36

Transformation and gel electrophoresis

- agarose
- polyacrylamide

DNA transformation

- DNA can be exchanged among bacteria by three methods:

(1) • **Transformation**: the most popular techniques of molecular genetics that first discovered in bacteria. Transformation works best when the donor and recipient cells are very closely related.

(2) • **Transfection** is process by which foreign **DNA** is introduced into a cell by a virus or viral vector and is used mainly for mammalian cells

(3) • **Conjugation**: introduction of gene strand through pilus

DNA ل cell wall للخلايا يكون ضعيف حتى تقدر تاخذ ال

competent cell خلية مضيفة

Transformation

1. • Chemical transformation, cell are made competent (able to take up exogenous DNA) by treatment with divalent cations such as calcium chloride, which make the bacterial cell wall more permeable to DNA.

2. • Heat shock is used to temporarily form pores in the cell membrane, allowing transfer of the exogenous DNA into the cell.

3. • Electroporation, a short electrical pulse is used to make the bacterial cell temporarily permeable.

4. • Particle bombardment, is typically used for the transformation of plant cells. Gold or tungsten particles are coated with the DNA construct and physically forced into the cell by gene gun.

Competent cells

- Prepare a small, overnight culture of the bacteria in LB broth. Grow at 37°C without shaking.
- Use 1.0 mL of the overnight culture to inoculate 100 mL of fresh LB broth. This culture is grown with rapid shaking at 37°C until it reaches an OD600 of(0.3-0.4) Transfer the culture to sterile plastic centrifuge tubes. Cool on ice for 10 min.
- Centrifuge at 5000 g for 10 min at 4°C using a refrigerated centrifuge.
- Pour off the supernatant and resuspend cells in 50 mL of cold 0.1M CaCl2. Leave on ice for at least 20 min.
- Centrifuge again as before and resuspend the cells in 20 mL of cold 0.1M CaCl2. Transfer the suspensions to sterile Eppendorf tubes as 0.1ml aliquots and store at -80 degrees C.

ligation
competat cell

DNA transformation protocol

- Thaw all reagents completely on ice.
- Add 1 µL of ligation reaction to thawed competent cells.
- Gently mix by tapping tube of competent cells.
- Incubate reaction on ice for 30 minutes.
- Heat shock the competent cell mixture by incubation for 30 to 60 seconds in a 42°C heating block.
- Incubate tubes on ice for another 10 minutes.
- Add 500 µL of LB media and incubate at 37°C with shaking at 250 rpm.
- Warm selection plates to 37°C an spread 50µL of transformed cells on selection plates.
- Incubate plates at 30°C overnight

مشاکل سے نبرد آزما ہونا بعملیۂ ا

Trouble shooting in transformation

• Few or no colony transformants

Cause	Solution
Wrong antibiotic was used or antibiotic concentration was too high	Ensure the correct antibiotic was applied to plates.• Use only concentration recommended by competent cell or • antibiotic manufacturer.
Competent cell viability is low	Thaw competent cells on ice and use immediately.• Check expiration date of cells.• Do not re-freeze cells.• Do not vortex cells - gently tap to mix.•
DNA insert encodes protein that is toxic to cells	Use a lower incubation temperature (25–30°C).• Use a cell strain and vector designed for tightly controlled • transcription.
Heat-shock incubation too long	Reduce incubation time from 45 to 25 seconds.•
Construct is too big	Use electroporation for vectors over 10 kb.•
Too much ligation mixture was used for the transformation	Ligation reaction components can inhibit transformation. Dilute • ligation reaction with TE buffer (up to 5 times).

Trouble shooting in transformation

بكون ملون لانه plate أزرق

(2) No Plasmid in colony tranformants •

Antibiotic concentration too low	Use antibiotic concentration recommended by manufacturer.
Antibiotic is degraded	•Aliquot working volumes of antibiotic and avoid freeze-thaw cycles. •Add antibiotic to liquid plate media after sufficient cooling.

(3) No insert in colony tranformants plasmids •

Vector re-ligation	•Vector insert ratio not optimal. Use a vector:insert molar ratio from 1:1 to 1:10. Use a DNA concentration of 1-10 µg/ml. •Dephosphorylate DNA with phosphatase to prevent re-ligation

	ligation

Trouble shooting in transformation

4

- **Sequencing of tranformants plasmid reveals wrong plasmid sequence**

DNA insert encodes protein that is toxic to cells ما ممكن يصير نتائج بالبروتين إلى mutation	•Use a lower incubation temperature (25–30°C). •Use a cell strain and vector designed for tightly controlled transcription.
Mutations introduced by initial PCR	•Use a high-fidelity polymerase. Bfu → بدل tac
Inconclusive sequencing artifacts	Repeat sequencing reaction.

Electroporation

- An instrument called an electroporator produces a brief electrical shock that introduces DNA into the cells without killing them

صعقة كهربائية

- Advantages
  - Rapid ✓
  - Requires fewer cells ✓
  - Can be used to introduce DNA into other cell types ✓
  - More efficient process ✓

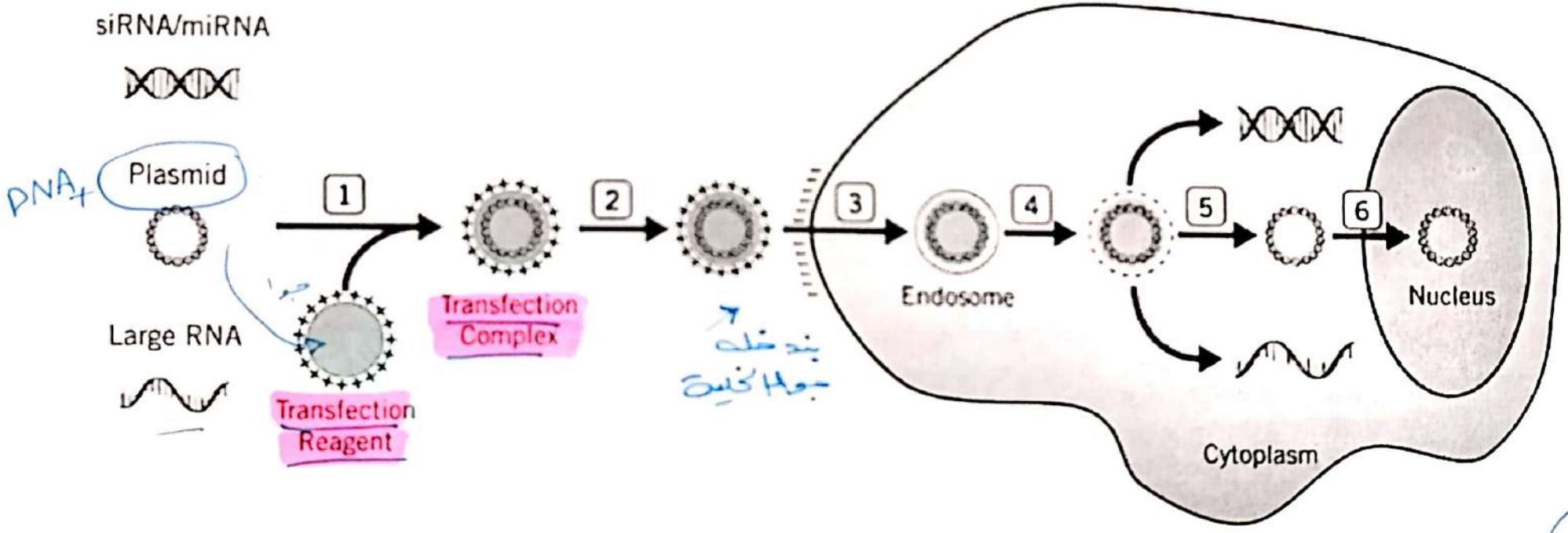
* insert الي عايزه حجم أكتر من 10 Kb بستخدم هاي الطريقة

Transfection

by virus or complex

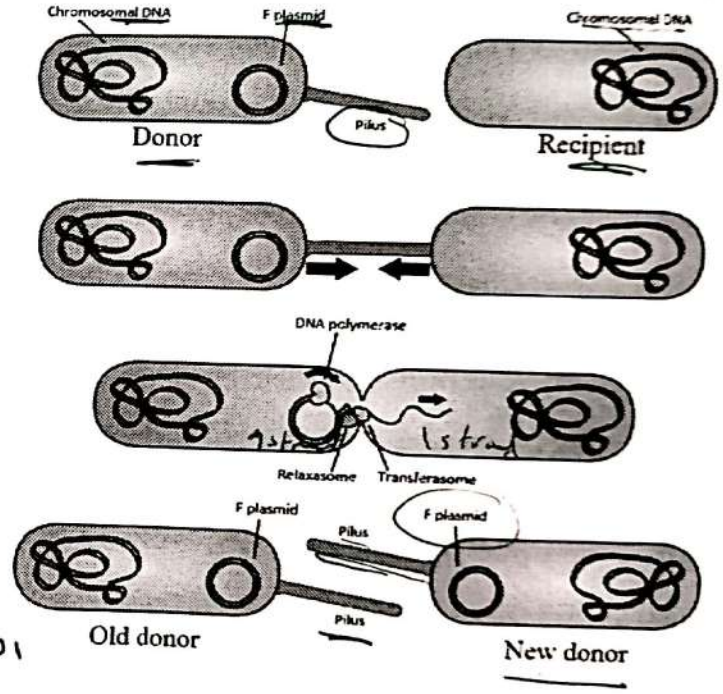
ribosome ال Polymer

- **Transfection** is the way to deliver exogenous nucleic acids such as DNA, RNA or oligonucleotides into cells.
- These nucleic acids can be transported by polymeric or lipidic **transfection** reagents that facilitate their cellular uptake



Conjugation

- Donor cell produces pilus.
- Pilus attaches to recipient cell and brings the two cells together.
- The mobile plasmid is nicked and a single strand of DNA is then transferred to the recipient cell.
- Both cells synthesize a complementary strand to produce a double stranded circular plasmid and also reproduce pili; both cells are now viable donor for the F-factor



Gel electrophoresis

- Electrophoresis a standard method used to separate, identify and purify nucleic acids
- Agarose or polyacrylamide gels can be used as both gels are porous in nature

Agarose gel electrophoresis (AGE)

- Agarose is a natural linear polymer extracted from seaweed that forms a gel matrix by hydrogen-bonding when heated in a buffer and allowed to cool.
- For most applications, only a single-component agarose is needed and no polymerization catalysts are required.
- Agarose gels are simple and rapid to prepare. They are the most popular medium for the separation of moderate and large-sized nucleic acids and have a wide range of separation but a relatively low resolving power, since the bands formed in the gels tend to be fuzzy and spread apart. This is a result of pore size and cannot be largely controlled.

Advantages and disadvantages

- **Advantages**
- Nontoxic gel medium ✓ هي عبارة عن polysaccaride
- Gels are quick and easy to cast ✓ سهلة
- Good for separating large DNA molecules ✓ المشاكل بالصغيرة ؛ !
- Can recover samples by melting the gel (1), digesting with enzyme agarose (2) or treating with chaotropic salts (3)

طرق التخلص منه ال gel والحصول على ال plasmid او insert.i

- **Disadvantages**
- High cost of agarose ✓
- Fuzzy bands ✓ معقدة — For low molecular weight sample „ small fragment
- Poor separation of low molecular weight samples ✓

Agarose concentration

- Agarose gels are normally in the range of 0.2% to 3%.
- If the aim is to separate large DNA fragments, a low concentration of agarose should be used, and if the aim is to separate small DNA fragments, a high concentration of agarose is recommended

Concentration of agarose (%)	DNA size range (bp)
0.2	5000-40000
0.4	5000-30000
0.6	3000-10000
0.8	1000-7000
1	500-5000
1.5	300-3000
2	200-1500
3	100-1000

Polyacrylamide gel electrophoresis

0.8	1000-7000
1	500-5000
1.5	300-3000
2	200-1500
3	100-1000

Polyacrylamide gel electrophoresis (PAGE)

- Polyacrylamide gels are chemically cross-linked gels formed by the polymerization of acrylamide with a cross-linking agent, usually N,N'-methylenebisacrylamide.
- The reaction is a free radical polymerization usually carried out with ammonium persulfate as the initiator and N,N,N',N'-tetramethylethylendiamine (TEMED) as the catalyst.

Polyacrylamide gel electrophoresis (PAGE)

- Although the gels are more difficult to prepare and handle, involving a longer time for preparation than agarose gels, they have major advantages over agarose gels. They have **a greater resolving power, can accommodate larger quantities of DNA** without significant loss in resolution and the DNA recovered from polyacrylamide gels is extremely **pure**
- The **pore size** of the polyacrylamide gels can be altered in an easy and controllable fashion by **changing the concentrations** of the two monomers.
- Polyacrylamide is a **neurotoxin** (when unpolymerized), but with proper laboratory care it is no more dangerous than various commonly used chemicals

- لما يكون unpolymerized مكونه neuro toxic (لأنه بيحتوي free radicals)

polymerization خلاص تكون safe.

Advantages and disadvantages

- **Advantages**
  - Chemically stable cross-linked gel
  - Sharp bands واضحة ومفصولة بشكل جيد
  - Good for separation of low molecular weight fragments
- **Disadvantages**
  - Toxic monomers
  - Gels are tedious to prepare and often leak and many time they need.
  - Need new gel for each experiment

أما بال agarose نفس الجل تقدر استخدمه أكثر من مرة ما دام في مكان نافع.

Polyacrylamide concentration

- With increasing the concentration of monomer in the gel, the pore size decreases in a nearly linear relationship.
- Researchers have settled on Concentration values of 5% (19:1 acrylamide/bisacrylamide) for most forms of denaturing DNA and RNA electrophoresis, and 3.3% (29:1) for most proteins, native DNA and RNA gels.

Acrylamide/Bis Ratio	Gel %	Native DNA/RNA (bp)	Denatured DNA/RNA (bp)
19:1 (5%)	4	100-1500	70-500
	6	60-600	40-400
	8	40-500	20-200
	10	30-300	15-150
	12	20-150	10-100
29:1 (3.3%)	5	200-2000	70-800
	6	80-800	50-500
	8	60-400	30-300
	10	50-300	20-200
	12	40-200	15-150
	20	<40	<40

كلما زاد ال conc. عنا كلما قل ال pore size بالتالي بنفصل أقل M.w, DNA, protein أكل

اذا بدي افصل Denatured DNA/RNA

اكثر ← اذا بدي افصل Native protein DNA/RNA

Electrophoretic buffer systems

- Effective separation of nucleic acids by agarose or polyacrylamide depends upon the **effective maintenance of pH within the matrix**. Therefore, buffers are an integral part of any electrophoresis technique.
- The electrophoretic mobility of DNA is affected by the **composition and ionic strength** (salt content) of the buffer. Without salt, electrical conductance is minimal and DNA barely moves. In a buffer of high ionic strength, electrical conductance is very efficient and a significant amount of heat is generated.
- Different categories of buffer systems for electrophoresis:
  - A dissociating and non-dissociating
  - B continuous and discontinuous.

Dissociating and non-dissociating buffer systems

- Separation on the basis of molecular weight requires the inclusion of denaturing agents, which unfold the DNA or RNA strands and remove the influence of shape on their mobility.
- The most commonly *dissociating buffer systems* used include **urea and formamide** as DNA denaturants.
- Denatured DNA migrates through these gels at a rate that is almost completely dependent on its base composition and sequence.
- Denaturing or dissociating buffer systems for proteins include the use of sodium dodecyl sulfate (SDS). In the SDS-PAGE system, proteins are heat- denatured with SDS before electrophoresis so that the charge-density of all proteins is made roughly equal with net negative charge

Dissociating and non-dissociating buffer systems

- When samples are electrophoresed, proteins separate according to mass alone, with very little effect from compositional differences.
- DNA molecules are negatively charged; therefore the addition of SDS in the gel preparations is only with the aim of enhancing the resolution power of the bands
- In the absence of denaturants, double stranded DNA (dsDNA), like a PCR product, retains its double helical structure, which gives it a rodlike form as it migrates through a gel.
- During the electrophoresis of native molecules in a *non-dissociating buffer system*, separation takes place at a rate approximately inversely proportion to the log10 of their size

Continuous and discontinuous buffer systems

- In the *continuous buffer systems* the identity and concentration of the buffer components are the same in both the gel and the tank. Although continuous buffer systems are easy to prepare and give adequate resolution for some applications, bands tend to be broader and resolution consequently poorer in these gels.
- These buffer systems are used for most forms of DNA-AGE, which commonly contain EDTA (pH 8.0) and Tris-acetate (TAE) or Tris-borate (TBE) at a concentration of 50mM (pH 7.5-7.8).
- TAE is less expensive, but not as stable as TBE. In addition, TAE gives better resolution of DNA bands in short electrophoretic separations and is often used when subsequent DNA isolation is desired. TBE is used for PAGE of smaller molecular weight DNA (MW<2000) and AGE of longer DNA where high resolution is not essential.

essential.

Continuous and discontinuous buffer systems

- *Discontinuous (multiphasic) systems* employ different buffers for tank and gel, and often two different buffers within the gel.
- Discontinuous systems concentrate or "stack" the samples into a very narrow zone prior to separation, which results in improved band sharpness and resolution. The gel is divided into an upper "stacking" gel of low percentage of acrylamide and low pH (6.8) and a separating gel with a pH of 8.8 and much smaller pores (higher percentage of acrylamide).
- The stacking gel prevents any high-molecular-weight DNA present in the sample from clogging the pores at the top of the running gel before low molecular- weight DNA has entered. Both, the stacking and the separating gels, contain only chloride as the mobile anion, while the tank buffer contains glycine as its anion, at a pH of 8.8. The major advantage of the discontinuous buffer system over continuous buffer system is that this gel system can tolerate larger sample volumes

Loading buffer

- This is the buffer to be added to the DNA fragment that will be electrophoresed. This buffer contains glycerol or sucrose to increase the density of the DNA solutions; otherwise, the samples would dissolve in running buffer tank and not sink into the gel pocket.
- The gel loading buffer also contains dyes that facilitate observation of the sample during gel loading and electrophoresis, such as bromophenol blue or xylene cyanol. Because these molecules are small, they migrate quickly through the gel during electrophoresis.
- The components and concentrations of the 6X loading dye usually used are: 0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol; or 0.25% bromophenol blue, 50 mM EDTA, 0.4% sucrose.

Voltage/current applied

- The higher the voltage/current, the faster the DNA migrates. If the voltage is too high, **band streaking**, especially for DNA≥12-15kb, may result. Moreover, high voltage causes a tremendously **increase** in buffer **temperature and current** in very short time.
- The high amount of the heat and current built up in the process leads to the melting of the gel, DNA bands smiling, decrease of DNA bands resolution and fuse blowout. it is highly recommended not exceed 5-8 V/cm and 75 mA for standard size gels or 100 mA for minigels.
- When the voltage is too low, the mobility of small (≤1kb) DNA is reduced and band broadening will occur due to dispersion and diffusion.

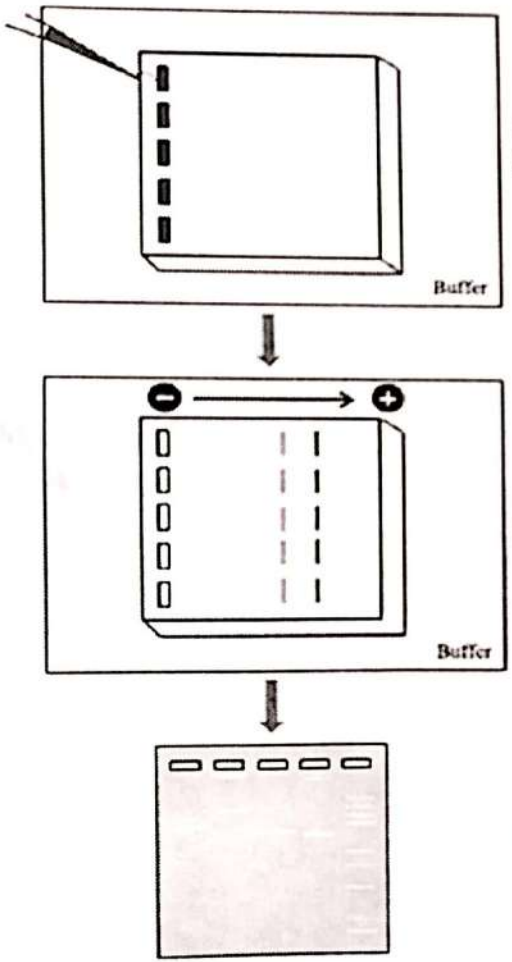
Visualizing the DNA

- After the electrophoresis has been completed there are different methods that may be used to make the separated DNA species in the gel visible to the human eye.

1. **Ethidium bromide staining (EBS)**

- The localization of DNA within the agarose gel can be determined directly by staining with low concentrations of intercalating fluorescent ethidium bromide dye under UV light. The dye can be included in both, the running buffer tank and the gel, the gel alone, or the gel can be stained after DNA separation.
- For a permanent record, mostly instant photos are taken from the gels in a dark room.
- Note that ethidium bromide is a potent mutagen and moderately toxic after an acute exposure. Therefore, handle it with caution.

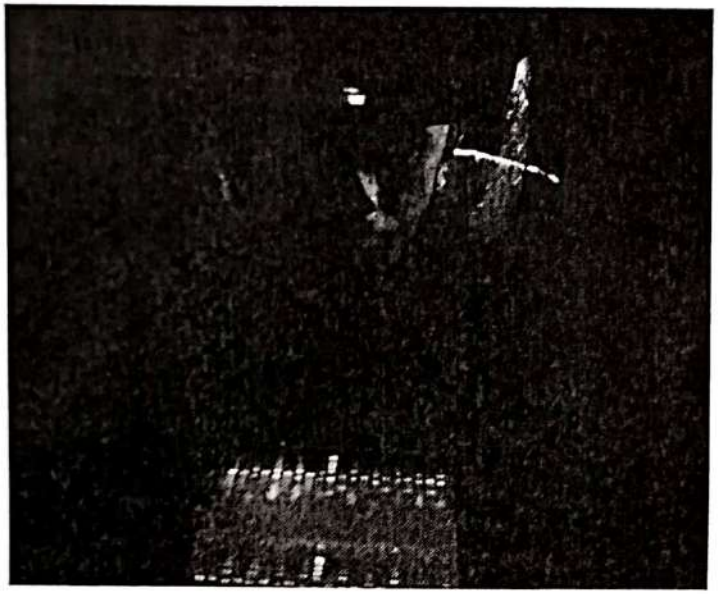
Ethidium bromide staining



DNA/RNA samples and marker loaded in the horizontal gel electrophoresis system

Direction of migration of DNA/RNA samples in horizontal gel electrophoresis system

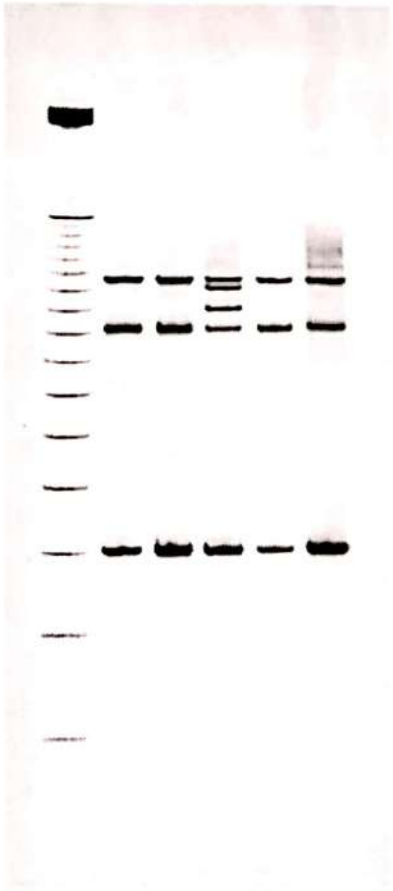
Agarose gel after ethidium bromide staining



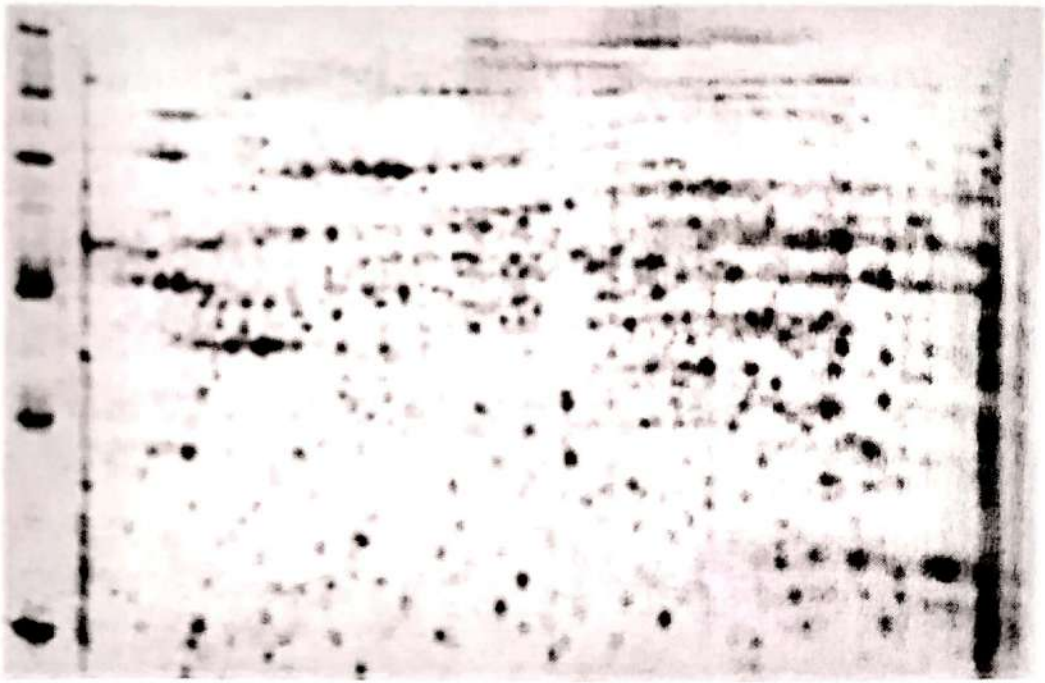
Silver staining (SS)

- Silver staining is a highly sensitive method for the visualization of nucleic acid and protein bands after electrophoretic separation on polyacrylamide gels.
- Nucleic acids and proteins bind silver ions, which can be reduced to insoluble silver metal granules. Sufficient silver deposition is visible as a dark brown band on the gel. Silver staining protocols include many steps:
- i) fixation to get rid of interfering compounds,
- ii) silver impregnation with either a silver nitrate solution or a silver-ammonia complex solution,
- iii) rinses and development to build up the silver metal image, and
- iv) stop and rinse to end development prior to excessive background formation and to remove excess silver ion

Silver staining



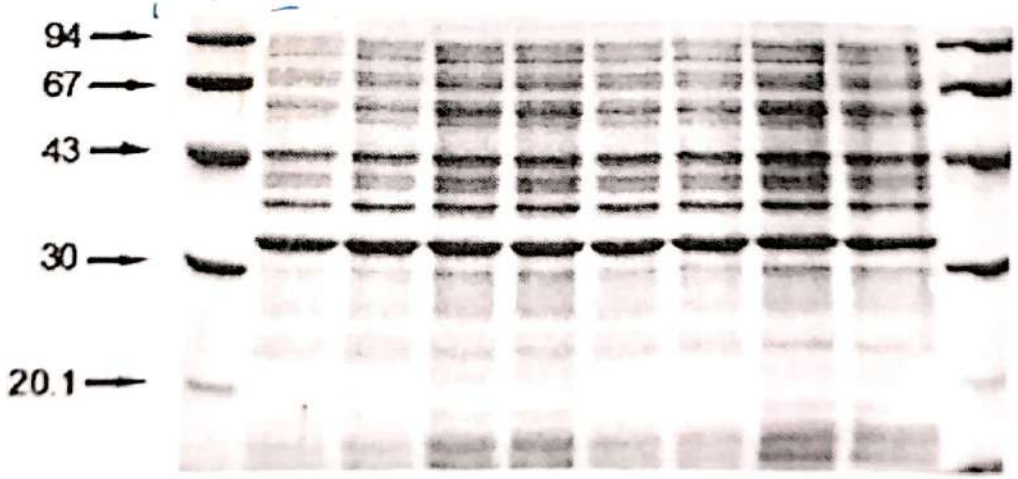
For DNA



For protein

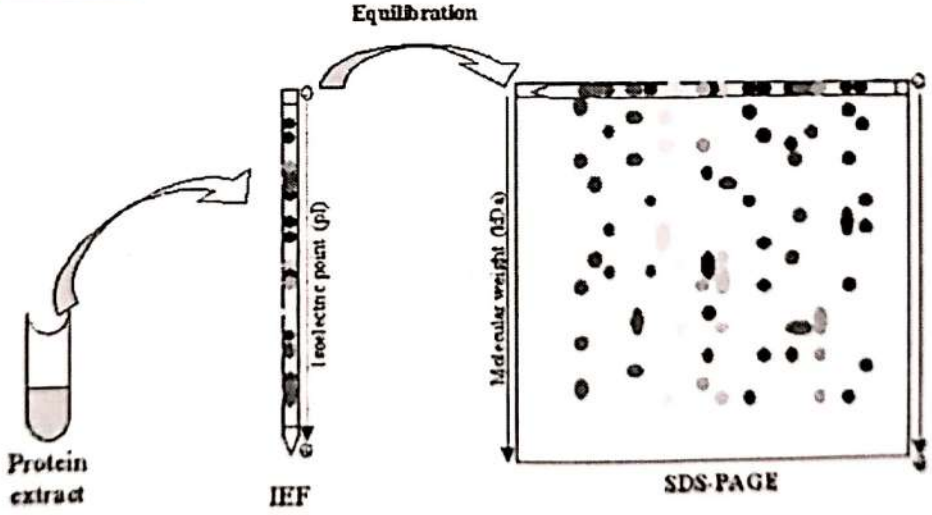
Coomassie staining

- The Coomassie blue staining allows detecting up to 0.2 to 0.6 µg of protein, and is quantitative (linear) up to 15 to 20 µg.
- It is often used in methanol-acetic acid solutions and is discolored in isopropanol-acetic acid solutions

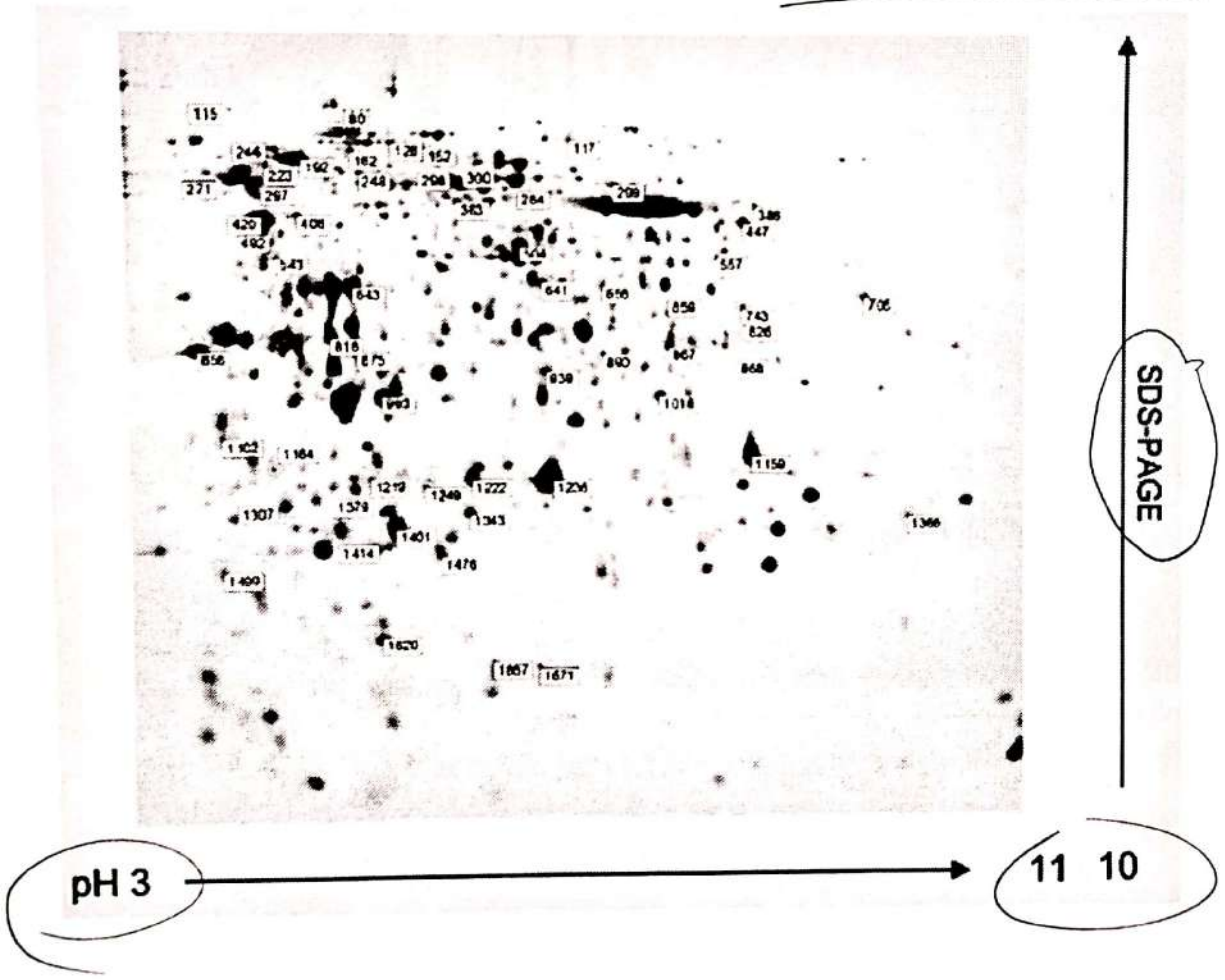


Two-dimensional gel electrophoresis

- Two-dimensional gel electrophoresis (2-DE) is based on separating a mixture of proteins according to two molecular properties, one in each dimension.
- The most used is based on a first dimension separation by
1. isoelectric focusing and second dimension according to
2. molecular weight by SDS-PAGE.



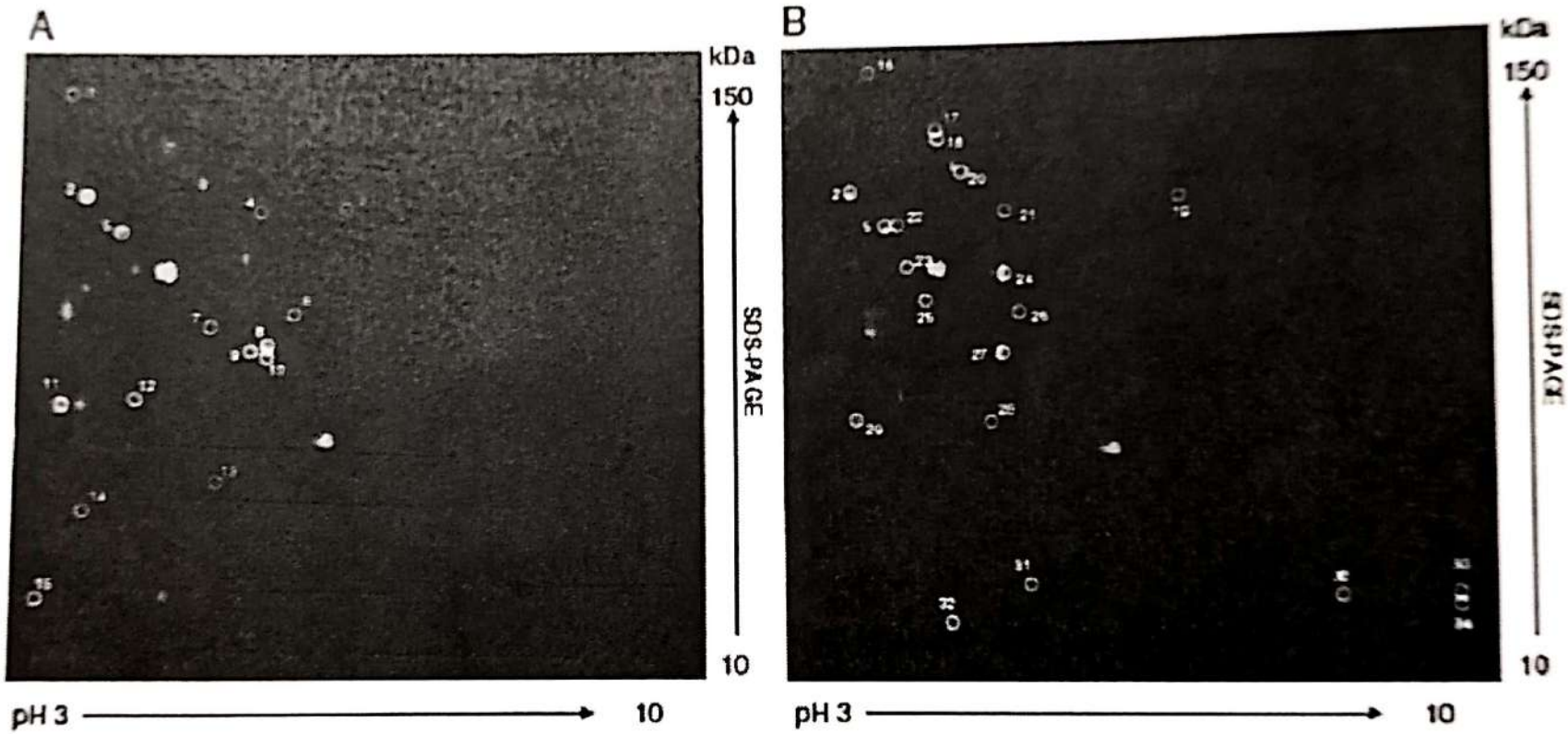
Two-dimensional gel electrophoresis organism protein fingerprinting



Two-dimensional fluorescence difference gel electrophoresis (2-D DIGE)

- A method that labels protein samples prior to 2-DE, enabling accurate analysis of differences in protein abundance between samples.
- The technology is based on the specific properties of fluorescent cyanine dyes that are spectrally resolvable and size- and charge-matched
- Identical proteins labeled with each of the three dyes (Cy2, Cy3 and Cy5) will migrate to the same position on a 2-DE gel. This ability to separate more than one sample on a single gel permits the inclusion of up to two samples and an internal standard (internal reference) in every gel.
- The internal standard is prepared by mixing together equal amounts of each sample in the experiment and including this mixture on each gel

Two-dimensional fluorescence difference gel electrophoresis (2-D DIGE)



Protein identification by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry

- Mass spectrometry is a technique to analyze with high accuracy the composition of different chemical elements and atomic isotopes splitting their atomic nuclei according to their mass-charge ratio (m/z).
- It can be used to identify different chemical elements that form a compound or to determine the isotopic content of different elements in the same compound