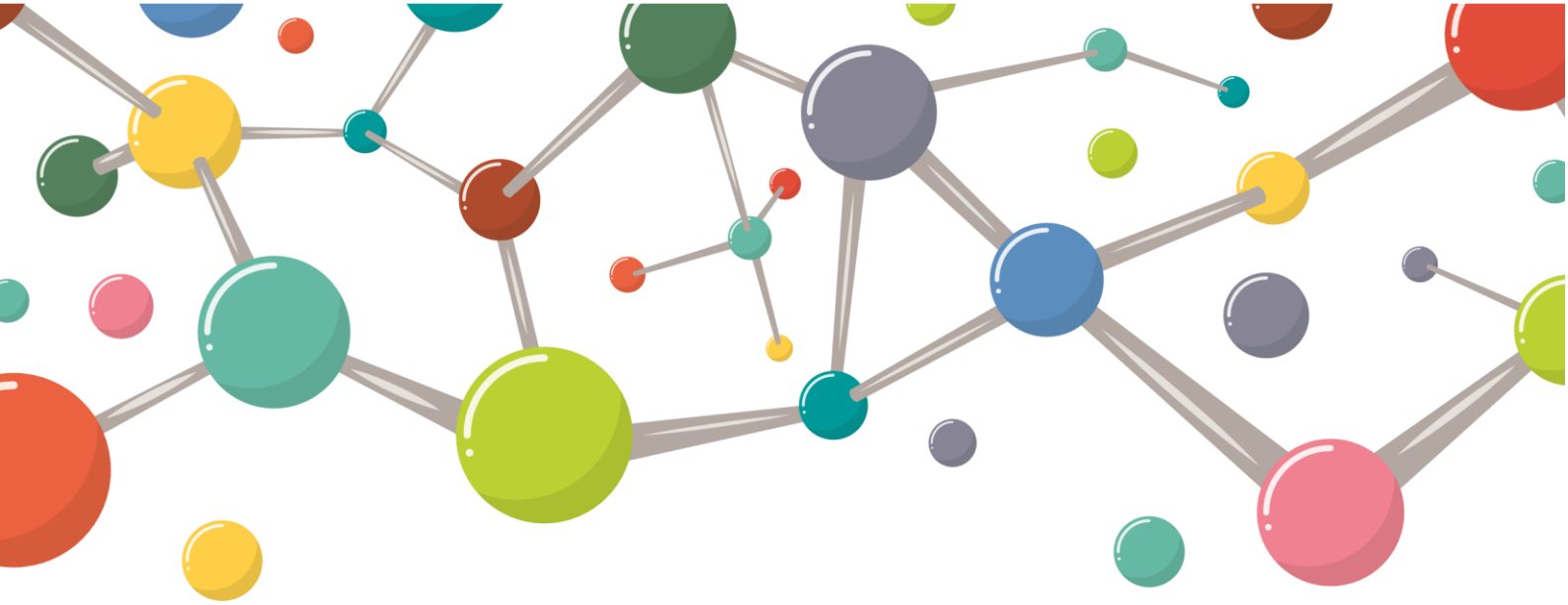

MOLECULAR BIOLOGY

SHEET (3)

WRITER: MOLECULAR BIOLOGY TEAM
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DOCTOR: DR. NABIL, DR. KHALDON

Black color: doctor's explanation, Navy color: slides , Blue color :extra information , Red color : Dr.khaldun's notes , highlighted sentence : modified , purple color: book notes

Non-B DNA Conformations:

These kinds are unequally distributed

We will discuss in this course about 3 types of DNA conformations: A-DNA, B-DNA and Z-DNA.

B-DNA: is the predominant form of DNA in living systems. It was Proposed by Watson and Crick. It has two Grooves: Major and Minor.
A-DNA: Was Proposed by Rosalyn Franklin. And is found mainly in RNA and in RNA-DNA Hybrid. It has two Grooves: Major and Minor.
Z-DNA: some of it is found within the B form of DNA, it is formed whenever there is a G-C (Purine, Pyrimidine) repeats. Z-DNA is important because it has some relationship with regulation of Gene expression, some diseases, it has connected with double break of DNA, and it represents a signal or a label in the genome for the existence of a gene. In other words it tells the cell that there is a gene in this region of the genome. It has only one type of Grooves. And it is called Z-DNA or Z form because the phosphate backbone has a zigzag apperance.

This Table shows some of the characteristics of DNA conformations that you should know.
(Helix sense means to which oriantation was the DNA Winded (Clockwise, Counter-Clockwise)).
(Axial Rise mean the space between every Base pair).

"سَتَذْكُرُ الأَمسَ بِالإِجلالِ مُبتَسِمًا

إِنَّ المَشَقَّةَ فِي خُطواتِنا رُتَبُ."

0.5 means half the distance between base pairs

TABLE 3.2 Comparison of Major Features in A-, B-, and Z-Forms of DNA

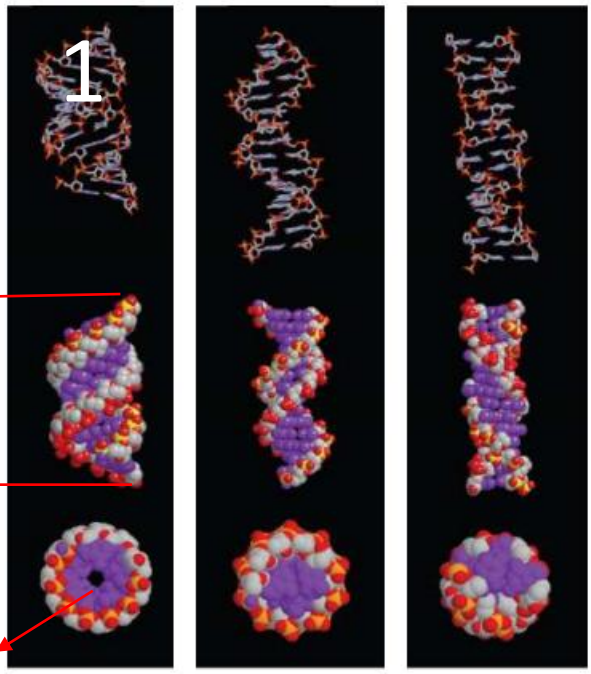
Parameter	A-DNA	B-DNA	Z-DNA
Helix sense (اتجاهية)	Right	Right	Left
Base pairs per turn	11	10.5	12
Axial rise per base pair (nm)	0.26	0.34	0.45
Base pair tilt (°)	20°	–6°	7°
Diameter of helix (nm)	2.3	2.0	1.8

Turn=يعني ي نرجع لنفس زاوية helix

Loosely packed

Tightly packed

Proven by Rosalind frankline



We start here

This is a turn

We finish here

There is a hole in the middle

Figure 03.17: DNA conformations. (a) A-DNA, (b) B-DNA, and (c) Z-DNA.

(Top structures from Protein Data Bank 213D. B. Ramakrishnan and M. Sundaralingam, Biophys. J. 69 [1995]: 553–558. Prepared by B. E. Tropp; Middle structures from Protein Data Bank IBNA. H. R. Drew, et al., Proc. Natl. Acad. Sci. USA 78 [1981]: 2179–2183. Prepared by B. E. Tropp; Bottom structures from Protein Data Bank 2ZNA. A. H. -J. Wang, et al., Left-handed double helical DNA . . . Prepared by B. E. Tropp.)

Z-DNA conformation was found in vitro that it is formed in High salt concentration, but in vivo we do not have the high salt concentration, so the formation of Z-DNA depends on the repeating sequence of G-C in B-DNA(B-DNA is formed at lower salt concentration).

Extra note about DNA conformations:

-left handed Z-conformation and right handed B-conformation are not mirror images but entirely different structures.

Extra note about A and B-DNA differences:

-A-DNA has an axial hole when you look on it from the above whereas B-DNA does not.

-A-DNA has a deep major groove and a very shallow minor groove, whereas both the major and minor grooves are about the same depth in B-DNA.

.Z-DNA:

-Formed with CA or TG repeats under high salt conditions (>2M) (NaCl conc.= 2M, MgCl2=0.7M).

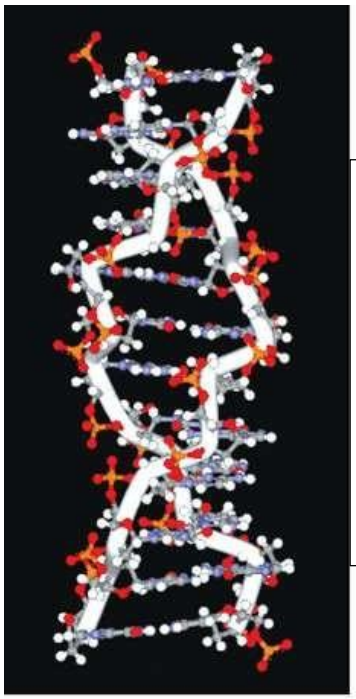
.Conditions for Z-DNA are never approached in vivo but:

-Z-DNA can exist in localized chromosomal regions.

.Z-DNA binding proteins exist

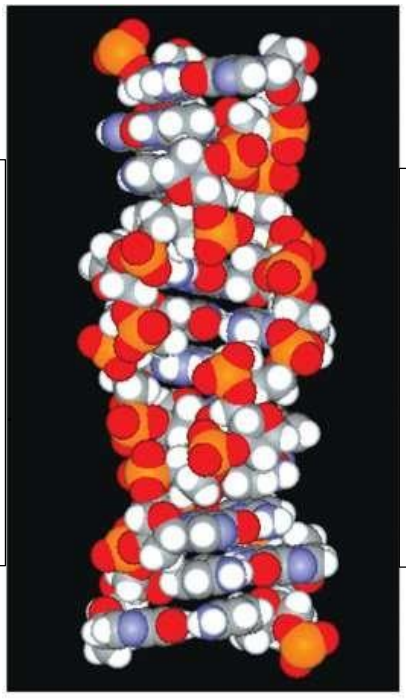
possible role in gene regulation(when and what amount of genes that will undergo transcription)

-potential hot spots for DNA double stranded breaks



(a) Z-DNA with zig-zag sugar phosphate backbone shown in white

Figure 03.18A:Z-DNA.
Z-DNA backbone shown as whitetubes. The bases ,sugars , and phosphates are shown as ball and stick structures.



(b) The same Z-DNA with the zigzag sugar phosphate backbone shown in space filling display

Figure 03.18B: Z-DNA. The same Z-DNA shown in a space filling display.

RNA Structure:

There are different types of RNA and they are very important such as:

r-RNA which constitutes the ribosomes, t-RNA which is very important in protein synthesis, and m-RNA which represents a message for each polypeptide's gene expression. In addition of these types of RNAs we have a small RNA (micro interfering RNA) which is important in gene expression.

Like Proteins, RNA structure is described as:

1-Primary Structure: Which the sequence of Ribonucleotides that constitutes the RNA molecule (base sequence).

2-Secondy Structure: some times within the single strand of RNA or between different RNA molecules there could be some complementary bases and they will base pair to each other(according to Watson-Crick base-Pairing rules). And it is important to Stabilize the RNA molecule and help it to form it's tertiary structure in order to do it's proper function .

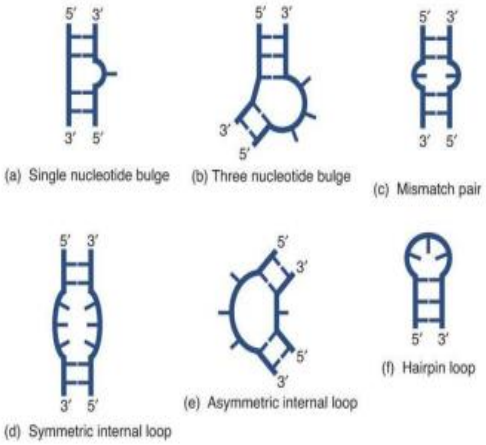


Figure 03.22: RNA loop and bulge secondary elements. (a) single nucleotide bulge, (b) three nucleotide bulge, (c) Mismatch pair, (d) symmetric internal look, (e) asymmetric internal loop, (f) Hairpin loop.

Secondary structure of the RNA consists of helical regions and various kinds of loops, bulges and junctions within the helical regions.

Some types of secondary structures:

A) **The Single bulge formation: is formed when there is no ribonucleotide to base pair with the existing nucleotide in the opposite strand.**

B) **Three nucleotides bulge: Just like the Single bulge formation but now there is no ribonucleotides to base pair with the 3 nucleotides in the opposite strand.**

C) **Mismatch pair: this type is formed when the Bases on both sides are not complementary with each other.**

D) **Symmetric internal loop (multiple mis-match pairs): is just like the one before but now there are 3 miss-matched bases (the number of bases is symmetrical).**

(Any mis-match region will interrupt the both regions before and after it) (this note is for points c and d)

E) **Asymmetric internal loop: has one difference than the one before which is that the number of bases on each side is not symmetrical.**

F) **Hairpin loop: has two parts: the stem (complementary base-pairs region), and the loop which has bases that has no pairs. And is called Stem-loop Structure.**

These secondary structures are very important in gene expression at the level of transcription as we will see.

" أتُقال فيك قصيدةٌ مرموقةٌ؟

أنت القصائدُ مطلعًا وختامًا"

3-Tertiary Structure: three dimensional structure between 2 or more Secondary Structures (coming from the interaction and folding between them)(by hydrogen bonding) and it is important for RNA to do it's function(it is stabilized by metal cations such as Na+,Mg2+ that offset charge repulsion).

Is the second (in figure b) structure stable?
You may think that this structure is not stable because of the negative charges on it.
But, don't imagine this structure like this exactly, because there is a micro-environment that could shield the negative charges and stabilize it, such as: metals, proteins or others.

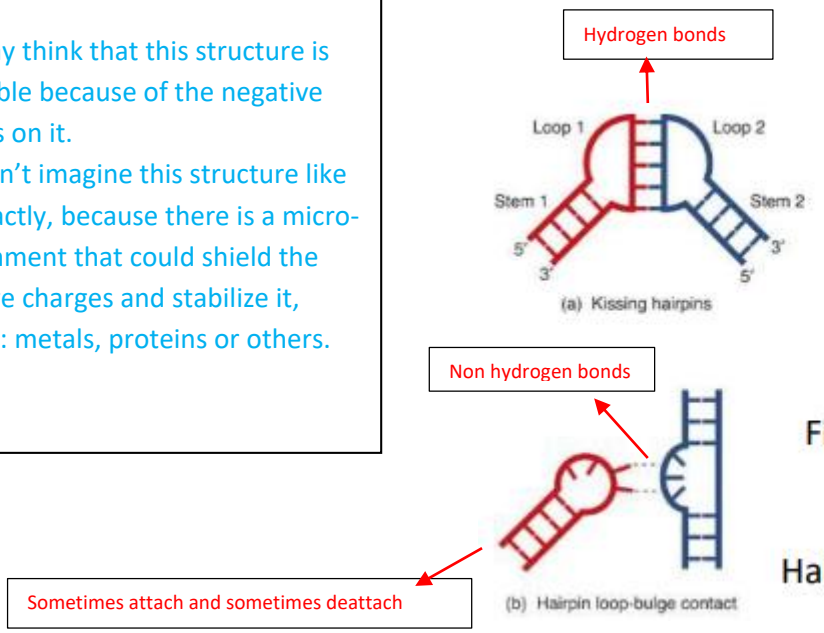


Figure 03.25A: Interactions that bring distant RNA segments together. (a) Kissing hairpin interaction.

Figure 03.25B: Interactions that bring distant RNA segments together. (b) Hairpin loopbulge interaction.

Two tertiary Structures are mentioned:

A) Kissing hairpins: Is formed by the interaction of the loops of 2 hairpin loop structures.

B) Pseudoknot: it is formed when the base sequence hairpin loop pairs with complementary single-stranded region that is adjacent to the hairpin loop.

4)quaternary structure: describe the arrangement of RNA molecule with respect to other RNA molecules or with proteins molecules.

خلصنا هذا الفصل !!

هانت هانت زملائي

Chapter 4:

In this chapter we will talk about important techniques in molecular biology, starting from Extraction of DNA from tissues to the separation of different macromolecules from these homogeneous tissues by centrifugation or electrophoresis, and we will talk about PCR and the DNA sequencing of any piece of DNA and the sequencing of human genome.

Nucleic acid (mainly DNA) isolation:

It means to extract DNA from the cell without its remaining components to use it in experiments.

You could extract DNA from different tissues(bacteria, viruses, plasmids, plants, mammalian cells)

Note: before any extraction we must understand the host that we will extract DNA from it.

The Techniques used to separate macromolecules depends on the type of cell you are using. But generally speaking, they all will follow one principle with some differences.

_Viral DNA (phenol extraction-removes proteins-) (sometimes chloroform or isoamyl alcohol are added to assist the phenol).

_Bacterial DNA:

(lysosomes to digest cell envelope, detergent (SDS) disrupts cell membrane, after alcohol precipitation chromosomal DNA can be spooled out)(we use the same method of viral DNA except that the spooled out DNA is dissolved in a buffer solution containing RNases to digest existing RNAs)

Note: after centrifugation we will have two layers of pellet and supernatant.

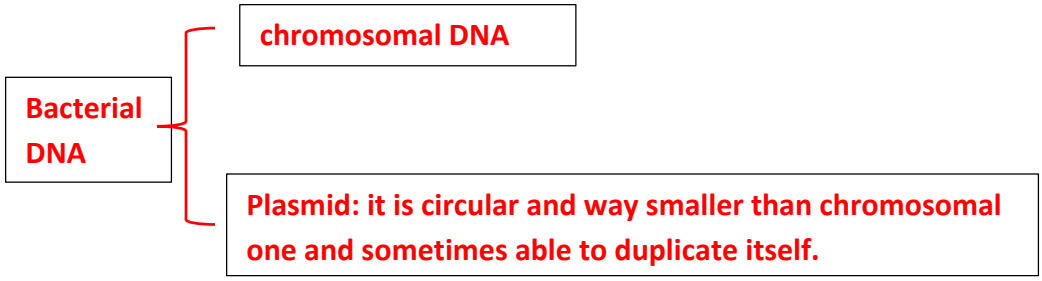
We remove RNA using RNases

The main principle of DNA extraction:

1-removal of proteins

2-DNA will now be in the aqueous layer

3-removal of DNA from the aqueous layer using alcohol, now DNA will be found precipitated at the bottom of the test tube(after centrifugation).



_plasmid DNA isolation: lysosome then SDS treatment along with NaOH(hydrolyzes RNA), neutralizes with acid, plasmid remains in solution while chromosomal DNA is in an insoluble aggregate.

_Yeast and fungal DNA: need to remove polysaccharide wall (that is resistance to lysosome. Other enzymes-such as cellulase that is isolated from snails- break down these cell walls) then similar to bacteria.

The principle in extracting macromolecules is as follows:

-First, you break the cell wall of the cell with lysosomal enzymes and then break the cell membrane with detergents such as SDS (sodium dodecyl sulfate).

-After breaking the cell, you centrifuge the cell and take the supernatant which contains the macro molecules (DNA, RNA or Proteins) then you mix it with phenol which will denature the Proteins (phenol extraction).

-While the Nucleic acids are still in the phenol phase, you aspirate the phenol phase that contains (DNA, RNA), then you precipitate DNA, RNA using Alcohol. You will see threads of Nucleic acids that will be formed upon addition of ethanol.

-Then you Centrifuge the nucleic acids, the nucleic acids will go into pellet, you discard the supernatant and then wash the pellet with aqueous buffer at the proper PH and mix it, and it's ready for using and doing many genetic tests on it.

Preparation of RNA is very tedious and crucial because RNA is subjected to degradation by RNases which are found everywhere so its extraction is harder than DNA extraction. In order to avoid degradation of RNA you have to:

– Freeze the tissue in liquid nitrogen then grind the frozen cells.

– Resuspend in acidic phenol/chloroform/sodium acetate/guanidinium thiocyanate.

– RNA present in the upper aqueous phase is precipitated with ethanol or isopropanol, most of the DNA and proteins are found in either the lower organic phase or the interphase.

To get your RNA as intact molecule not degraded. If you didn't take any precautions while preparing RNA you will get degraded molecule. The same thing goes for DNA as well.

physical techniques used to study macromolecules:

Centrifugation can separate macromolecules based on density.

Ultra Centrifugation:

It's a tool used to separate the homogenous tissue contains macromolecules (such as DNA, RNA, Proteins, ETC…) from the supernatant that we get after preparation.

You take the sample which contains all these molecules and subject it to very high centrifugal force (more than 800000 x Gravity).

sedimentation rate depends on molecular size and shape.

-if two particles have the same shape, the one with larger mass sediments more rapidly than the one with the smaller mass

-if two particles both have the same molecular mass, the one with the more compact shape sediments more rapidly.

-shape influences sedimentation rate because the more compact a particle is, the less frictional drag it experiences as it sediments through a solution.

Th ratio between sedimentation velocity and centrifugal force is called sedimentation coefficient (s)

S=velocity/centrifugal force

The value of s depends on the molecular mass and shape

-it cannot be determined by simply adding the s value of the two smaller particles. for example: ribosome is composed of small subunit which have 30 S value and large subunit which has 50 S value, but if we put the two subunits in a test tube we can not say that the S value=80 .

-in honor of theone svedberg, the ultracentrifuge's inventor, 10^-13 second is called one svedberg or 1S.

one of the techniques used is called:

sucrose gradient centrifugation.

In this technique you do your centrifugation on a centrifuged tube that has a sucrose's gradient(sucrose gradient: gradual differences -increase or decrease- in the sucrose concentration).

To prepare a sucrose gradient solution, you have to bring 2 containers. One contains a high density solution of sucrose while the other contains a low density solution of sucrose and connect them to one another. You start filling tube with drops of sucrose from the higher concentration side. Each drop falling from the high concentration container will be replaced by another drop coming from the low concentration container filling its place and lowering the general concentration of the first container. You keep doing that until the tube is filled with a gradient concentration of sucrose.

you put your mixture on the top of the sucrose solution in the centrifuge tube carefully and then you put it in the swinging bucket rotor which is a

type of centrifuge head that contains bucket that can swivel on pins, the rotor is placed in the centrifuge and then subject it to high speed of centrifugations and the different macromolecules will be separated to a different bands as you see in the test tube. after centrifugation each macromolecule will stay at the sedimentation that is equivalent to the concentration of sucrose at any point.

Remember: that each macromolecule has a sedimentation rate which is a fixed physical property which depends on the shape and mass of that macromolecules and is measured by svedberg unit (s units).

once macromolecule will set in the sucrose concentration that is compatible with its S unit and no more sedimentation will take place.

And after separating the macromolecules with the centrifuge a tiny hole is punched in the bottom of the centrifuge tube, and drops of the solution are collected in separate tubes. These drops representing successive layers of solution in the tube, are analyzed to determine the macromolecule's concentration along the tube.

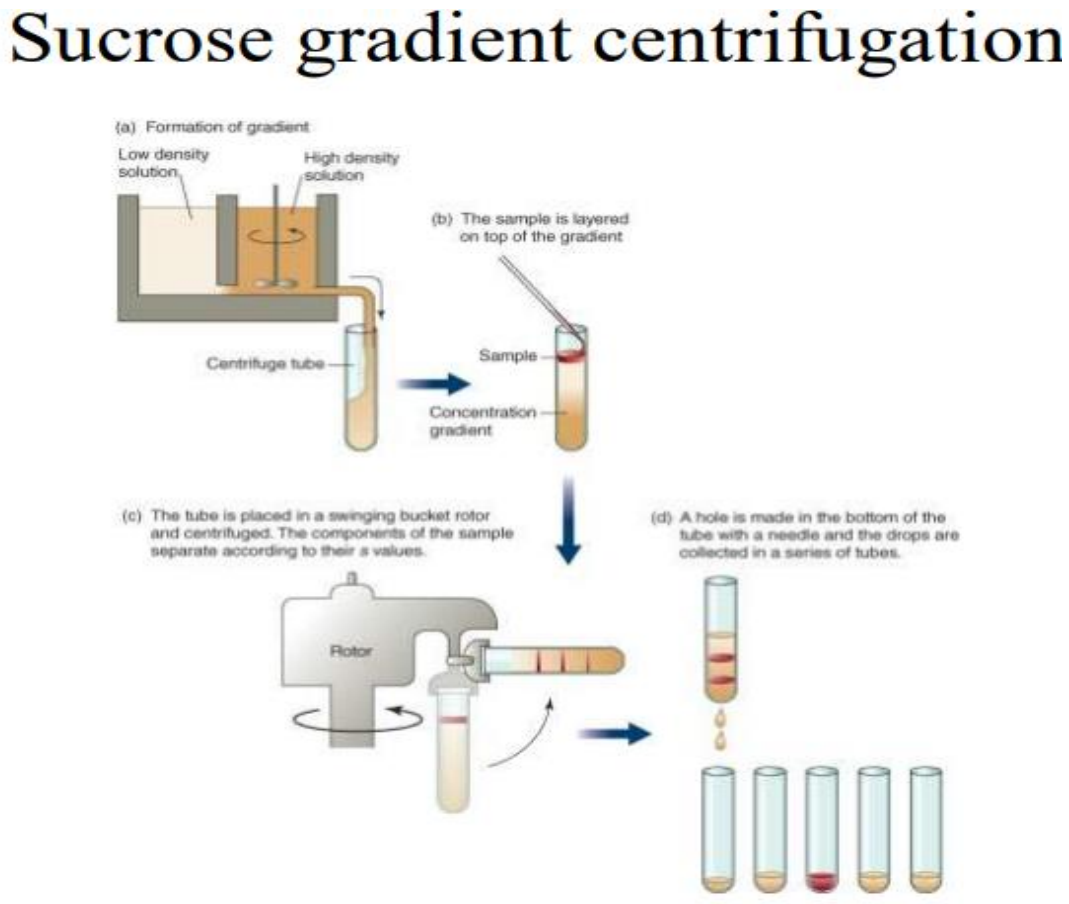


Figure 04.05: Sucrose gradient centrifugation.

Equilibrium density gradient centrifugation

_nucleic acids are suspended in CsCl (its density approximately equals the density of DNA)solution (or Cs2SO4)

_High speed centrifugation generates a near linear concentration gradient

_macromolecules seek their neutral density.

Another Technique is to use CsCl instead of sucrose. Which is able to separate a single strand DNA from a doubled strand DNA. And it could separate heavy single strand DNA from lighter ones.

We will talk about this technique later when we talk about DNA replication.

Gel Electrophoresis: (توجيهي)

It is another technique used to separate macromolecules based on their migration rate in an electric field.

It has two types: agarose gel electrophoresis, and polyacrylamide gel electrophoresis.

What is involved here? we will know by name.

Gel comes from the use of gel. electro comes from using electrical power and phoresies comes from the migration of molecules in the gel while you use the electrical power.

So we use this technique to separate molecules from each other according to their size, shape and sometimes the charges.

– Nucleic acids typically separated on agarose or polyacrylamide gels.

– Nucleic acid gels are often stained with ethidium bromide.

How to obtain the gel ?

We put agarose with a specific buffer and then we heat them and mix them and then leave them to cool down and then place it in a cast (between glass plates).

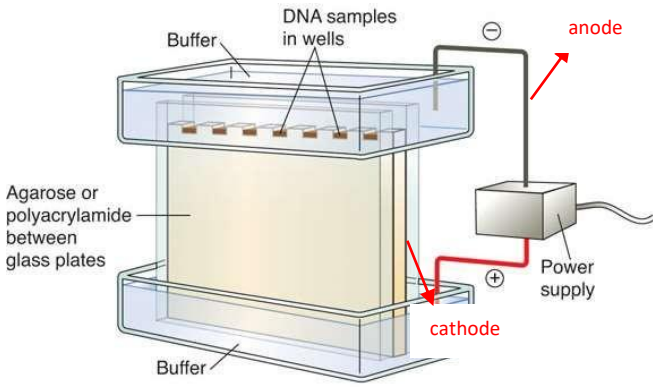
Generally speaking this technique works as follows:

You get 2 reservoirs containing buffer on either side and glass plates filled with gel (agarose, polyacrylamide solution) between them to solidify. Before solidification you put a mold at the top of the gel and immerse it in the gel before solidification. After solidification you remove that mold in order to make spaces(wells) to put your samples in. After putting the macromolecules in the wells, you will use a power supply as a force to move the samples to migrate in the gel according to their size for some time.

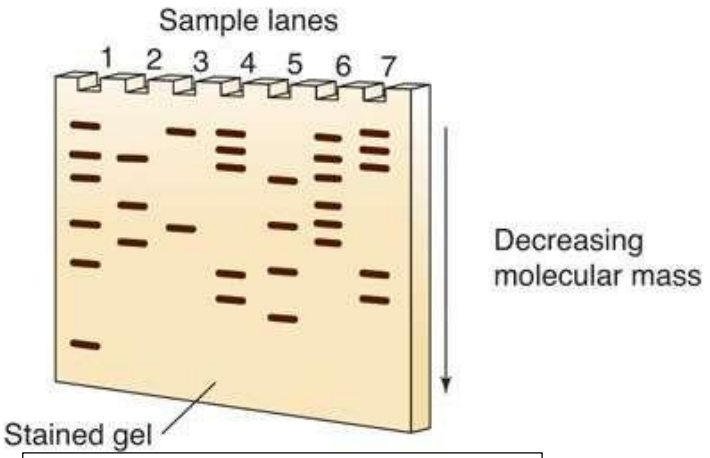
Smaller DNA (negatively charged) fragments will move more distance in the gel (toward the positive charge), why? Because they face less resistance.

after you finish the process, you will take the gel and stain it(for DNA you stain it with ethidium bromide which will fluoresce when you look at the gel under U.V).

In the figure of the stained samples, each band represents a DNA fragment. The wells are in different lanes, each lane is from different DNA samples that will show you their contents of DNA fragments.



Apparatus of gel electrophoresis capable of handling several samples simultaneously.



The separated components of a sample appear as bands, which may either be colored or fluoresce.

DNA molecules with the same number of base pairs will move the same distance, so if we see two molecules and we know that the first has 20 bp and then we see that the two molecules have moved the same distance then we will know that the second has also 20 bp.

_electrophoresis can be used to determine a polypeptide's molecular mass (an example is above in red)(it can also be used to separate topoisomers) however:

-unlike DNA, native proteins do not have a uniform charge or shape

-requires a specialized electrophoretic technique

(because ethidium bromide is carcinogenic, many workers prefer to work with commercially available safer substances)

-polyacrylamide gels can separate single stranded molecules that differ in size between 5-750 nucleotide long.

Agarose gels have lower resolving power but separate DNA molecles that range in size from 200 bp to about 50000 bp.

Disadvantages of gel electrophoresis:

1-slow(requires hours tocomplete a run)

2-difficult to automate

3-does not easily provide quantitave data

So an alternative mean for separating DNA in an applied electric field, called capillary gel electrophoresis, does not suffer from these disadvantages.

_SDS-PAGE(sodium dodecyl sulfate poly acrylamide gel electrophoresis)(الدكتور خلدون حكى مش داخل)

وكأنى بالرمح اضربُ قائلاً

الأرض أرضى والزمان زمانى

لا تنسوا أخواننا فى غزة من دعائكم !

Enzymatic techniques used to modify DNA:

Tools used in molecular biology:

Mainly these tools are used to make recombinant DNA or amplified DNA or to sequence DNA, those tools are enzymes of most of the cases.

We are talking about some types of enzymes:

Nucleases: 1) DNases 2) RNases 3) general nucleases

Nucleases: group of enzymes that will degrade or cleave or break down nucleic acids (which could be DNA or RNA).

Nucleases: Enzymes that digest polynucleotides by cleaving the 5'→3' phosphodiester bond.

Some DNases are specific for double stranded (ds) and others act only on single stranded (ss) DNA and others for both.

DNases are two types:

1) Endonucleases (-endo = inside)

2) Exonucleases (-exo = outside)

* Endonucleases: a group of enzymes that will cleave DNA(break phosphodiester body)from inside the DNA molecule " they will cleave the internal phosphodiester bond between deoxynucleotides"(an eample on it is S1 endonuclease).

Nucleases that act within a strand, some are base sequence specific

** Exonucleases: enzyme going to cleave the external phosphodiester bond starting from 5 prime or 3 prime of the DNA .

if it starts from 3' it will be called (3' Exonucleases) but if it starts from 5' it will be called (5' Exonucleases).

Nucleases that act only at the end of a strand • 5' →3' or 3' →5

Specific type of endonucleases (Restriction endonucleases): enzymes that found in bacterial cells and not found in mammalian cells, they are used in bacteria to attack any foreign DNA invading that bacteria like virus DNA

(trypsin or other specific proteases used to cut polypeptide chains)

A class of nucleases that cleave (restrict) viral DNA that enters a cell and Cleave within a specific nucleotide sequence (restriction site).

once the virus or its DNA enter the bacterial cell restriction endonucleases start to degrade the viral DNA but they are not cleaving the bacterial DNA , although both DNA are the same but the bacterial one is protected by labeling it with methyl group so the enzyme will understand that it is belong to the bacterial host cell while the non-methylated DNA belongs to the foreign DNA coming from viruses as an example.

Host DNA is protected through methylation of restriction site (so the active site of the enzyme cannot be attached to it)

These restriction endonucleases could be purified (Extracted) from different types of bacteria and they are used in DNA recombinant technology and in molecular biology for various purposes (diagnostic, cloning and DNA recombinant and the production of different recombinant drugs).

cloning role: uses DNA restriction enzymes to cut a vector and an insert at specific locations so they can be easily joined together by the enzyme DNA ligase to create recombinant DNA.

The use of restriction endonucleases in recombinant DNA and in molecular biology in lab:

The restriction endonucleases they cleave double stranded DNA ,some of them will cut from the middle and produce blunt (flush) end(نهاية غير لزجة)

HpaI: a cleavage enzyme isolated from a bacteria.
H: the first latter of the bacterial species.

HpaI and SspI these types of restriction enzyme when they cleave the internal phosphodiester bond they form blunt end, while PstI form cohesive or sticky end.

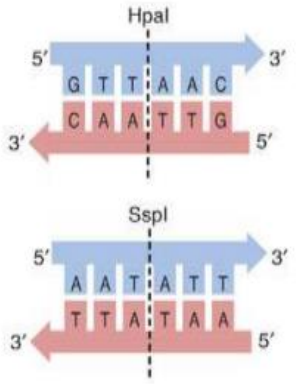


Figure 04.10A: Restriction endonuclease generated ends. Some restriction endonucleases such as HpaI and SspI cut on the line of symmetry to produce blunt ends.

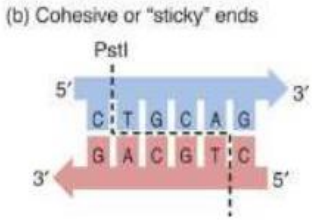


Figure 04.10B: Restriction endonuclease generated ends. Other restriction endonucleases (PstI) cut on either side of the line of symmetry to produce cohesive ends.

When we read the two sticky ends from the same side for example from 5' to 3' they are the same 5'TGCA3' this similarity called palindromic

These restriction enzymes recognize specific palindromic sequences and they are very specific, they do not cleave any where.

If you have very long DNA molecule you could insert foreign DNA between these steaky ends and re-ligate them and produce recombinant DNA molecule using these restriction enzymes.

Example of enzymes produce sticky ends: EcoR1, HindIII(III = 3 'Latin numbers') PstI and BamHI

- **Type II restriction enzyme recognition sites are palindromic**
- **Some generate flush or blunt ends**
- **Others generate cohesive or sticky ends**

important techniques:

- **Southern Blotting**

  Northern Blot, Western Blot

- **PCR**

Southern Blotting (according to scientist name) is used to detect specific DNA fragments and can be used to detect both partial and complete deletions of a gene.

You have the whole genome (prepare the DNA first) in the lane and electrophores it, if you are interested in specific gene and you want to detect in which lane is it.

After you prepare your DNA located on agarose gel electrophoresis you transfer DNA from the gel to a Nitrocellulose paper by specific technique, after all DNA has been transfered to the paper then this paper is put in denaturation solution (NaOH) in order to make all the double stranded in the paper into single stranded then take it and hybridizes it with a specific probe(short sequence of nucleotides that has complementary regions with the gene sequence that you are interested in) after hybridization for specific time you wash the non-hybridized probes and then you look at your nitro cellulose paper to see where the hybridization spots have been found, then you could tell for example that a gene in a specific lane is the gene you are interested in because it has hybridized with a specific probe that has a complementary sequence to its sequence. To understand more read the following paragraphs, assume we have known a gene in a fish for example(we know its sequence, protein, and its chemical properties) and we want to know if this gene is found in human or not, how? We use southern blotting.

We get the genome that we want to study from its organism (human in this case) by DNA extraction, and we can also cleave it into smaller fragments by restriction enzymes and then we make gel electrophoresis for it and then we will place it on a membrane. How do we transfer it?

1)get a tray that is filled with a buffer

2)place a sponge on the base so that the buffer can move upwardly through the sponge

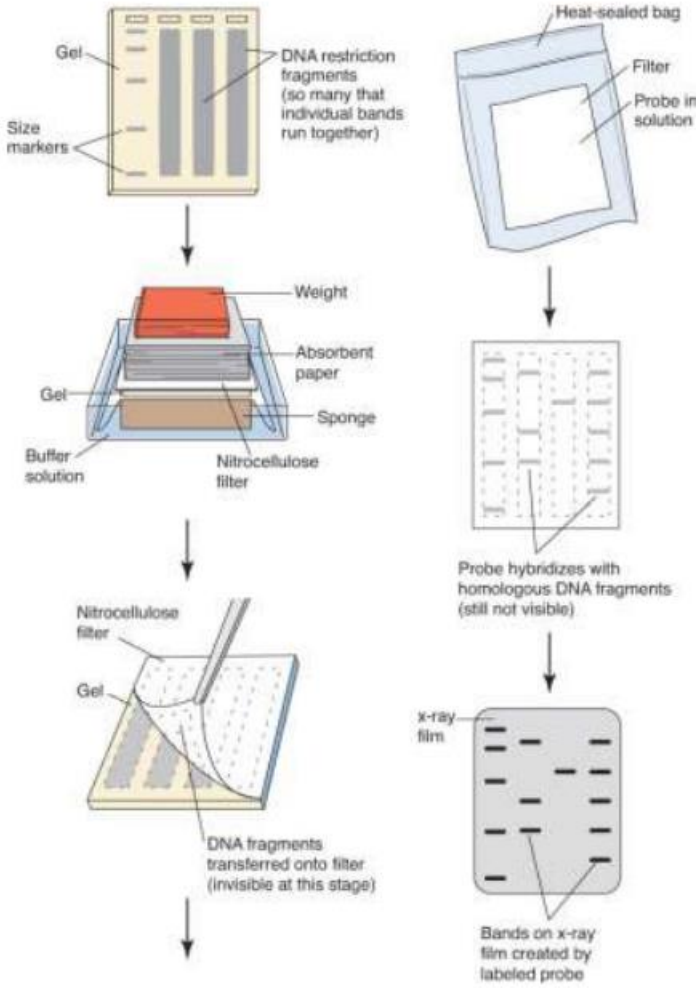
3)add a nitro cellulose filter or a nylon membrane and beneath it we will put the gel

4)place an absorbent paper and add some weights on top of it to compress the paper and create a column of buffer that moves (by osmosis) through the sponge and the across the gel carrying the DNA with it transferring it to the nylon membrane.

5)after that we place this membrane in a device called cross linker contains U.V light so it binds DNA to this membrane

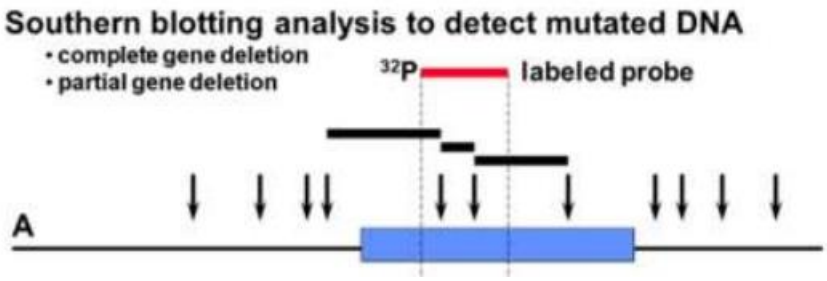
6)now we get the probe (in our case it has complementary sequence for fish one) and prepare it (may be using PCR) and label it, so we will be able to see it through X-ray or dyes, and then we place the probes on the cellulose paper so they will bind with the gene that we want to study if its found in the sample.

Figure 04.14: Southern blot analysis: an experimental method for identifying a specific DNA fragment in a gel.

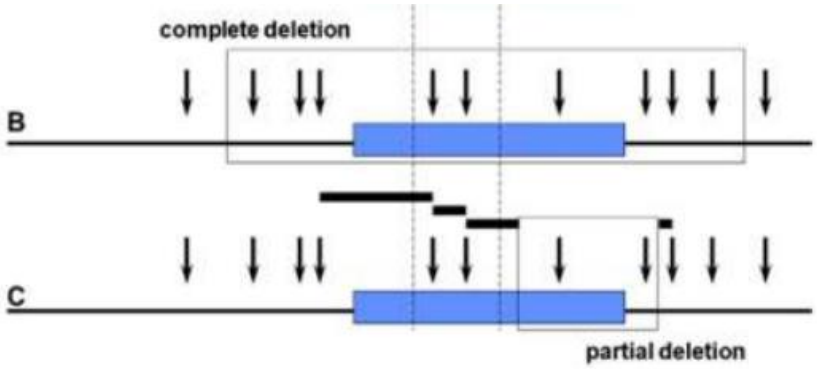


Application of the southern blotting technique:

((Used to detect mutant DNA))

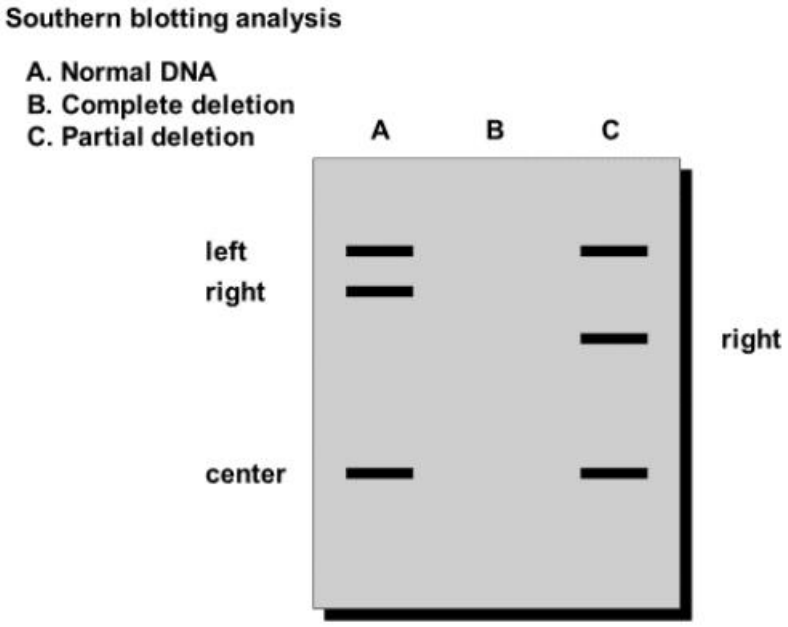


Arrow show the recognition side for the restriction enzyme



Subject the DNA to digestion by this restriction enzyme(will cut down at every arrow)

,then get the fragments and electrophores them then you do Southern Blotting on the gel by hybridizing the Nitrocellulose paper with specific probe labeled by (32p), it will hybridize with all (three)fragments.



We take a Nitrocellulose paper and expose it to x-ray, emission will start at the places where the hybridization take place. A: healthy gene (no defect, no mutation). Upon digestion by specific restriction enzyme then southern blot produce a pattern of three fragments for that gene.

B: the whole gene is deleted from its genome and if the same experiment is done, the hybridization will not take place so no fragments will appear.

C- partially deleted: three fragments will appear but not in true sizes (small than the normal) so get different places in the paper

The importance of this technique is to determine the mutation and deletion from the gene that causes genetic diseases (a lot of genetic diseases are diagnosed by Southern Blot).

(Northern Blot, Western Blot)

Northern Blot:(RNA gel blot) the same principle of Southern Blot but it is used for the detection of the RNA, and the probe in RNA is complementary DNA or complementary RNA sequence.

Western Blot (protein gel blot): the same principle of Southern Blot but it is used to detect the protein, and the probe in proteins is labeled antibodies of the specific protein that we are looking for.

Student question: the protein technique: take total homogenate protein from a cell and electrophories it then put it on a nitro cellulose paper and then hybridise them with labeled antibodies that react specifically with the protein we looking for, so we will know that it exists in the lane.

The Polymerase Chain Reaction (PCR)(invented by kary B.Mullis): it is used to amplify DNA.

amplify: make copies of DNA (more than one million from one sample).

What is the difference between replication and amplification? Replication happens in vivo and amplification happens in vitro using PCR

Importance of the copies: represent enough material from gene or DNA molecules to do a lot of molecular biology tests for different purposes (diagnoses of viral diseases, infectious disease, genetic diseases and cancers).

From one pair you can amplify DNA and has enough material for genetic testing.

It is used in Forensic Medicine in order to discover criminals.

If we have a sample of DNA that we sure it contains the criminal DNA but it is so small, we use PCR in order to increase its concentration to ensure that it belongs to the suspect or not.

The principle of this amplification:

this technique is composed of many cycles (each cycle requires only 4-6 minutes) and the routine one that we are running every action 30 time (cycle) and each cycle composes of :

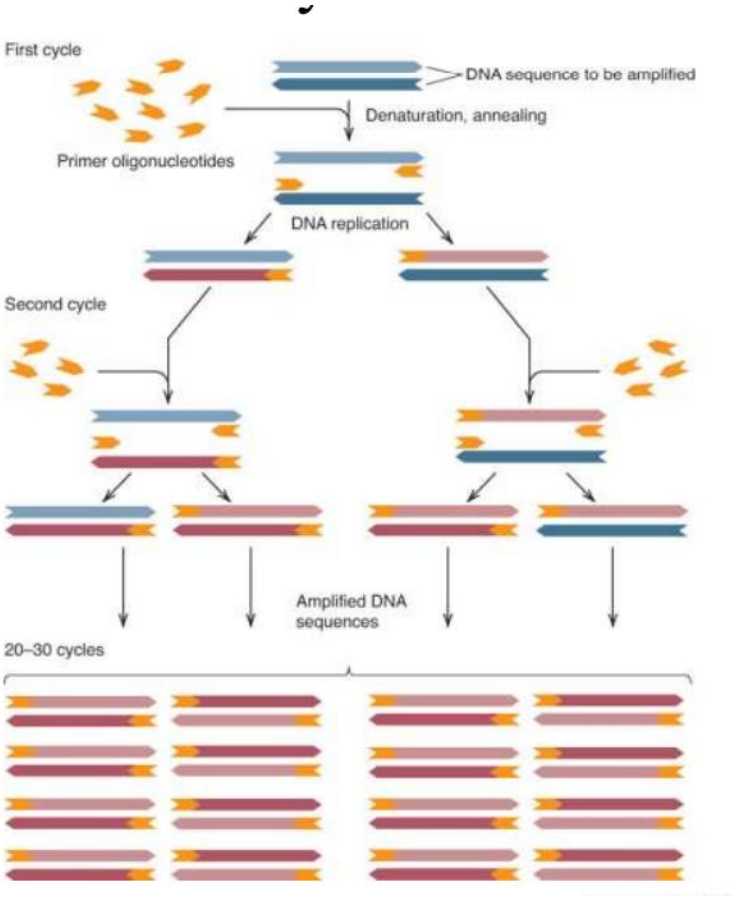
1. **Denaturing linear duplex DNA that you want to amplify it.**
2. **Annealing an oligonucleotide primer to it (the primer oriented with their 3'-ends directed to each others).(oligo: less than 20-25) Remember: DNA polymerase can not build DNA from zero in replication so we use primer)**
3. **Extending the DNA primer with heat stable DNA polymerase. (repeating DNA polymerase 1 additions at each cycle can be avoided by using a thermostable DNA polymerase such as Pfu or vent DNA polymerase)**

By doing the first cycle we convert the one molecule double stranded DNA into two molecules DNA double stranded

Repeated the cycle 30 times will form one billion copies of the first molecule double stranded DNA.

After n cycles the amplification yield would be 2^n

PCR is known as thermal cycler which are programmed to shift their temperature up and down during different stages of the cycle.



After denaturation by heat (94-95) the primer will attach to each side to flanking the region to amplify (the region contain the gene you need).
attaching (binding) of the primer to flanking region of the templet (gene) name annealing.
Then DNA polymerase (taq1: stable at high temperature) it will extend the primer in the 5' to 3' direction in both strands(it will add to primer's 3', to remember: free three prime).

Primer: short oligonucleotides (20 deoxynucleotides) with complementary sequence to the sequence of the templets.

PCR can amplify a specific DNA region in only a few hours

Importance:

- **Amplification of a segment of DNA for genetic engineering**
- **Rapid detection of pathogenic bacteria and viruses**
- **Detection of inborn errors of metabolism (genetic diseases)**
- **Detection of tumors**

DNA sequencing

DNA sequencing using method called The Sanger-Chain Termination Method,(Dideoxynucleotide Sequencing),(we use it to discover the DNA sequence).

In this method, small amounts of dideoxynucleotides will be incorporated during synthesis to cause termination of primer chain extension

Dideoxynucleotide means the absence of oxygen on both carbons 3' and 2'. As in the figure:(04.24)

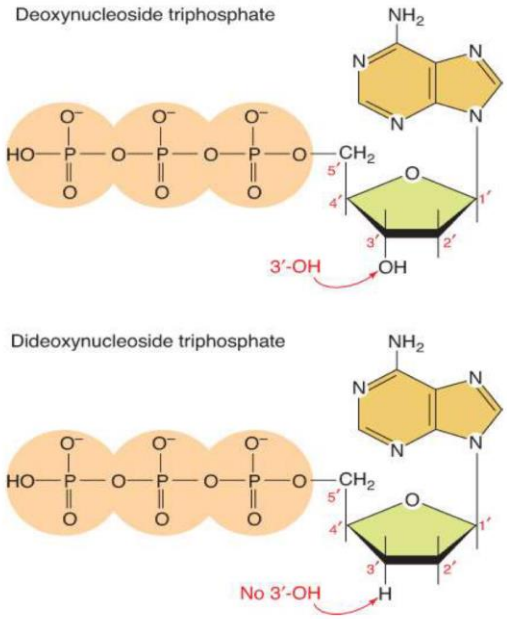


Figure 04.24: Comparison of deoxy- and dideoxynucleoside triphosphate structures.

If you use dideoxynucleotides to extend the primer, DNA polymerase will not form phosphodiester bonds because of the absence of hydroxyl group on the 3' carbon.

If we mix 1% of dideoxynucleotides with 99% of deoxynucleotides, there will be an incomplete extension of the primer, because the 1% of the time for the reaction will be terminated and the 99% of the time of the reaction will be extended.

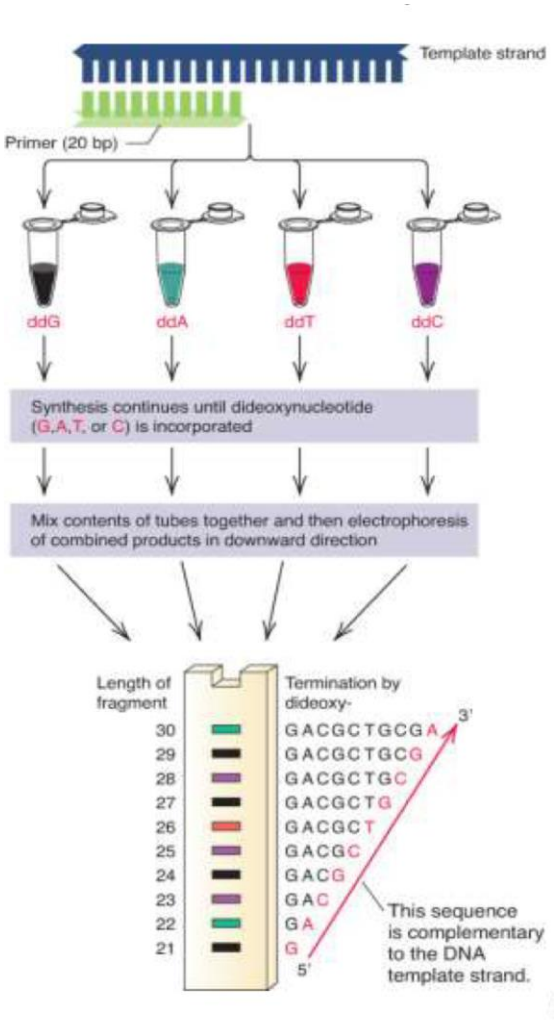


Figure 04.25: Chain termination method for sequencing DNA. Lengths of the terminated DNA fragments are shown at the left of the gel.

In this figure(04.25)

we have four tubes and in each tube we will put a DNA template(great amount of it), complementary primer chain, DNA polymerase, deoxynucleotide triphosphates (all four types: dATPs, dGTPs, dCTPs, dTTPs) and dideoxynucleotide (just one of the four types in each tube: ddA , ddG, ddC, ddT)(labeled with fluorescent dyes).

let just talk about one tube, we stain the contents of the tube with a color

After that, the DNA polymerase will extend the primer and stop 1% of the time when it reaches the dideoxynucleotides and it will continue to extend due to the presence of enough amount of the deoxynucleotides. (if we have ddA the reaction will complete until we reach A, and if we have ddG it will continue until we reach G and so on). ,(we will undergo the DNA sequencing reaction and it will continue until the dideoxynucleotide binds to the template then the sequencing is terminated, but we may find many A in the sequence, so the ddA may bind in the first site and terminate the reaction and may bind in other sites so the length of the strand will differ, and in other tubes the same thing will happen and we will get different strands with different lengths and terminated by G,C,T,A)

We mix all components of the tubes together, (each tube has a specific color), and then electrophorese the mixture by using capillary electrophoresis or polyacrylamide gel electrophoresis, after all that there is a reader in the computer that reads these colors.

Each color represents a nitrogen base sequence, so the reading from the bottom to the top gives the sequence that It is used to complete the DNA series, and the reading is from the 5' to the 3'.

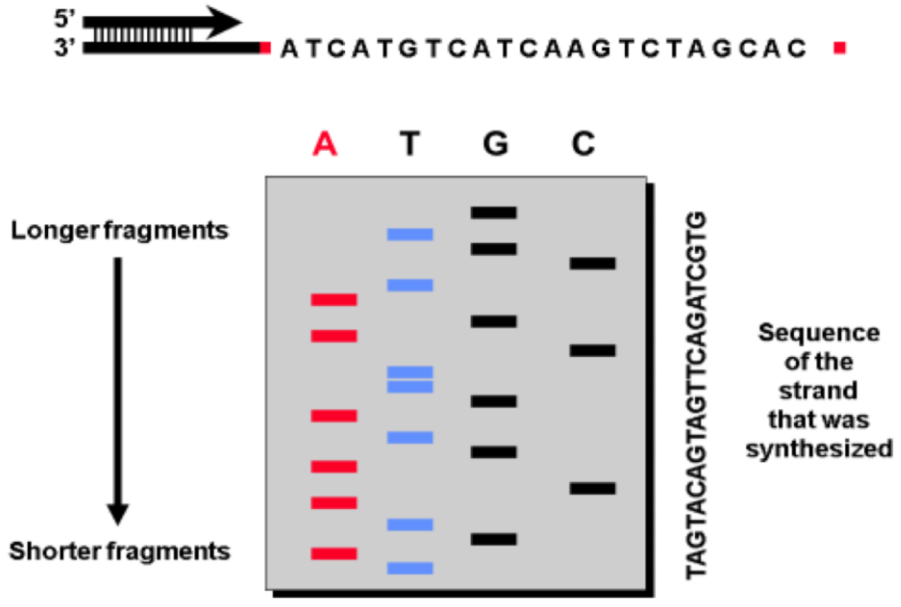
(strands with different ends has a different colors because they came from different tubes, so we will take the mixture of all tubes and electrophorese it to orientate the strands from the shortest to the longest and the computer will see that each strand has a specific color which means that it is terminated with a specific nucleotide, we increase the length by one nucleotide as we go up in the gel, and we can know each end of each strand by its color so we can know the template sequence)

Remember:

-In these reactions, the synthesis continues until the dideoxynucleotide whether (G,C,A or T) is incorporated in the newly synthesized strand, but other reactions continue.

-Each tube contains a collection of strands that ends at its dideoxynucleotide(the dideoxynucleotide that is found in that tube), and each strand in all of these tubes defers from the other by one nucleotide, and according to the colour, we know the end of the sequence.
Now days we use a laser scanner, which reads the colours faster and gives us the sequence directly.

The traditional method to know DNA sequencing requires tedious work where we make a half meter long gel and put 4 lanes and use p32(phosphorus) to detect the emission of the radioactivity then we start reading according to the X-Ray field(as in the figure above) from the



shorter stretch to the to the longer stretch from 5' to 3'.
Because the product is amplified, dideoxy-terminator cycle sequencing requires less DNA than the standard technique.
In this way, they were reading 250 base pairs per day, in the mid-eighties, but now, after the great development in the dideoxynucleotide

sequencing, and by using laser scanner, they are reading 60 billion base pairs in ten minutes.

A major advance in dideoxynucleotide sequencing using a thermal cycler:

-Thermo stable DNA polymerase

-fragments separated by capillary electrophoresis.

This technique is used to discover the human genome sequence, but without mixing the colours in the tubes. computer will read them in separate, but not manually reading.

The computer will read the colors and give you peaks according to the colour so if you do a sequence for a normal gene and compare a sequence for a mutated gene you will see differences in sequences so you could discover the mutation and discover the cause of the genetic disease.

The Human Genom Sequence

***The Human Genome Project completed initial sequencing in 2001.**

-> 2.85 billion of the 3 billion nucleotides have been sequenced.

~Error rate estimated to be less than 1bp in every 10000 bp.

~Work continues to fill in the remaining gaps.

-> Contains 20000-25000 protein coding genes.

-> Almost identical (99.9%) in all people.

-> Invaluable reference but requires extensive annotation that continues even today.

(This paragraph was not given any attention by the doctor)

"وربَّ امتحانٍ سهرت لأجلهِ
حتى تُجاوِزَ علامتُكَ الستّينا"

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