Athar Batch

Genetics

Lecture: 26

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LECTURE 26

Polymerase chain reaction PCR

By

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PCR

- PCR is an **in vitro DNA amplification** *procedure in which* millions of copies of a particular sequence of DNA can be produced within a few hours.
- The flanking sequences of the gene of interest should be known.

- *PCR: polymerase chain reaction*
- *To produce millions of copies for specific gene, we have to know the sequence of nucleotides at the ends of that gene.*

Material required

1-**Target DNA** .

2- Two synthetic oligonucleotide primers:

These primers are complementary to the end of each strand of target DNA to be amplified. The selection of primer requires the knowledge of the flanking sequences of the gene of interest.

- **Two DNA primers** of about 20- 30 nucleotides with complementary sequence of the flanking region can be synthesized.(just at the edges of the region to be copied)

- *PCR is the same of DNA replication, but PCR is in vitro.*
- *A PCR tube is filled with all needed material for PCR (target DNA, primers, polymerase…)*

3- Heat stable DNA polymerase:

This enzyme is derived from bacteria Thermus acquaticus that can tolerate high temperatures. Therefore the enzyme is not denatured at high temperature. This polymerase is not denatured even at temperature around 95°C.

4- All four deoxyribonucleotide triphosphates (dNTPs).

- *Heat stable DNA polymerase = Taq polymerase.*

- The first step in PCR is the separation of the two DNA strands by heat. Heat stable DNA polymerase can tolerate the high temperature, and it is not denatured.
- Deoxyribonucleotide triphosphates: dGTP, dATP, dCTP, dTTP.

Technique steps

Step 1: Separation (Denaturation):

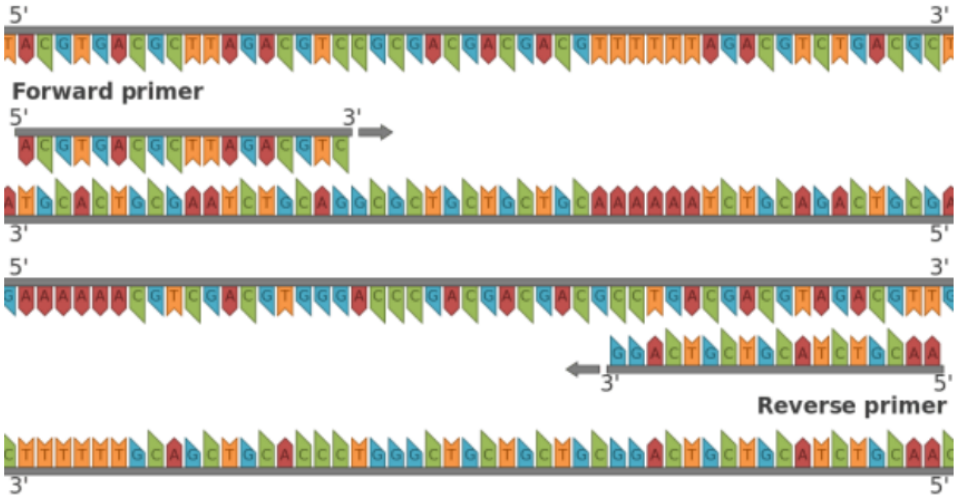
DNA strands are separated (melted) by heating at 95°C for 15 seconds to 2 minutes.

Step 2: Priming (Annealing):

The reaction mixture is cooled to about 50-65°C for about 1-3 minutes. The temperature chosen for cooling is usually about 2-3 below **Primer melting temperature (Tm).** the temperature at which half of the DNA duplex would dissociate to become single stranded.

- Melting temperature of Primer (Tm) means the temperature at which primers get fall off from the DNA.

- After separation of the two strands, we should decrease the temperature to help the primers to bind with the single strands.
- The companies that produce different primers define the melting temperature of the primer. In the second step of PCR, the temperature must be less than the melting temperature about 2 to 3 degrees.
- Melting temperature of the primer: the temperature at which the primer will separate from the complementary strands.



•Forward primers anneal to the antisense strand of the double-stranded DNA, which runs from 3′ to 5′ direction, whereas reverse primers anneal to the sense strand of the double-stranded DNA, which runs from 5′ to 3′ direction.

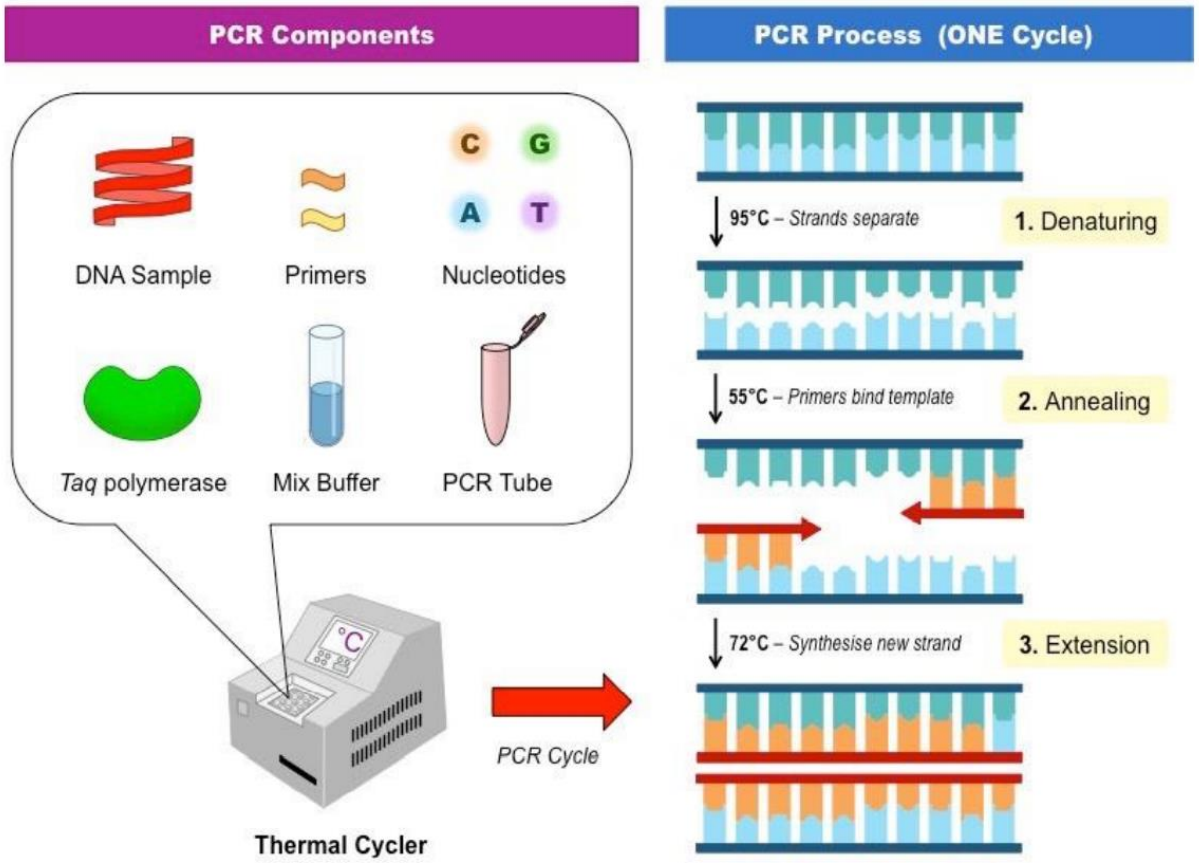
Step 3: Polymerization (extension) (elongation):

New DNA strands are synthesized by **Taq polymerase.** The polymerase reaction is allowed to take place at 72°C for 30 seconds in presence of dNTPs (all four deoxy ribonucleotide triphosphates).

4-The steps of 1,2 and 3 are **repeated.**

In each cycle, the DNA strands are doubled. Thus, 20 cycles provide for 1 million times amplifications. These cycles are generally repeated by automated instrument, called **(Tempcycler or thermalcycler).**

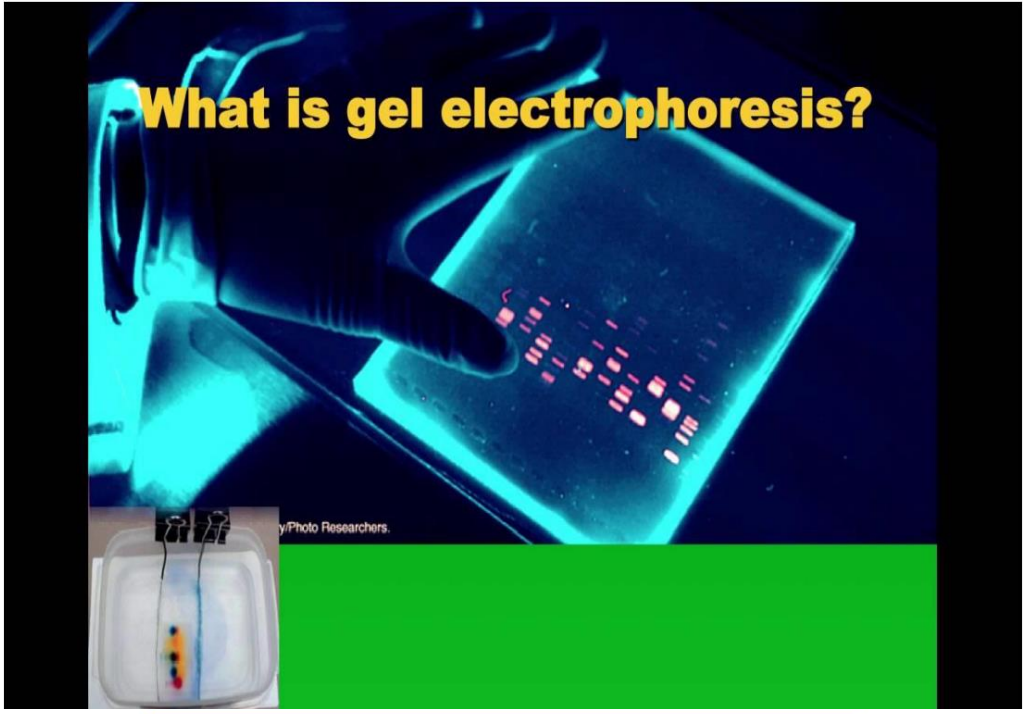
- *The cycles may be 30 or 35 cycles…*
- *In each cycle, each single DNA molecule is doubled.*
- *This is called exponential amplification.*



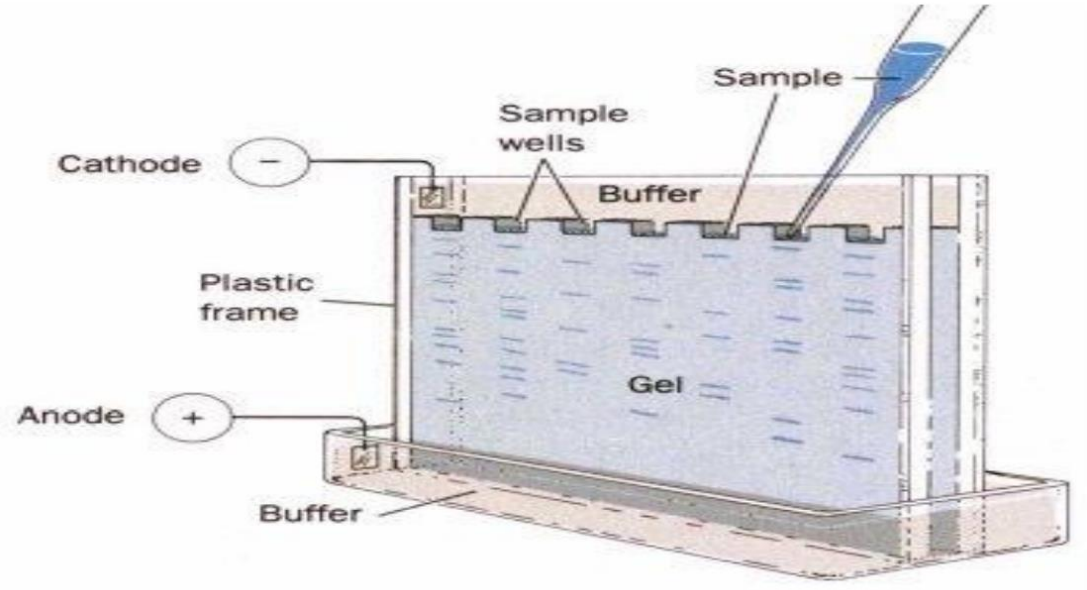
Identification of the PCR product

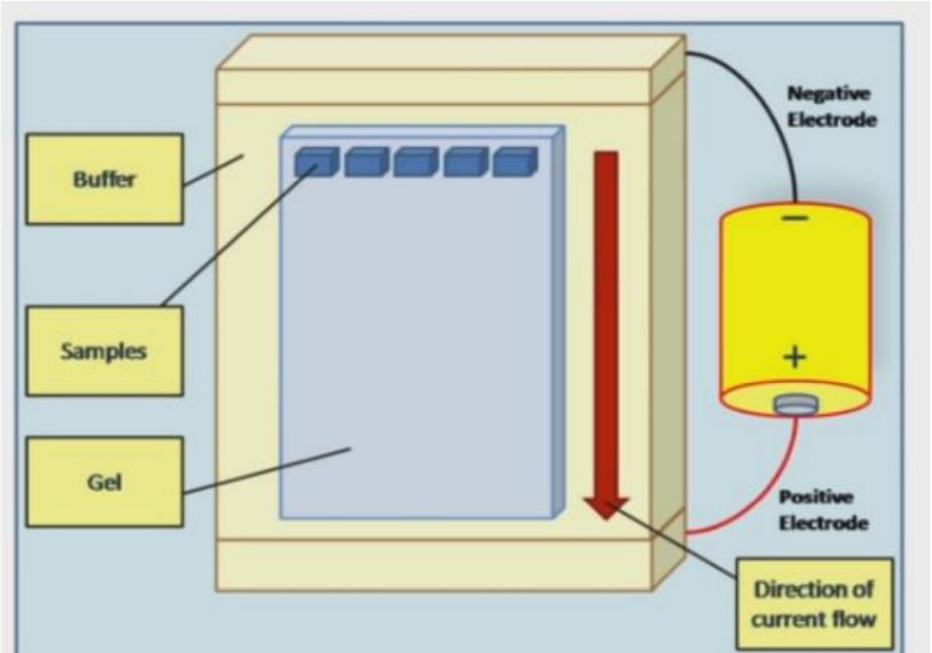
- Agarose gel electrophoresis is a basic and essential technique in molecular biology. It is routinely used for analysis of PCR products. It is the first step for analysis of specific DNA and RNA fragments by northern and Southern blots.

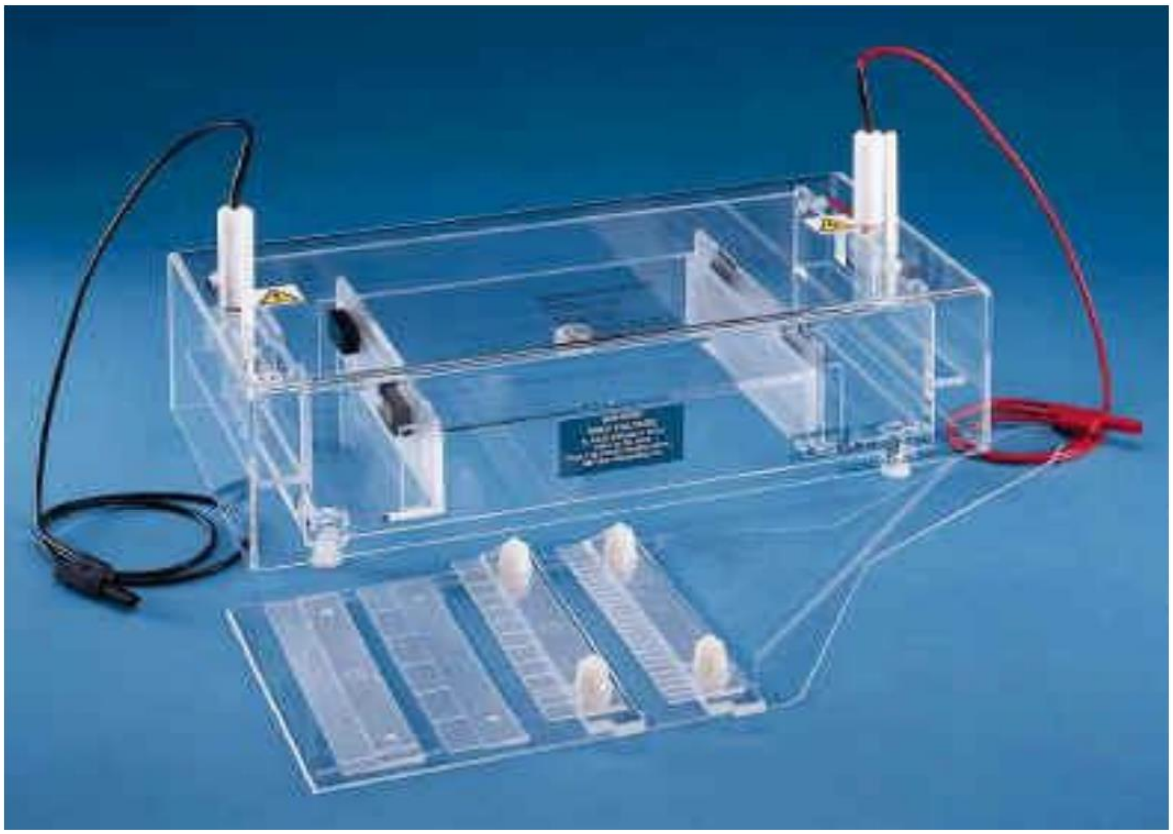
- *We cannot detect the amplification of DNA in PCR without using an identification technique.*
- *Electrophoresis:* الفصل الكهربائي

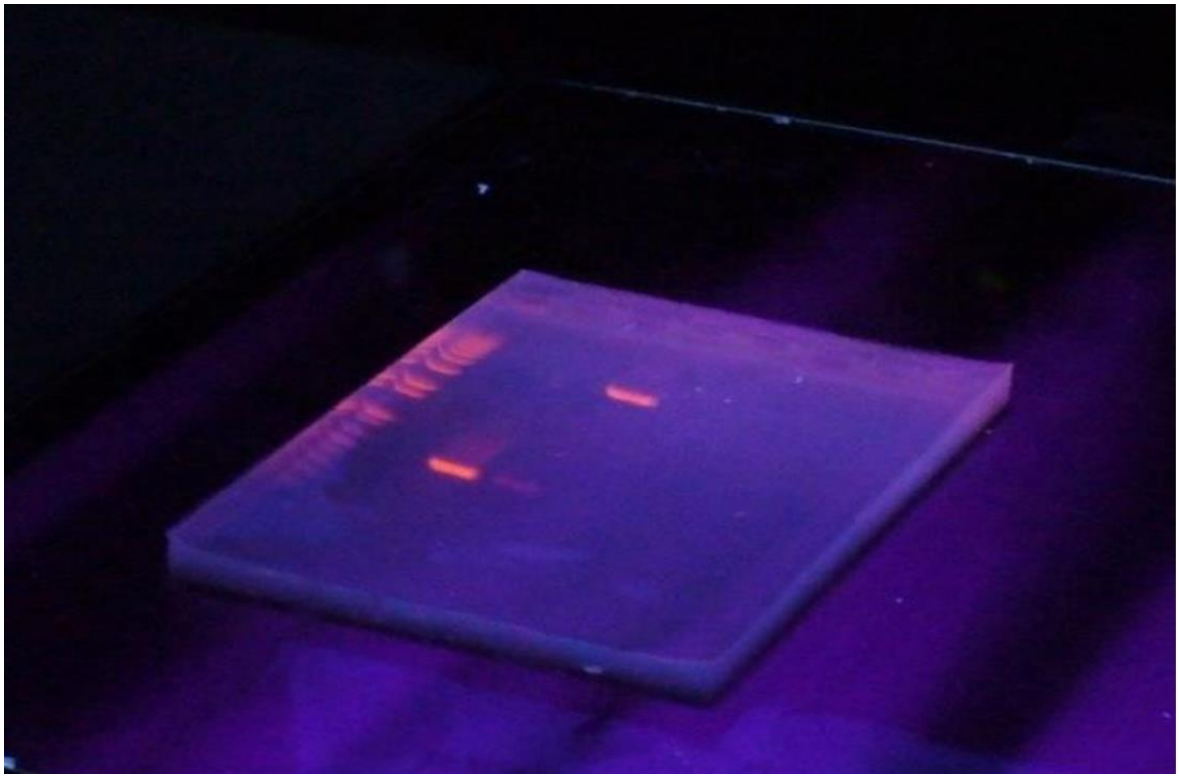


Electrophoresis









رح نعمل فتحات في احد اطراف الجل مشان نحقن المنتج اللي طلعلي من ال PCR

1) After preparation of the agarose gel, we put it in the electrophoresis device.
2) Then immersed in buffer solution.
3) Fill the wells (holes) in the agarose gel with PCR products
4) Connect the device with electricity source → one pole of the device is positive, and the other is negative.
5) Since DNA is negatively charged, DNA segments will travel from the negative pole to the positive pole.
6) During movement of DNA segments, they will bind to a dye called ethidium bromide that is already exist in the gel.
7) When the gel with its contents is exposed to ultraviolet light, the DNA segments will appear as fluorescent bands.

- اذا بنتطلع بالصورة اللي فوق ، عنا اشرطة مصبوغة مشعة مرتبة ورا بعضها بشكل منتظم. طبعا هاي الاشرطة بنسميها DNA ladder و هي عبارة عن قطع من ال DNA بمقاييس مختلفة و نحقنها بالبداية بأول well من الموجودين على طرف المادة الهلامية و بعد ما نوصلهم بالكهرباء راح يتحركوا من القطب السالب الى القطب الموجب... هاي القطع بستخدمها مشان اقيس عليها قطع ال DNAالناتجة من ال PCR كونها ممعروفة الحجم و الوزن.

Reverse Transcriptase PCR (RT-PCR)

- Refers to utilization of mRNA for the formation of target DNA using **reverse transcriptase enzyme**.
- Essentially normal PCR is preceded by reverse transcription (to convert the RNA to cDNA). This is widely used in expression mapping, determining when and where certain genes are expressed.
- **Presence of HIV RNA in blood can be detected as early as 4 weeks after infection.**

- We use RT-PCR to know the expression state of particular gene.
- RNA extraction rather than DNA extraction.
- cDNA: DNA produced by RNA.
- After production of cDNA, we use PCR to amplify cDNA.
- Corona, HIV and hepatitis C viruses are all RNA viruses. We use reverse transcriptase PCR to detect these viruses.
- We can detect the viral infections (that take long time to detect without using PCR) with in less than 4 weeks

Quantitative PCR (Q-PCR)

- Used to measure the quantity of a PCR product (commonly in real –time) QRT-PCR. It quantitatively measures starting amounts of DNA, cDNA or RNA.
- Quantitative real time PCR has a very high degree of precision.
- QRT-PCR methods use fluorescent dyes or DNA probes to measure the amount of amplified product in real time.

- Quantitative PCR can b used to identify the viral load (quantity).
  - مثلا لو بدأ ال amplification بعد ال cycle رقم 5 فيبدأ ال fluorescence يطلع ← معناته ان ال viral load عالي
  - و لو بدأ ال fluorescence بعد مثلا 30 cycle فيعني انه ال viral load مش عالي
- Quantitative PCR is very accurate except if e made mistakes during the process.

Clinical Applications of PCR

- **1. Diagnosis of bacterial and viral diseases:**

  In early phases of tuberculosis, the sputum may contain only very few tubercle bacilli, so that usual acid fast staining may be negative. But PCR could detect even one bacillus present in the specimen. Any other bacterial infection could also be detected similarly. The specific nucleotide sequences of the bacilli are amplified by PCR and then detected by Southern blot analysis.

- Reverse PCR is widely used in the diagnosis of viral infections like **Hepatitis C**, and **HIV**.

- *Tubercle bacilli: cause tuberculosis.*

- **2. Medicolegal cases:**

❖ **DNA profiling**

- Modern-day DNA profiling is also called **short tandem repeat (STR) analysis.** It uses the polymerase chain reaction (PCR)to produce many copies of specific STR sequences.
- In STR analysis the primers used in the PCR are designed to attach to either end of the STR sequence of interest.
- Short tandem repeats (STRs) are short tandemly repeated DNA sequences that involve a repetitive unit of 1-6 bp with the number of repeats varying among individuals, making STRs effective for human identification purposes.

- *DNA profiling:* البصمة الوراثية
- *99% of DNA is common in all people, 1% of DNA differs from one person to another.*
- *Sort tandem repeats: sequences of DNA that are different from one person to another. E.g: TTAGTTAGTTAGTTAG*
- *STRs are only identical in identical twins*

3. Diagnosis of genetic disorders:

The PCR technology has been widely used to amplify the gene segments that contain known mutations for diagnosis of inherited diseases such as sickle cell anemia, beta thalassemia, cystic fibrosis, etc.

4. PCR is especially useful for prenatal diagnosis

of inherited diseases, where cells obtained from fetus by amniocentesis are very few.

- *Diagnosis of genetic disorders:*

لو استخدمنا *primers* خاصة بالجين الطبيعي و صار *amplification* فيعني ان الجين ما فيه *mutation* و لو استخدما *primers* خاصة بال *mutated gene* و صار *amplification* فنعرف انه الجين *mutated*

- *Prenatal diagnosis: detection of genetic disorders of the fetus.*

ما باخذ العينة من ال *fetus* لأنه ممكن يسبب *abortion* بس ناخذها من ال *amniotic fluid* و اللي بيحتوي على عدد قليل من خلايا الجنين

5. Cancer detection:

PCR is widely used to monitor residual abnormal cells present in treated patients. Similarly identification of mutations in oncosuppressor genes such as p53, retinoblastoma gene, etc. can help to identify individuals at high risk of cancer

6. Fossil studies:

DNA can be isolated and PCR amplified from fossils and is used to study evolution by comparing the sequences in the extinct and living organisms.

- Fossil studies: دراسة الحفريات

7.Quantification of gene expression.

8.Tissue typing for transplanting, by PCR and detection of genetic variants.

- A very specific set of genes is examined when DNA testing is used for tissue typing. On chromosome 6 resides a large set of genes in the so called "Major Histocompability Complex," or MHC. These genes are very polymorphic (different) between individuals, and they code for the production of specific glycoprotein antigens located on the surface of many cells called the "human leukocyte antigens" or HLA.
- It is these antigens that allow our immune system to "recognise" our own organs and tissues from those of another individual. These antigens have the ability to provoke an immune system response that results in organ or tissue rejection if the tissue looks foreign.
- In tissue typing, the genes for a number of different HLA molecules are carefully compared between donor and recipient to ensure that they are as similar as possible to minimise the chance of a rejection.

9.In sex determination of embryos, also useful to detect sex-linked disorders in fertilized embryos.

- Quantification of gene expression:

expression فالجين يصير اله amplification اذا صار اله و معين بجين الخاص mRNA استخلص ال

- Major histocompability complex: تختلف اختلاف كبير من شخص لآخر
  - جهاز المناعة ما بيهاجم الجسم لأنه بيتعرف على ال HLA للشخص نفسه بينما بيهاجم اعضاء شخص آخر لأن ال HLA بتكون مختلفة عنده
- Certain genes are present in X chromosome, if these genes are detected, the embryo is male.