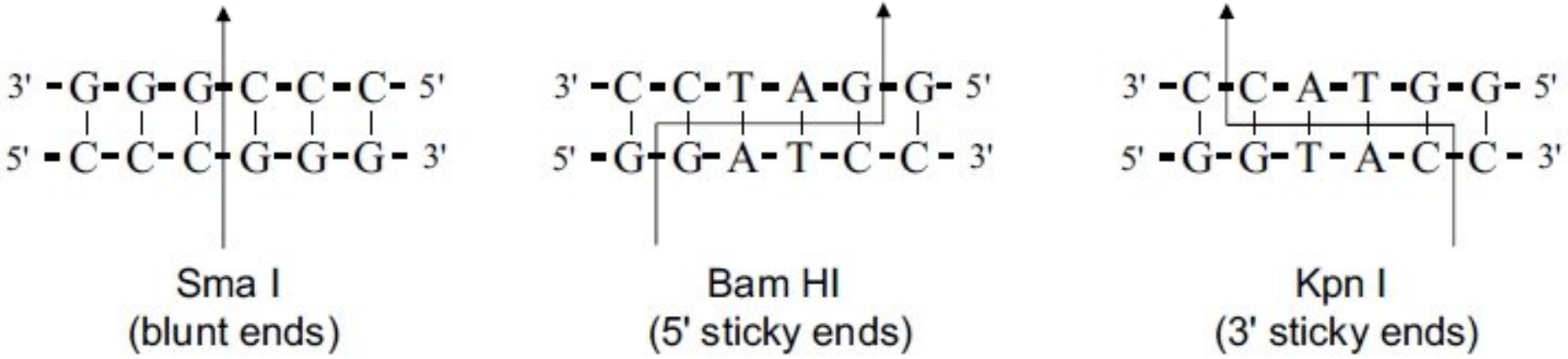
Experimental Techniques

Elementary Techniques

Restriction Enzymes and Gel Electrophoresis

- Genes of a genome are in chromosomes – to isolate a single gene the long DNA must be cut into pieces.
- Restriction endonucleases recognize specific short sequences of DNA and cut them at these specific positions – mostly palindromes.

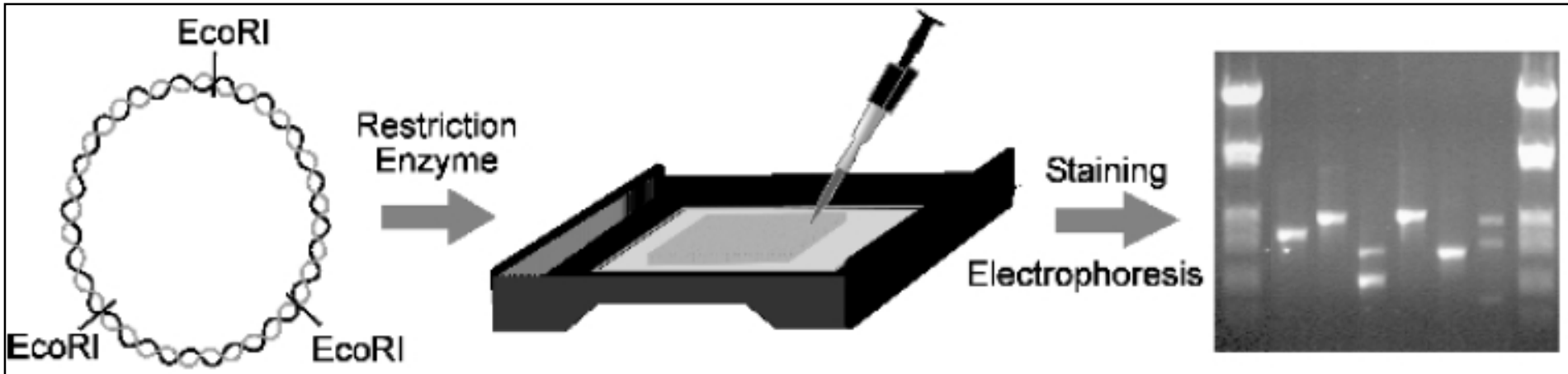


- Sticky ends have short stretches of single stranded DNA – useful for joining two DNA.
- REs - named after the organism in which they where discovered.

Name	Organism	Recognition sequence
Alu I	*Arthrobacter luteus*	5′ A G ↑ C T 3′
Bam HI	*Bacillus amyloliquefaciens*	5′ G ↑ G A T C C 3′
Bgl II	*Bacillus globigii*	5′ A ↑ G A T C T 3′
Eco RI	*Escherichia coli*	5′ G ↑ A A T T C 3′
Eco RV	*Escherichia coli*	5′ G A T ↑ A T C 3′
Hind III	*Haemophilus influenzae*	5′ A ↑ A G C T T 3′
Kpn I	*Klebsiella pneumonia*	5′ G G T A C ↑ C 3′
Pme I	*Pseudomonas mendocina*	5′ G T T T ↑ A A A C 3′
Sau96 I	*Staphylococcus aureus*	5′ G ↑ G N C C 3′
Sma I {Isoschizomers}	*Serratia marcescens* {same seq. diffrnt. cut}	5′ C C C ↑ G G G 3′
Xma I	*Xanthomonas malvacearum*	5′ C ↑ C C G G G 3′

- REs generate reproducibly specific fragments from large DNA.
- Restriction fragments, when separated according to size, form a specific pattern that represents a fingerprint of the digested DNA – can be used to orthologous genes from different organisms.
- By using the right RE, it is possible to cut the fragment with a gene of interest.
- Such fragments can be separated using electrophoresis.

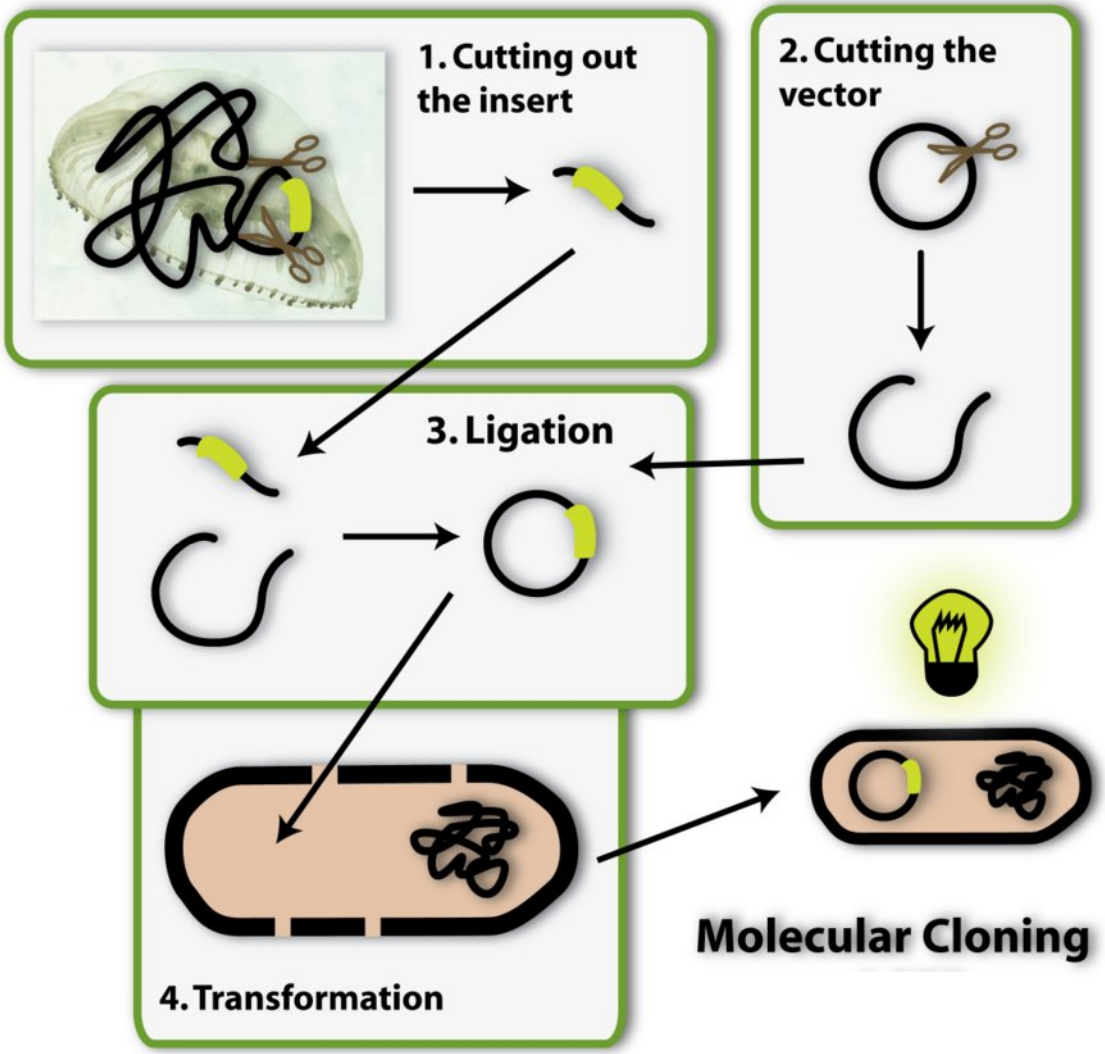
- Electrophoresis separates molecules that differ in size or charge - by applying an electrical field to the charged molecules.
- Electric field is created using two electrodes.
- Nucleotides of DNA (or RNA) are negatively charged and hence move from ~~the anode to the cathode~~ cathode to anode.
- positively charged cations move towards the cathode -negatively charged anions move away from it
- Separation is done using a gel matrix which gives a sieving effect.
- Pore size of the gel controls the size of the DNA fragments that can be separated.
- Agarose gels are used for DNA sizes between 0.5 kb and 20 kb - polyacrylamide gels could be used for smaller DNA.
- Pulse-field electrophoresis – Large DNA fragments are separated using this technique – varies the direction of the electric field periodically.
- Because of the oscillating field, the molecules have to re-orientate themselves – easier for the smaller fragments, but the larger ones lag behind and get separated.
- Dye for staining – ethidium bromide. Intercalates between DNA bases and when exposed to UV light fluoresces a bright orange.



Cloning Vectors and DNA Libraries

- Cloning – create an identical copy of a DNA molecule (or) isolate a specific DNA fragment from the total DNA content of a cell – and amplify the DNA.
- For amplification, a restriction fragment has to be inserted into a self-replicating genetic element. – A virus or a plasmid.
- The genetic elements are called cloning vectors and the amplified DNA is said to be cloned.
- Plasmids as vectors – The insertion process requires that the DNA to be cloned and the plasmid used are to be cut with the same restriction enzyme – further, the plasmid vector should have only one recognition site for this enzyme.

- Restriction digestion creates a linearized plasmid that has the same type of sticky ends as the DNA to be cloned.
- Vector and the digested DNA are now mixed at the right concentration and temperature – the complementary sticky ends base pair and form a new recombinant DNA molecule.
- Initially, the resulting molecule is held together only by hydrogen bonds. It is made permanent by ligase which makes covalent bonds between the phosphodiester backbones of the DNA.
- Vector is introduced into bacterial cells, which are grown in culture. Every time the bacteria double the recombinant plasmids also double.
- See fig. in next slide
- Upper size limit for the DNA one can clone into a plasmid vector is about 10 kb - other vectors are required
- Lambda phage – 20 kb, Cosmids – 45 kb, Yeast artificial chromosomes (YAC) – one million bases.
- DNA library – cloning all fragments of DNA into vectors. Shot-Gun cloning – every gene will be in a vector.

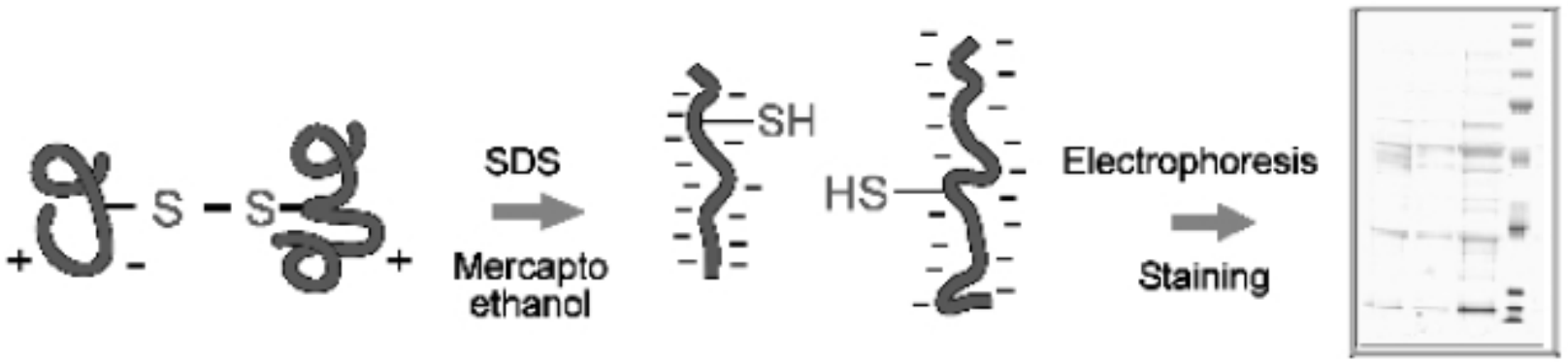

1. Cutting out the insert
2. Cutting the vector
3. Ligation
4. Transformation
Molecular Cloning

- Genomic DNA library – directly created from the genetic material of an organism. Restriction enzymes cut DNA in random, hence there is a possibility that a gene of interest may be cut in the middle.
- cDNA library, circumvents the problems. This technique uses the mRNA pool of the cells or tissue of interest.
- mRNA molecules represent the coding regions of the genes and contain neither introns nor inter-gene junk DNA.
- Using the enzyme reverse transcriptase, mRNA can be converted into complementary DNA (cDNA).
- cDNAs are important because,
  (1) they contain only coding regions;
  (2) they are tissue-specific since they represent a snapshot of the current gene expression pattern; and
  (3) the frequency of specific clones in the library is an indicator of the expression level of the corresponding gene.

1D and 2D Protein Gels

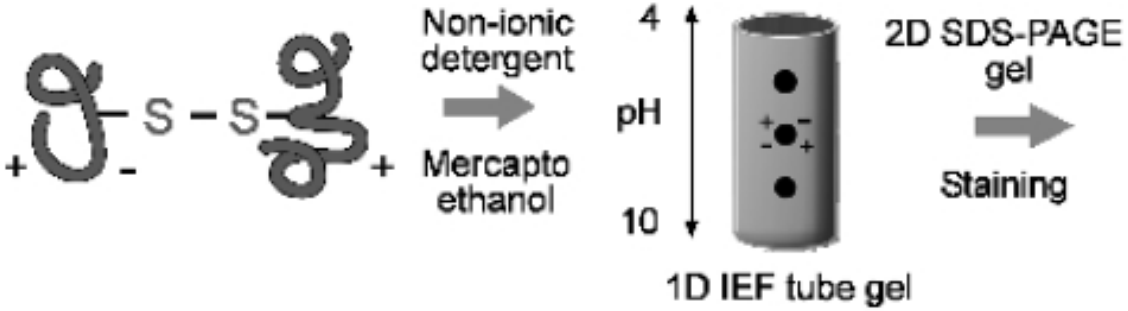
- DNA molecules carry a negative that is proportional to the length of the DNA, since the charge is controlled by the phosphodiester backbone. Small DNA – low negative charge, big DNA – big charge
- For proteins, the net charge varies as it depends on the amount and type of charged amino acids that are in the protein.
- If proteins are separated in native form, their velocity is difficult-to-predict.
- To make the negative charge dependent on size, a detergent – sodium dodecyl sulfate (SDS) is used.
- (1) the negative charge of the protein/detergent complex is proportional to the protein size because the number of SDS molecules that bind to a protein is proportional to the number of its amino acids,
- (2) all proteins denature and adopt a linear conformation, and
- (3) even very hydrophobic, normally insoluble proteins can be separated by gel electrophoresis.
- Under these conditions the separation is based on size.

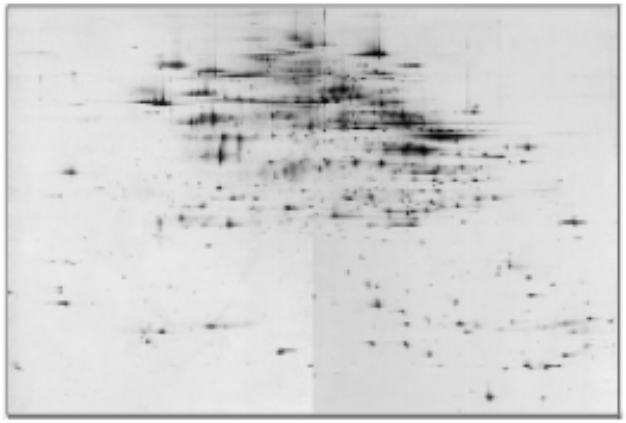
- For proteins, Acrylamide monomers are polymerized to give a polyacrylamide gel.
- During polymerization, the degree of cross-linking and thus the pore size of the gel can be controlled, based on the size of protein.
- Proteins often contain sulfide bridges between the same or different polypeptide.
- A reducing substance, mercaptoethanol, is often added, which reduces the sulfide bridges to sulfhydryl groups.
- This linearizes single peptides and separates multi-subunit complexes into the individual proteins.



- However, a cell or subcellular fraction contains hundreds or thousands of different proteins.
- Hence, individual bands overlap if they have the same size and proteins cannot be separated clearly.
- Two-dimensional polyacrylamide gel electrophoresis - to separate the proteins in a second dimension according to a property other than size.
- Isoelectric focusing (IEF) is such a separation technique.
- The net charge of a protein depends on the number of charged amino acids, and also on the pH of the medium.
- At low pH proteins get a high positive charge and at high pH proteins get a high negative charge.
- Accordingly, for each protein a pH exists that results in an equal amount of negative and positive charges. This is the isoelectric point of the protein, at which it has no net charge.
- For isoelectric focusing, the proteins are treated with a nonionic detergent so that the proteins unfold but retain their native charge distribution.

- They are placed onto a rod-like tube gel, which has been prepared such that it has a pH gradient from one end to the other.
- After a voltage is applied, the proteins travel until they reach the pH that corresponds to their isoelectric point.
- For the second dimension, the tube gel is soaked in SDS and then placed on top of a normal SDS slab gel.
- A voltage is applied perpendicular to the direction of the first dimension and the proteins are now separated according to size.
- The result is a two dimensional (2D) distribution of proteins in the gel – makes it possible to separate all proteins of a typical prokaryote in a single experiment!.



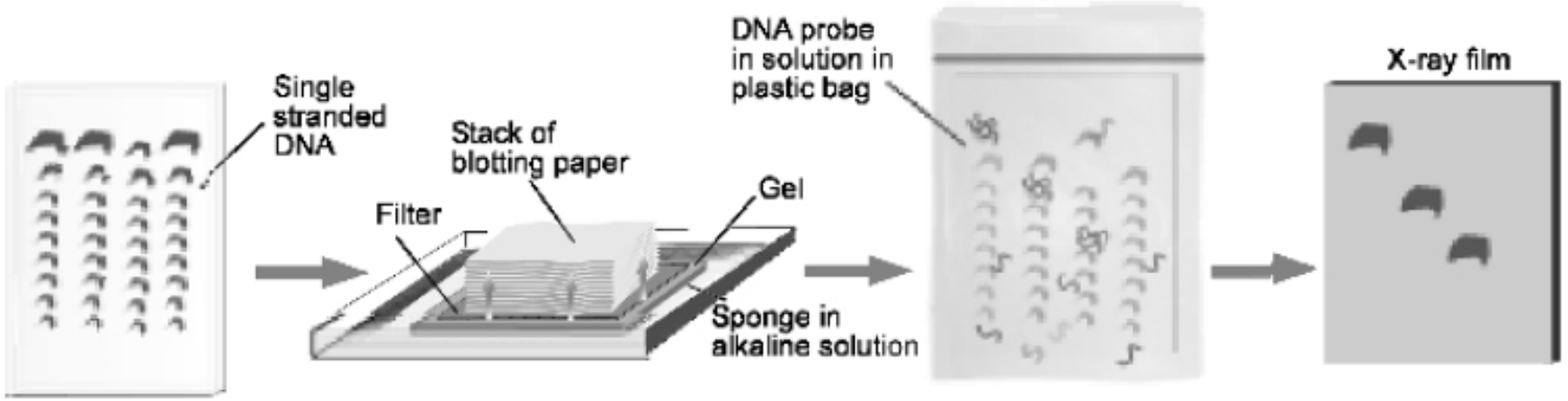


Hybridization and Blotting Techniques

- Used for the specific recognition of a probe and target molecule.
- A short fragment of DNA, the probe, is labeled in such a way that it can later easily be visualized (radioactive or fluorescent labels).
- The probe is incubated with the target sample and allowed for the complementary base-pairing of the probe and target molecule.
- Then, the location of the probe shows the location and existence of the sought-after target molecule.

Southern Blotting

- Used to analyze complex DNA mixtures – (method developed by Southern).



- Following gel electrophoresis, the DNA fragments are treated with an alkaline solution to make them single-stranded.
- The nitrocellulose or nylon membrane is sandwiched between the gel and a stack of blotting paper and the DNA is transferred onto the membrane through capillary forces.
- Finally, the membrane is incubated with the labeled DNA probe (here, radioactive labeling) and the bands are then visualized by X-ray film exposure.

Northern Blotting

- Northern blotting is very similar to Southern blotting. The only difference is that mRNA, not DNA, is used for blotting.
- Used not only to verify the existence of a specific mRNA but also to estimate the amount of the corresponding protein via the amount of mRNA.

Western Blotting

- For proteins – antibodies that are against the desired protein are used for blotting.

- Once the protein is transferred to the nitrocellulose membrane, it is incubated with the primary antibody.
- The primary antibody recognizes the protein and forms antibody-protein complex with the protein of interest.
- Then, the membrane is incubated with the so-called secondary antibody, which is an antibody against the primary antibody.
- If the primary antibody was obtained by immunizing a rabbit, the secondary antibody could be a goat-anti-rabbit antibody.
- This is an antibody from a goat that recognizes all rabbit antibodies.
- The secondary antibody is chemically linked to an enzyme, such as horseradish peroxidase - catalyzes a chemiluminescence reaction.
- Exposure of an X-ray film finally produces bands, indicating the location of the protein-antibody complex.
- The intensity of the band is proportional to the amount of protein.
- The secondary antibody serves as signal amplification step. The enzyme is not linked directly to the primary antibody.

Further Protein Separation Techniques

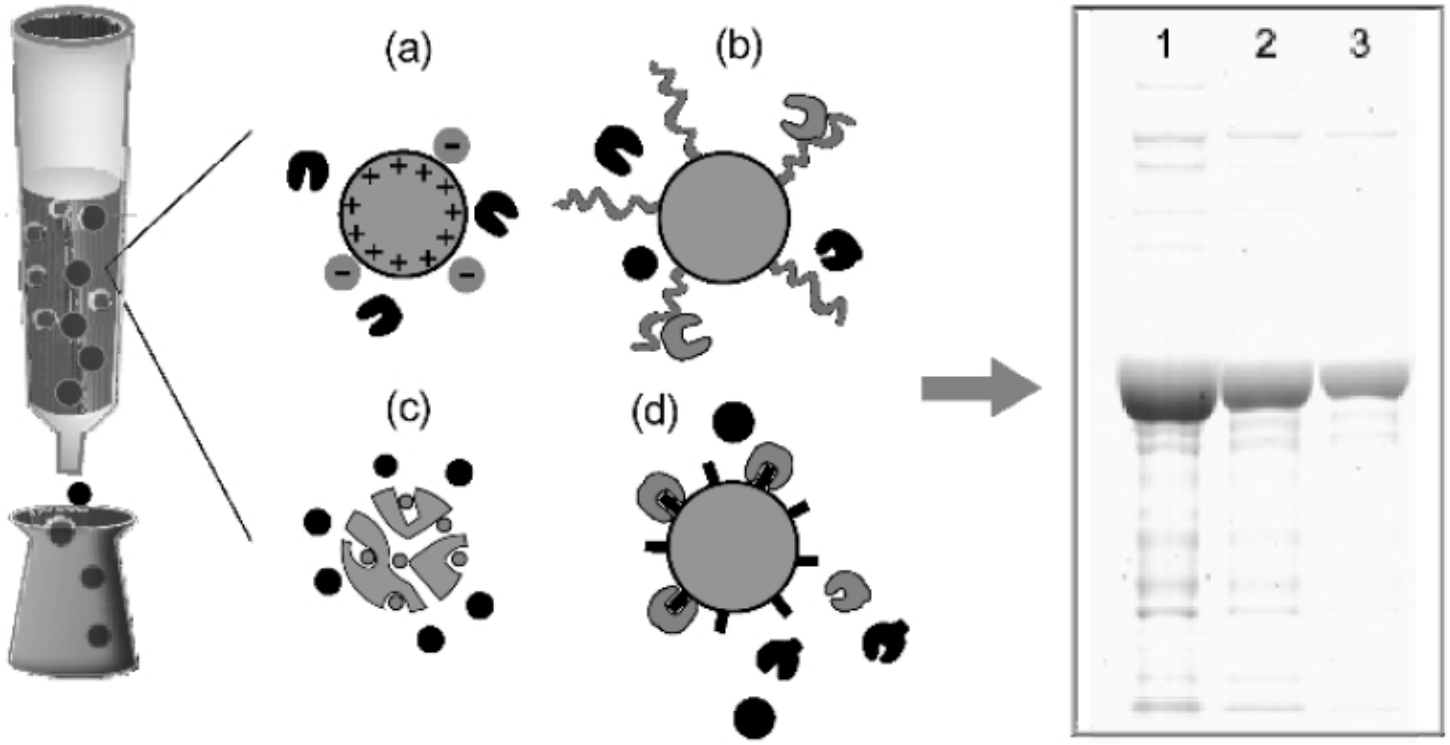
Centrifugation

- For the separation of cell components. Larger the object, faster it moves to the bottom.
- Low-speed centrifugation (around 1000-fold gravitational acceleration, g) collects cell fragments and nuclei in the pellet.
- Medium speeds (50,000 g) cell organelles and ribosomes are collected,
- Ultrahigh speeds (up to 500,000 g) - typical enzymes are in pellet.
- Sedimentation rate for macromolecules is measured in Svedberg units, S, after Theodor Svedberg, (invented ultracentrifugation).
- Ribosomal subunits, got their name from their sedimentation coefficient (40S subunit and 60S subunit).
- Because the friction is controlled not only by the size of the particle but also by its shape, S values are not additive.
- The complete ribosome (40S plus 60S) sediments at 80S and not at 100 S.

- Sedimentation rate is zero if the densities of the particle and the surrounding medium are identical.
- Basis for the equilibrium centrifugation - the medium forms a stable density gradient (caused by the gravitational forces)
  – the density of the studied particles should lie within the density range of the gradient.
- The necessary density gradients are typically formed with sucrose or cesium chloride (CsCl).

Column Chromatography

- A column (glass, a few centimeters wide and a few dozen centimeters tall) is filled with a solid carrier material and the protein mixture is placed on top of it.
- Then a buffer is slowly washed through the column and takes the protein mixture along with it.
- Different proteins are held back to a different degree by the column material and arrive at different times at the bottom.
- The eluate can be fractionated and tested for the presence of the desired protein.



- Column chromatography - a protein mixture is placed on top of the column material and then eluted with buffer.
- Different types of material are available to separate the proteins
- according to (a) charge, (b) hydrophobicity, (c) size, or (d) affinity to a specific target molecule.
- For affinity chromatography the column is loaded with the protein mixture in the first step. The proteins of interest bind, while the other proteins pass through the column.

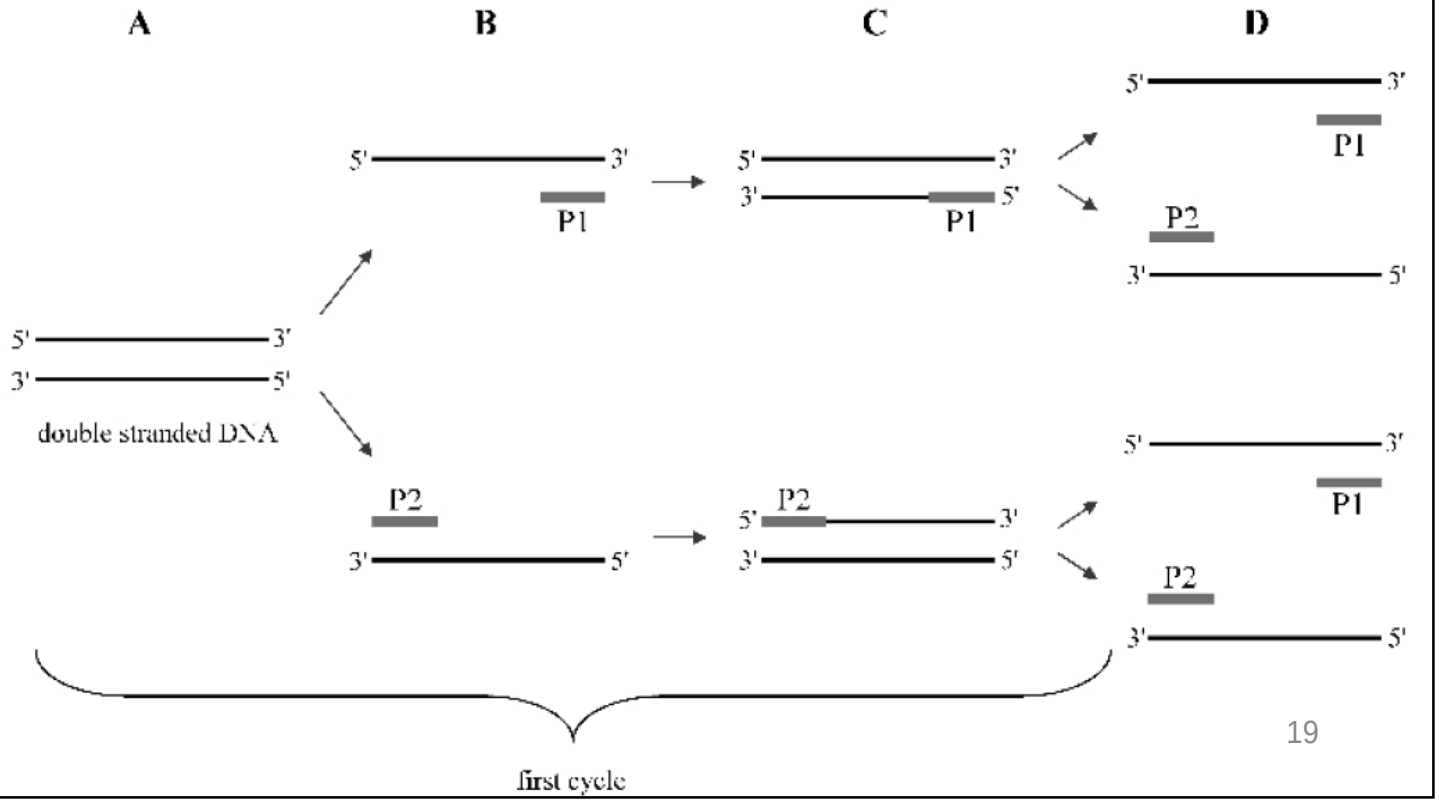
- In the second step, the elution process is started by using a high-salt or high-pH buffer that frees the bound protein from the column.
- Major improvement regarding speed and separating power is achieved through the high performance liquid chromatography (HPLC).
- The columns are much smaller and the carrier material is packed more densely and homogenously.
- To achieve reasonable buffer flow rates, very high pressures (up to several hundred atmospheres) are needed.

Advanced Techniques

- Polymerase chain reaction (PCR) allows billion-fold amplification of specific DNA fragments (typically up to 10 kbp).
- A pair consisting of short oligonucleotides (15–25 bp), the primers, is synthesized chemically such that they are complementary to an area upstream and downstream of the DNA of interest.

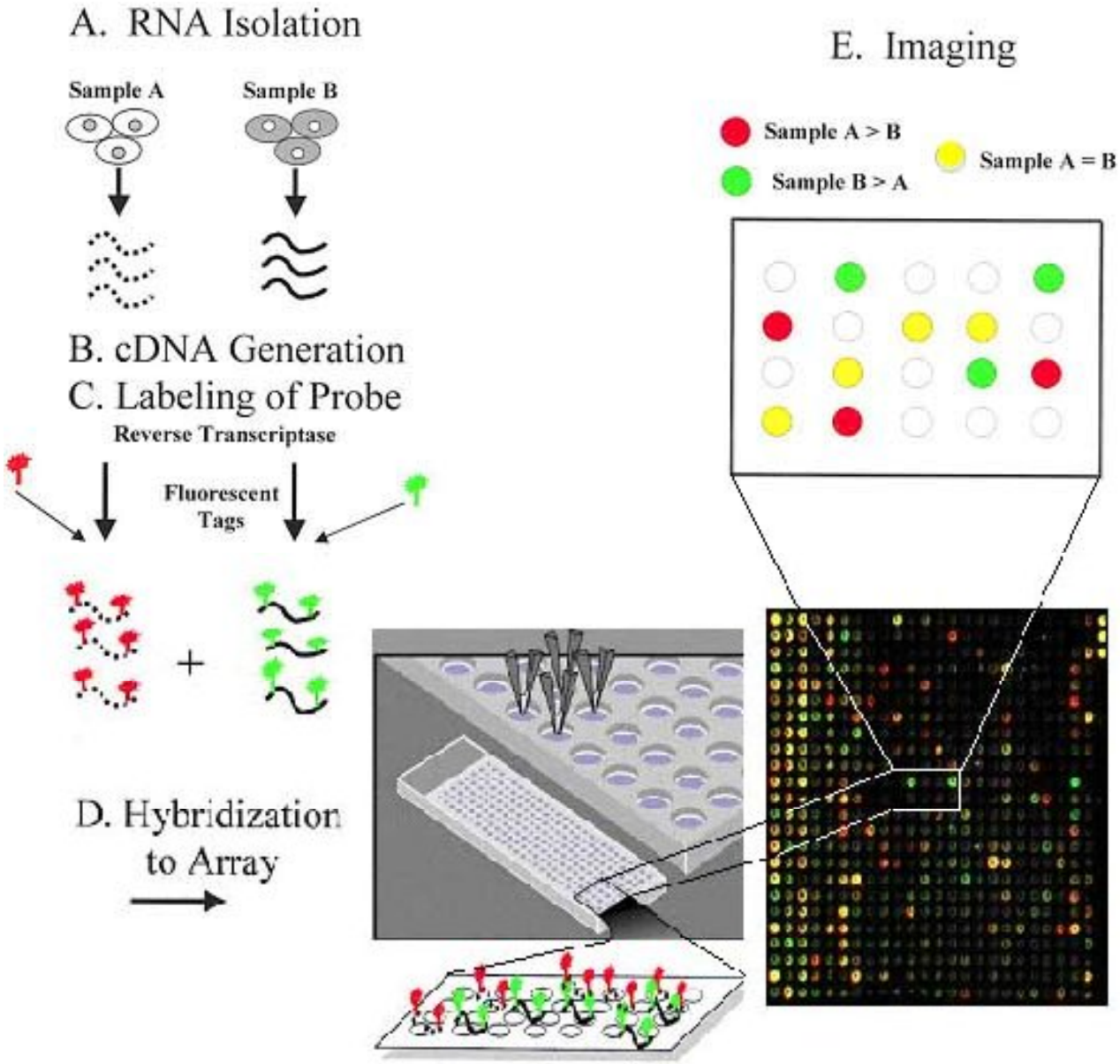
- DNA is made single stranded by heating (denaturation),
- During cooling phase primers are added to the mixture - Primers hybridize to the single-stranded DNA (annealing),
- DNA polymerase extends the primers, doubling the copy number of the desired DNA fragment (amplificatin). This concludes one PCR cycle.
- Each additional cycle (denaturation, annealing, and amplification) doubles the existing amount of DNA that is located between the primer pair.

P1 and P2 are primers upstream and downstream of the required gene.



DNA chips (microarrays)

- High-throughput analysis of gene expression
- Allow to monitor the expression of several thousand genes in a single experiment.
- A global picture of the cellular activity – idea is similar to that of systems biology – hence important.



- Construct the chip from a DNA library – Inserts of individual clones are amplified by PCR and spotted in a regular pattern on a glass slide or nylon membrane.
- Extract total mRNA from two samples that we would like to compare (e. g., yeast cells before and after osmotic shock).
- Using reverse transcriptase, transcribe the mRNA to cDNA and label with different fluorescent dyes. (red and green dyes)
- Incubate the cDNAs with the chip where they hybridize to the spot that contains the complementary DNA fragment.
- Wash – then measure fluorescence intensities for red and green.
- Red or green spots indicate a large excess of mRNA from one or the other sample, while yellow spots show that the amount of this specific mRNA was roughly equal.
- Very low amounts of both mRNA samples result in dark spots.
- Further, the intensities can be quantified and used for constructing clustergrams – useful to test whether related genes are expressed togather .

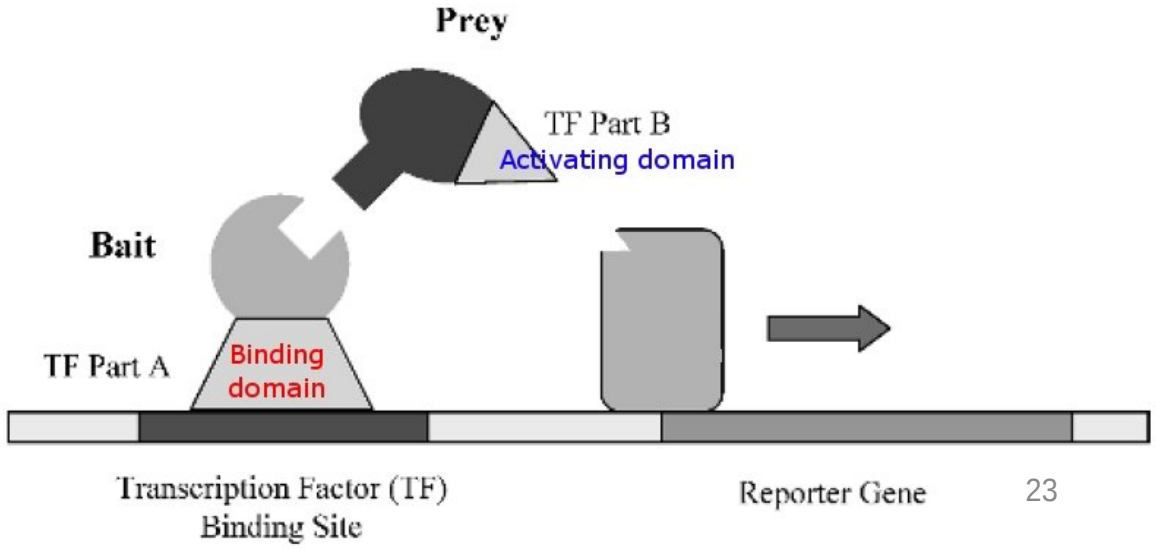
Protein chips

- The function of the genes is through proteins and not by the mRNAs – hence, Proteins chips are better than DNA chips.
- Proteins are fixed on a glass slide and incubated with interaction partners like,
  (1) other proteins (to study protein complexes),
  (2) antibodies (to identify the recognized antigens),
  (3) DNA (to find DNA-binding proteins),
  (4) drugs.
- More problems – proteins are not as uniform as DNA – recombinant proteins may not be expressed in sufficient quantity – different proteins react with different conditions like temperature, ionic strength etc.
- Protein chips do not give reproducible results – a lot of false positives result.
- Hence, the technique needs further improvement.

Yeast Two-hybrid (Y2H) system

- High-throughput detection of protein-protein interactions.
- Some transcription factors (TF) (like yeast Gal4 gene) have a modular design - DNA-binding domain is separated from the activating domain.
- To test whether two proteins (named bait and prey) interact, bait is fused to the DNA-binding domain and prey is fused to activating domain.
- If bait and prey interact, the two domains of the TF come close enough to stimulate the expression of a reporter gene.
- If bait and prey do not interact, the reporter gene is silent.

Although the detection occurs in yeast, the bait and prey proteins can come from any organism

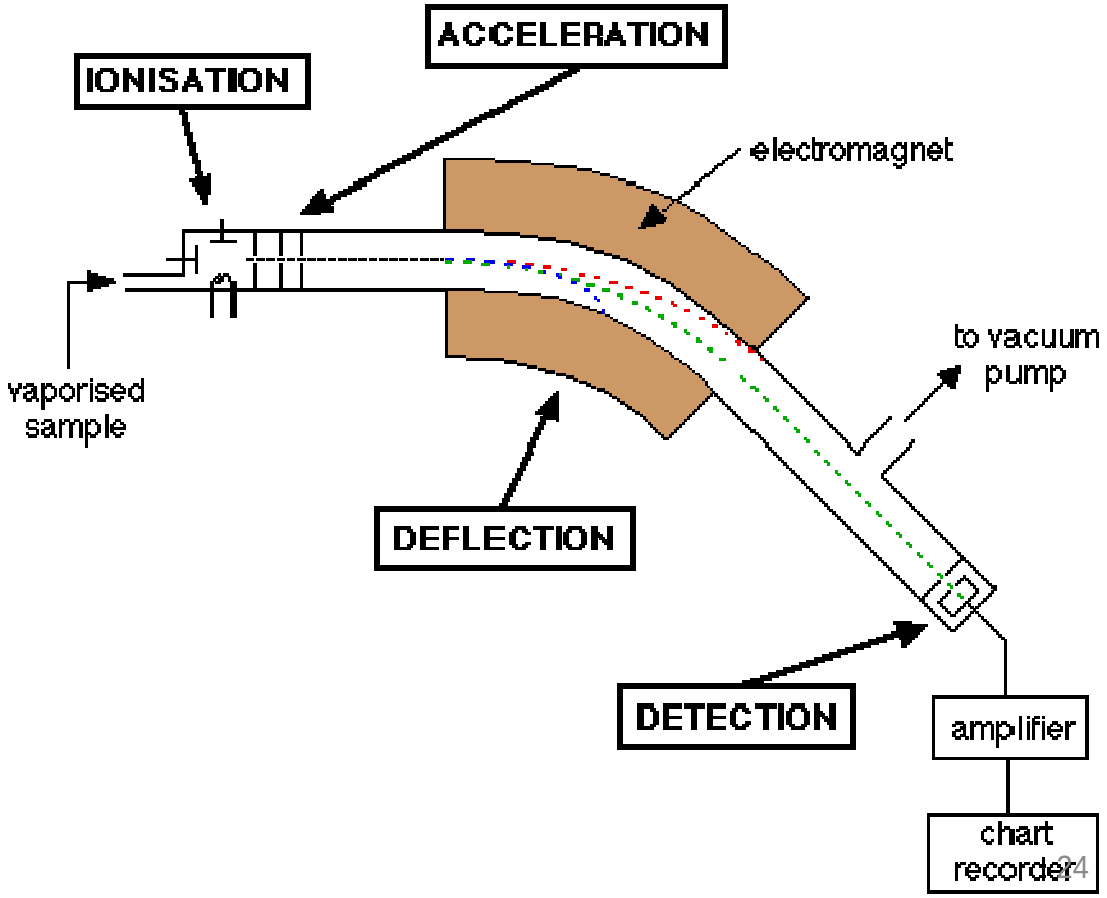


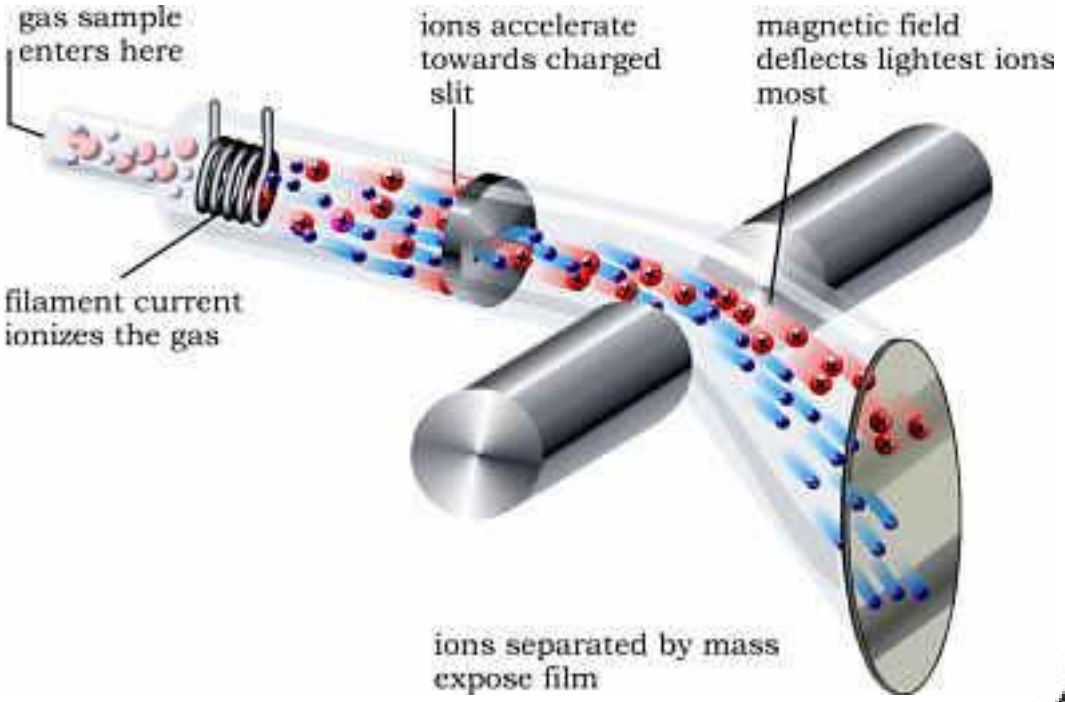
Mass Spectrometry (MS)

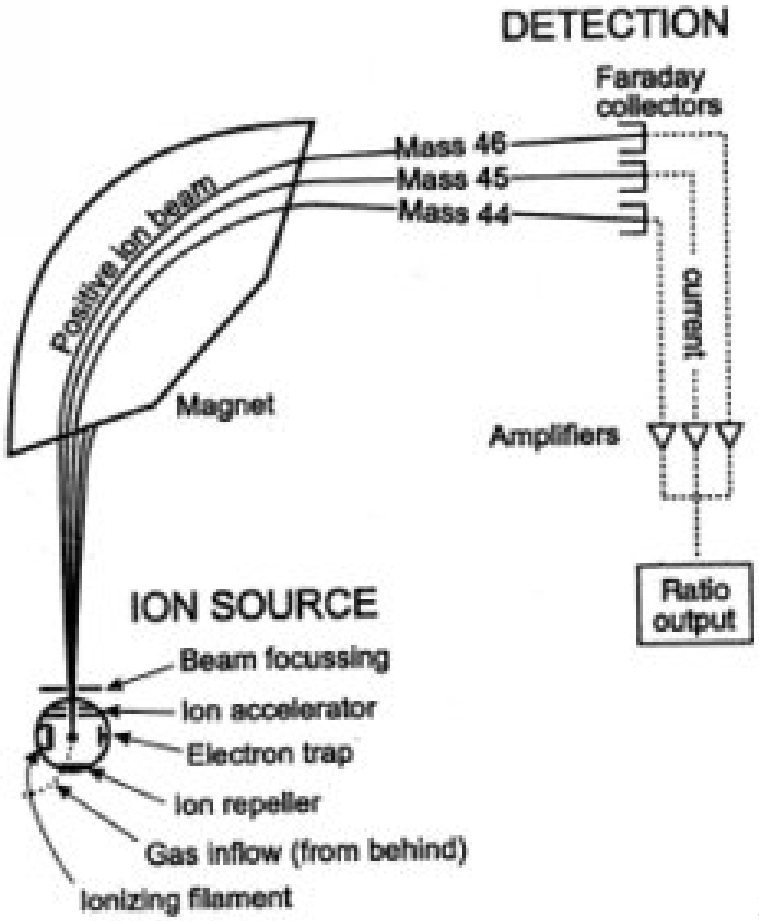
- An analytical technique that measures the mass-to-charge ratio of charged particles.
- Used for determining masses of particles in order to find the elemental composition of a molecule, and for elucidating the chemical structures of molecules (peptides).

Procedure

1. **A small sample is vaporized**
2. **Gas sample is ionized, usually to cations by loss of an electron.**
3. **Ions are accelerated and allowed to pass a magnetic field.**
4. **Ions get sorted and separated according to their mass and charge.**

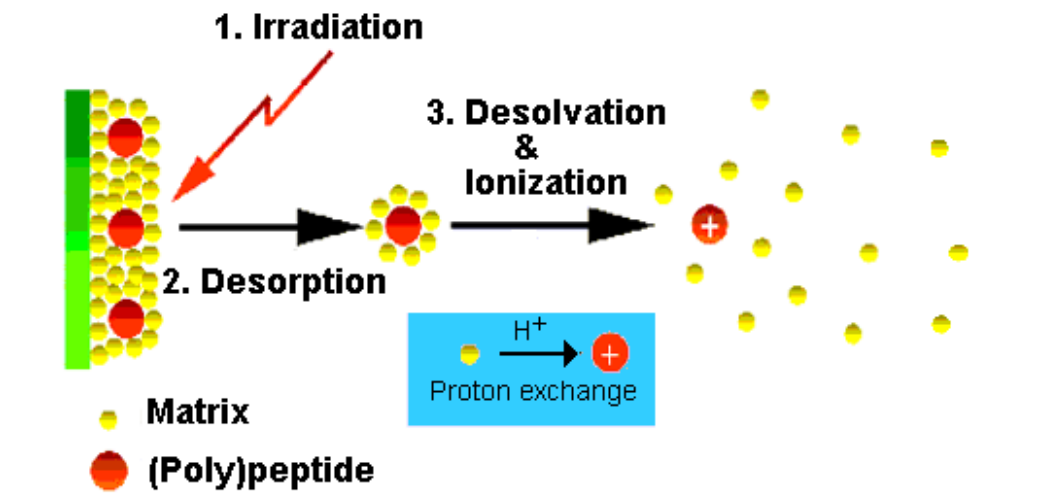


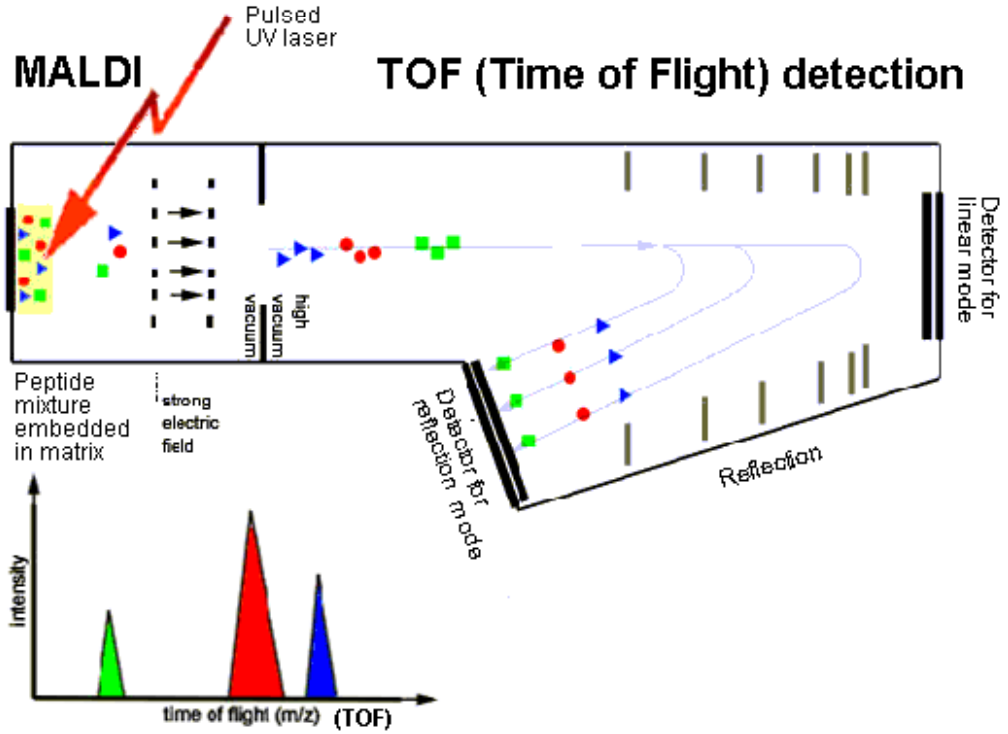

gas sample enters here
ions accelerate towards charged slit
magnetic field deflects lightest ions most
filament current ionizes the gas
ions separated by mass expose film


DETECTION
Faraday collectors
Mass 46
Mass 45
Mass 44
Positive ion beam
current
Magnet
Amplifiers
Ratio output
ION SOURCE
Beam focussing
Ion accelerator
Electron trap
Ion repeller
Gas inflow (from behind)
Ionizing filament

- Biological molecules are mostly fragile and not volatile - solved with the matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) technique.
- A co-precipitate of an UV-light absorbing matrix and a biomolecule is irradiated by a nanosecond laser pulse.
- Most of the laser energy is absorbed by the matrix, which prevents unwanted fragmentation of the biomolecule.
- The ionized biomolecules are accelerated in an electric field and enter the flight tube.
- During the flight in this tube, different molecules are separated according to their mass to charge ratio and reach the detector at different times.
- Smaller ions arrive in a shorter time at the detector than massive ions.
- In this way each molecule yields a distinct signal and hence identified.

MALDI (Matrix Assisted Laser Desorption Ionization)



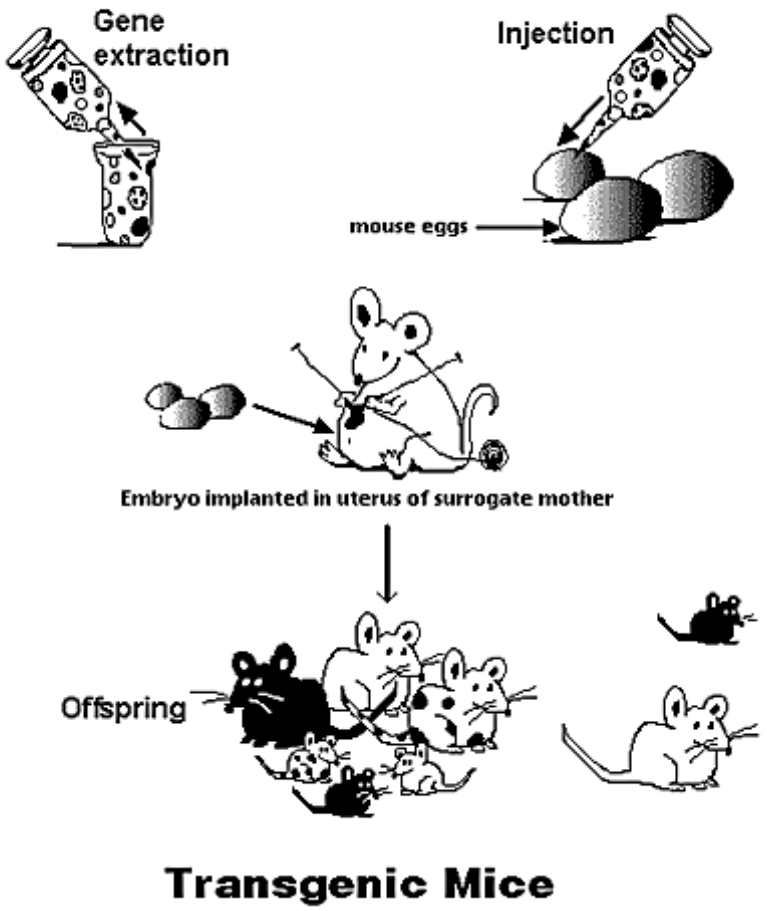


- MS can also be used for sequencing of peptide fragments.
- Two mass spectrometers are used in succession.
- The first one separates the peptides (according to their time of flight) and feeds them individually into the second MS
- Second MS further fragments the peptides at their peptide bonds.
- Hence, the output of the second spectrometer is the mass peaks of a single amino acid.
- This can be used to construct the sequence of the peptide.

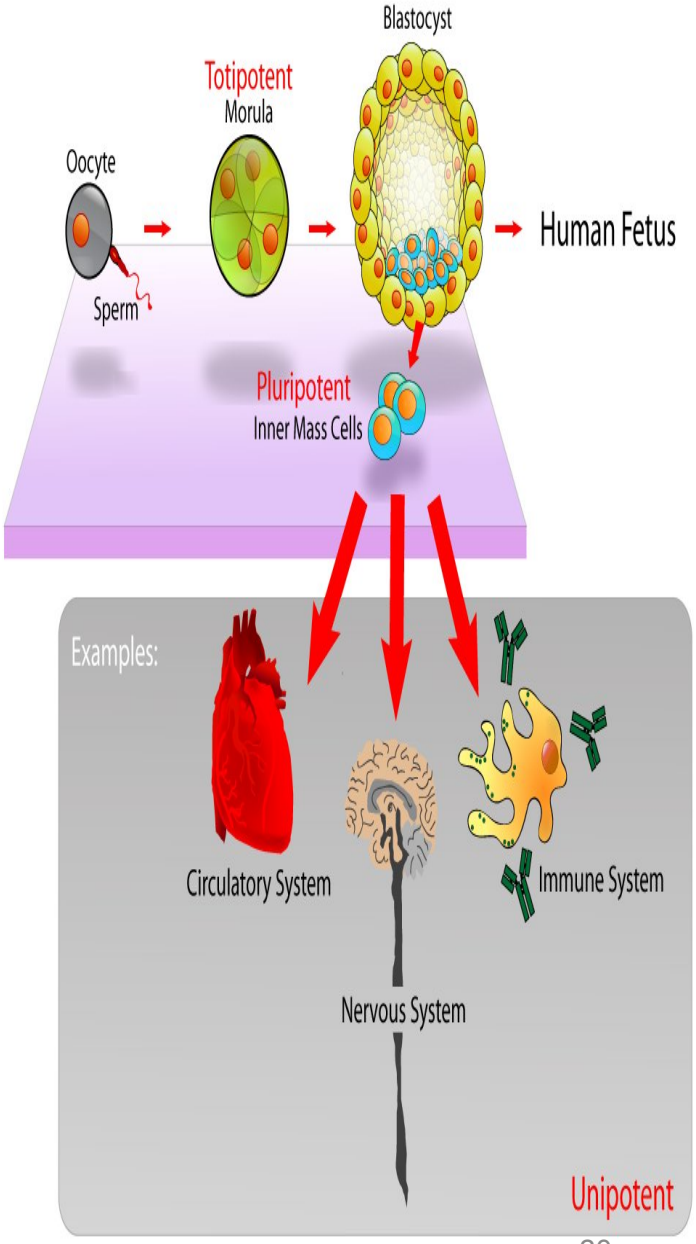
Transgenic Animals

- Genetic material can be introduced into single-celled organisms – it automatically passes through generations – difficult to do it in higher organisms.
- The first method applied successfully to mammals was DNA microinjection (Gordon and Ruddle 1981).
- In mammalian cells linear DNA fragments are rapidly assembled into tandem repeats which are then integrated into the genomic DNA.

- This integration occurs only at a single random location – will not be in all the cells.
- Hence, a linearized gene construct is injected into the pronucleus of a fertilized ovum.
- Then it is introduced into foster mothers – embryo containing the foreign DNA in some cells of the organism develop – chimera.
- Some animals of the daughter generation (F1 generation) will carry the transgene in all of their body cells and a transgenic animal has been created.



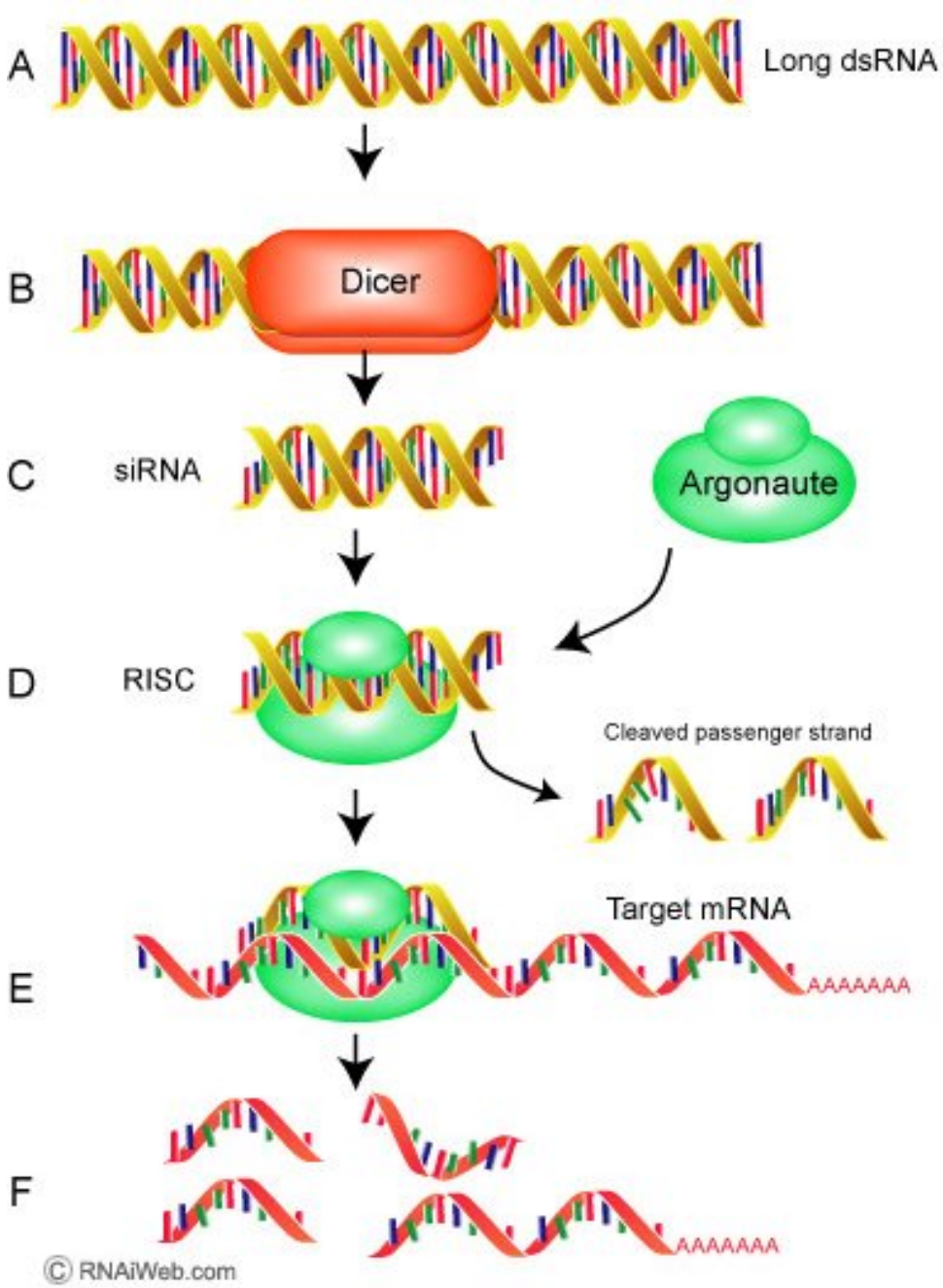
- Advantage – microinjection is applicable to a wide range of species. Disadvantage – integration is a random process and the insert is unpredictable.
- Often the expression of the recombinant DNA is suppressed by silencers or by an unfavorable chromatin structure.
- In rare cases, the integration of a gene variant into the genome does not occur randomly, but replaces the original gene via homologous recombination.
- Either modifies or inactivates any gene - knockout animals.
- In this technique the gene construct is introduced into embryonic stem cells (ES cells) – omnipotent – can give rise to any cell type.



- With the help of PCR or Southern blotting, the few ES cells that undergo homologous recombination can be identified.
- Some of these cells are then injected into an early embryo at the blastocyst stage, which leads to chimeric animals that have wild-type cells and manipulated ES cells.
- ES cell–mediated transfer works particularly well in mice and is the method of choice to generate knockout mice, which are invaluable in deciphering the function of unknown genes.

RNA interference (RNAi)

- Highly evolutionarily conserved mechanism of gene regulation.
- RNAi is an RNA-dependent gene silencing process that is
  - initiated by short double-stranded RNA molecules
  - controlled by the RNA-induced silencing complex (RISC)
- A convenient method for silencing selected genes
- First discovered in 1998 by Andrew Fire and Craig Mello in the nematode worm *Caenorhabditis elegans* – later found in a wide variety of other organisms, including mammals.



A. Endogenously transcribed or exogenously introduced long dsRNA acts as a trigger.

B. It is first processed by the RNase III enzyme Dicer.

C. Dicer cuts long dsRNA into 21-23 nt Short interfering RNA (siRNA) with 2-nt 3' overhangs.

– siRNA can also be synthesized outside and introduced into a cell.

D. siRNA are incorporated into the RNA-inducing silencing complex (RISC)- which consists of an Argonaute (Ago) protein.

– Ago cleaves and discards the passenger (sense) strand.

E and F. The remaining (antisense) strand of the siRNA duplex serves as the guide strand

– guides the RISC to its homologous mRNA, resulting in the endonucleolytic cleavage of the target mRNA.

Thank you