’ ability that allows a crisp image at a given depth into the sample that in turn enables the 3D imaging.

Examples in Confocal Microscopy

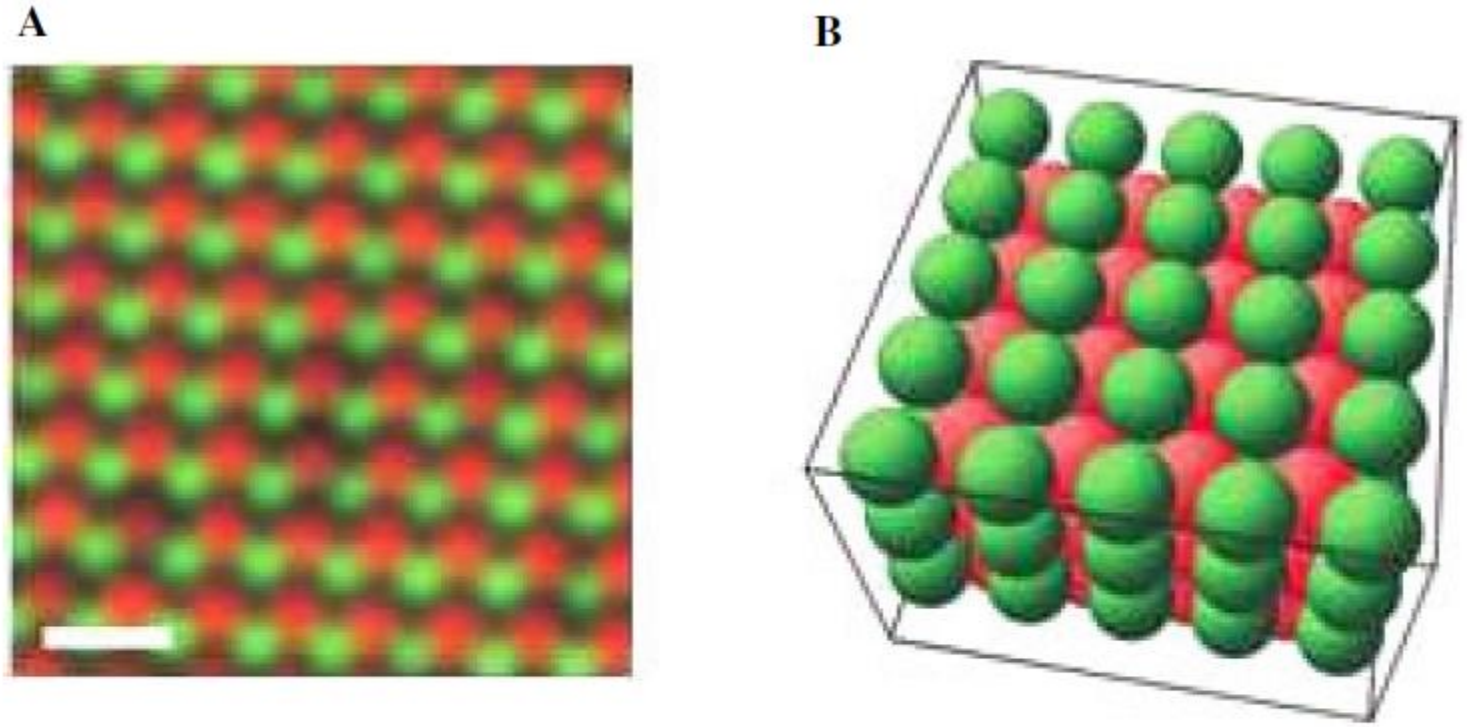
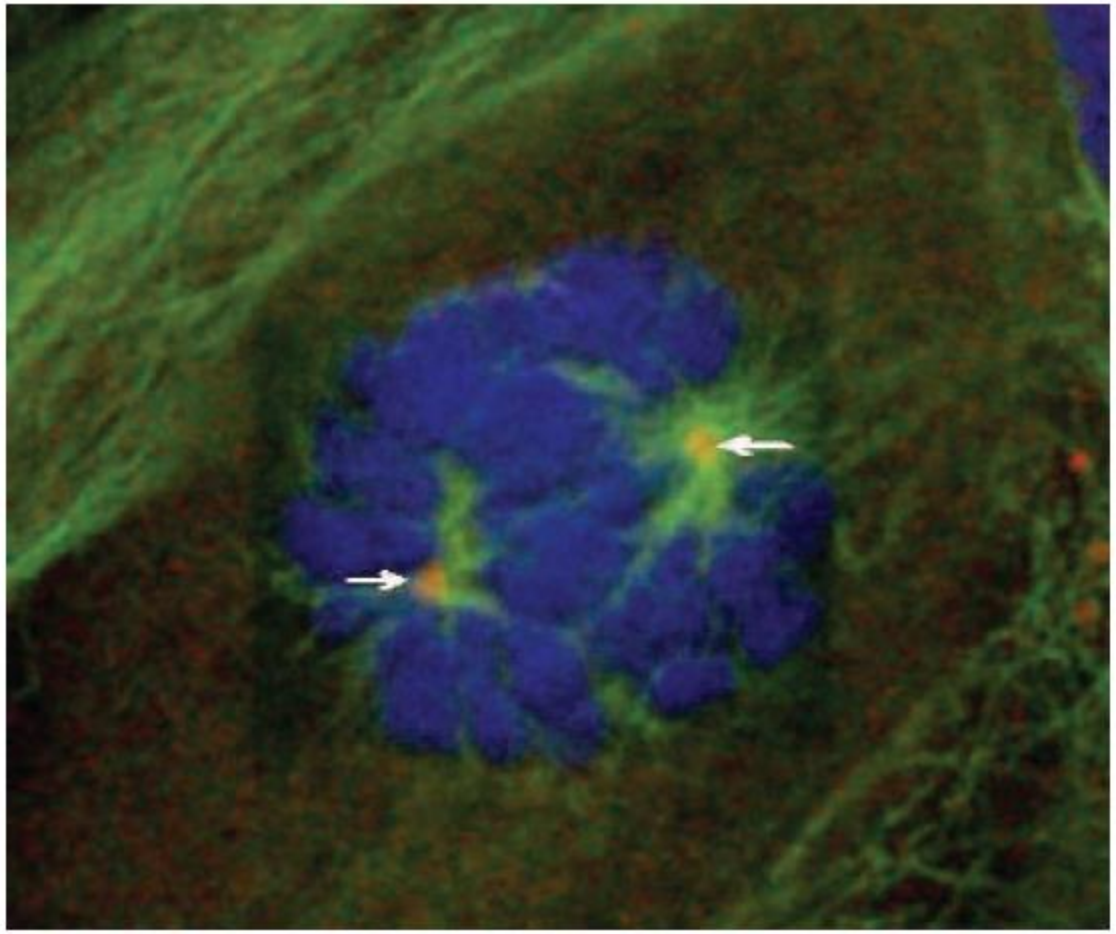


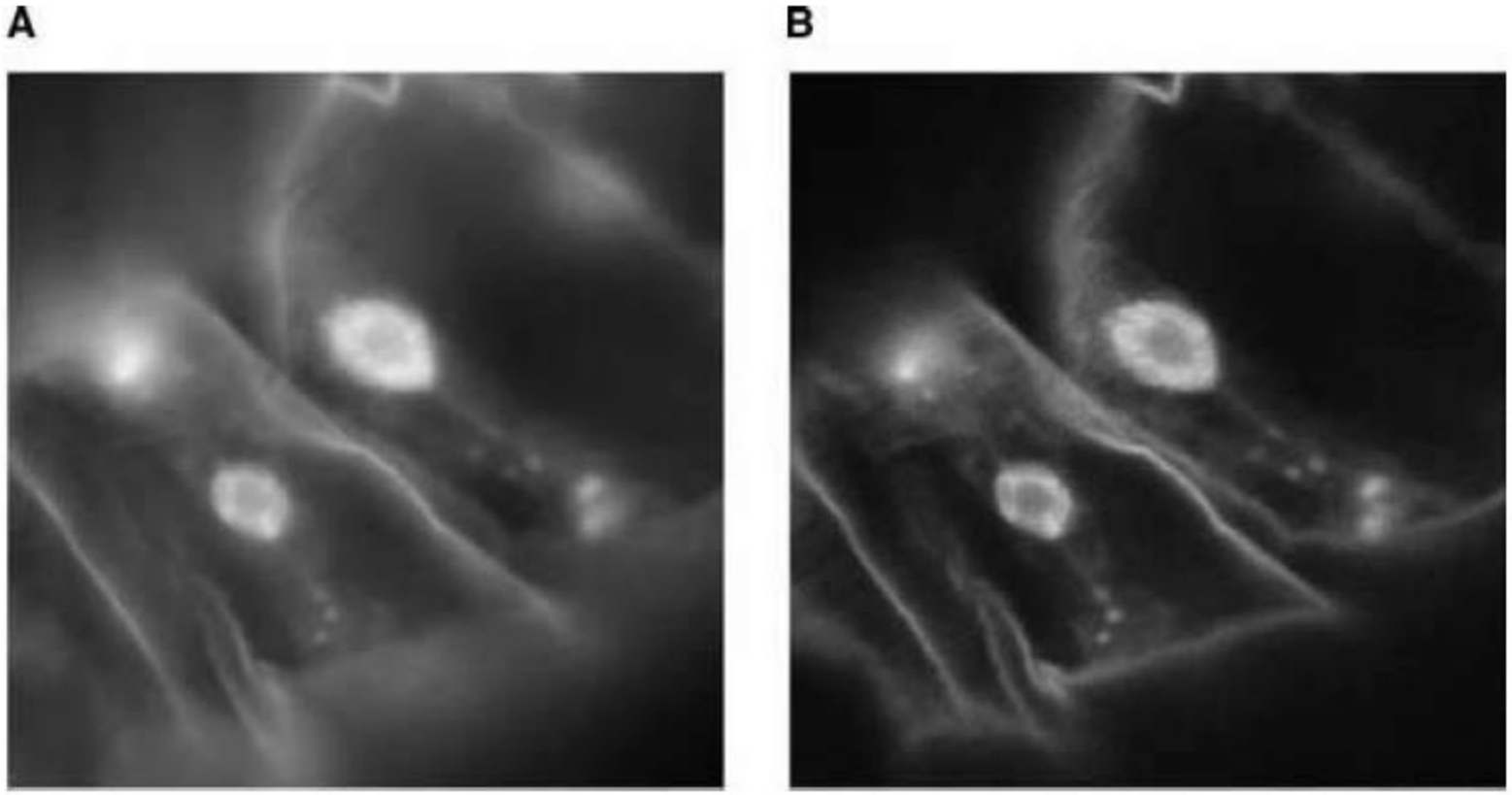
Figure 7. Reprinted with permission from Macmillan Publishers Ltd: [157] copyright 2005. (A) Confocal slice of the (100) plane of a colloidal mixture that forms a CsCl lattice. The red and green spheres represent the two similar sized colloids with opposite charges. The scale bar on the image is 10 μm. (B) Three-dimensional model of what the structure looks like.

Examples in Confocal Microscopy



- Combined confocal and two-photon micrograph of a dividing cell in cultured smooth muscle cells.
- Blue channel – DNA of the dividing nucleus (DAPI), green channel – microtubules (anti-alpha tubulin, FITC), red channels – microtubule organizing centers (arrowed) at the spindle poles (anti-gamma tubulin, Cy3).

Examples in Confocal Microscopy



- Images of cells of spirogyra generated with and without optical sectioning.
- The image in (B) was created using a slit rather than a pinhole for out-of-focus light rejection.
- Most of the haze associated with the cell walls of the filamentous algae is absent, allowing clearer distinction of the different parts.