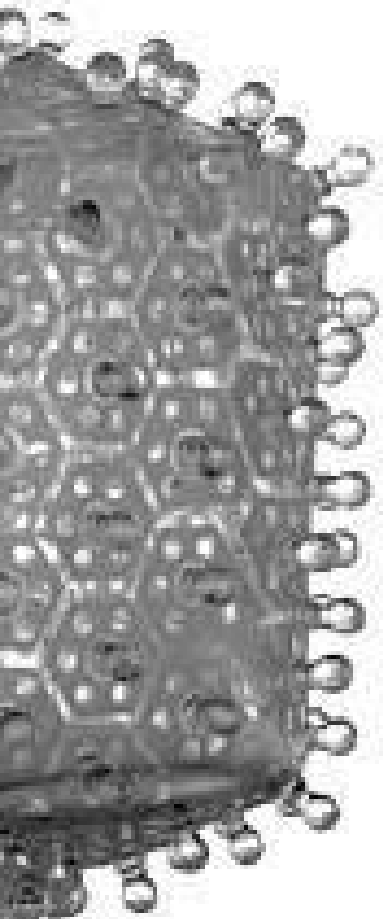


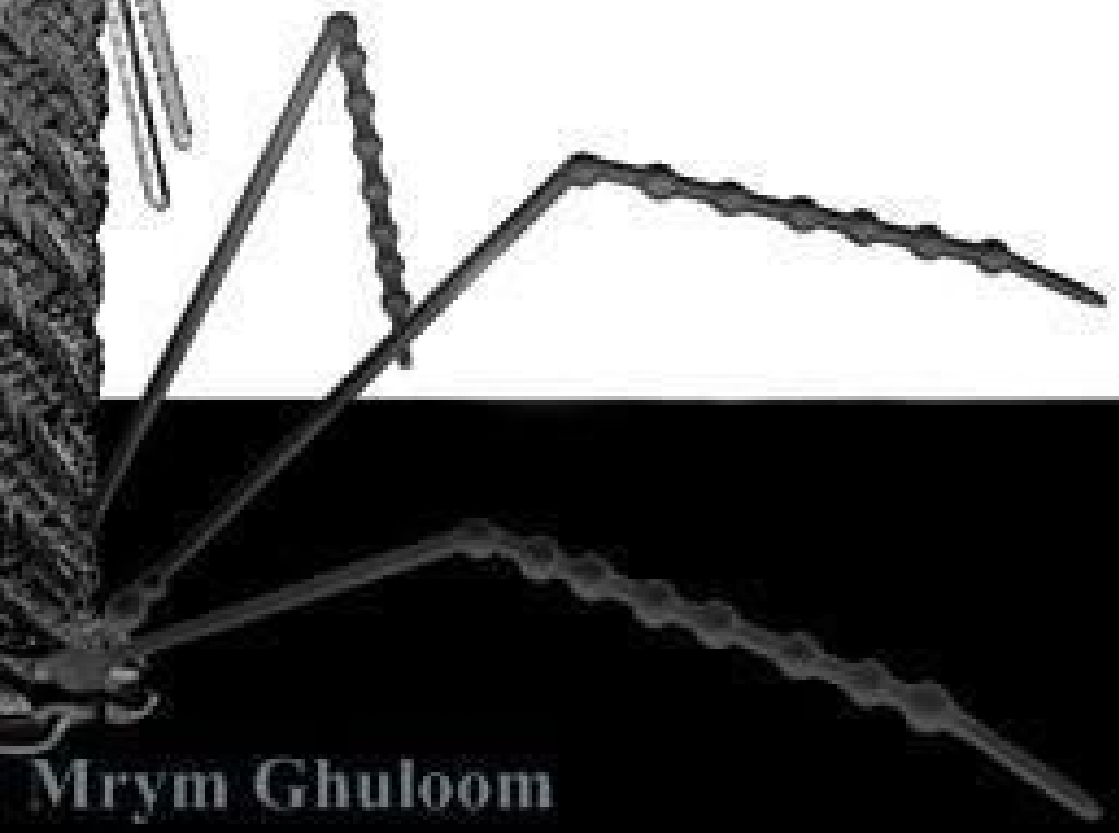
Microbiology

Lecture No:...24 (8-viro)

Dr Name:...Dr. Hamed....

Done by:...Farah Bilal....

Sheet ■ Slide □



Mrym Ghuloom

Diagnosis (2)

Detection

Diagnosis might take 3 lectures we don't want to sum up with it because it's necessary to understand it very well. Today our lecture is a continuation for diagnosis and next lecture will be about it too. After that we'll start talking about antiviral chemotherapy or treatment. And it might take 2 lectures.

Today we'll talk about **detection** of viral genomes by nucleic acids **amplification** methods.

It's very common and **highly sensitive**, sensitivity might reach up to 99% in detecting the causative agent, and it's used to **detect** and **quantify**.

Detection is different from quantification, you detect to know that this thing is there, but to know how many copies of this virus, this is called quantification (كمي ونوعي).

DNA virus such as HIV provirus, Hepatitis B, CMV –CytoMegaloVirus- and HPV – human papilloma virus- can be detected in clinical samples. Also RNA virus such as HIV, Hepatitis C and influenza viruses.

However, before you amplify the genetic material of RNA viruses you have to transcribe it into double stranded genetic material, so if it is a DNA it's already a double stranded and you don't have to do anything before, and we will see later on why it makes a different.

If it's a double stranded that's fine, But if it's a single stranded we can't amplify it. As a rule of thumb if we're using PCR, we have to copy it into a double stranded using a reverse transcription step.

((If it's single stranded you have to re-copy it into double stranded genetic material.))

Why HIV is mentioned in both in term of clinical detection? ** Because it has RT so it can be RNA or DNA.

All diagnostic methods nowadays depend on **molecular** and **genetic material** and **amplification** –PCR-.

Written by Farah Bilal

- **So if you detect and quantify the genetic material, this will help you in diagnosis**, you will know:

1- How many copies of the virus are in the sample.
2- The treatment follow up.

When you treat a viral infection like a chronic infection (hepatitis B), one of your goals is to decrease the number of viral copies that exist in the serum of the sample blood. So it's a parameter for you to follow up your success. If it's successful or not and if there's a resistance that developed during the treatment.(PCR will help you)

- **It is prone to false positive results due to contamination, means that there is no virus but the result of the test says that there is, due to contamination.**

This means that, when you do the test the result you get is positive, but actually the virus you're testing is not there. That happens because there's contamination with another genetic material in your sample which gives you this "false positive" result.

Because you are dealing with a very sensitive technique and with a genetic material, so contamination with another genetic material might lead to false positive result.

To avoid the false positive, for example I work in this lab on a specific line of viruses, the other lab is working with another line, maybe plasmids, any contamination which is easy to be transmitted from someone to another, only by coming to my lab and using my tools he might bring plasmids from his lab and this will contaminate my stuff and lead to false positive results. To avoid the contamination and the false positive result for consequently **strict control** and **aseptic techniques are necessary**.

Where's always contamination, there will always be **aseptic techniques** (wearing gloves, separating rooms, separated mixtures...).

Separate places for DNA and mixture preparation. Because PCR basically is two parts:

1) Prepared buffer/mixture, base pairs of nucleotides and enzymes.
2) Genetic material from any sample (blood, serum, skin, hair, etc).

Both should be in separated places, although we will eventually mix them together, because I might work today in this lab on a diagnostic method for the influenza virus with its genetic material and the next day I might work on the diagnostic method for other virus with its buffer, so if there's a contamination from the DNA of the influenza in this room this will contaminate my mixture. That's why it's prohibited to move the genetic material to a room where the other part of the reaction is happening.

And each room had its own gloves, gowns, pipettes and other equipment. And they all should be sterile, and there must be traffic.

*From Slides:

To avoid false positive results due to contamination:

1- Separate places for DNA and mixture preparation.
2- Independent colour coded ventilated rooms, each with its own sterile gloves, gowns pipettes, and other equipment.
3- In the case of adjoining rooms, the direction of flow of activities must always be from entrance to exit. –traffic-

PCR, Polymerase Chain Reaction:

- Component:

1- **Primers**: consist of a specific sequence of base pairs/nucleotides which will attach and hybridize or anneal to a specific sequence in a virus genetic material.

To come back to the positive-negative, the primers usually we have 10 base pairs and we have 2 primers, their function is to attach to a complementary sequence in the virus **-so the primers are positive, and the complementary sequences are negative small pieces in the virus genetic material-**. We're talking about 10 base pairs for each primer and the sequence of viral genetic material could be 1000 bp so they will attach to specific sequences, and since we have 2 primers each of them will attach to a part of the genetic material.

2- **Taq polymerase:** is an enzyme its function is to **amplify.** This enzyme is from a bacterium called thermophillus aquaticus, which lives in hot springs so we can conclude that this enzyme (taq polymerase) will *tolerate high temperatures*. (we will see the importance of this later on in this lecture)

Imagine that each primer has attached to specific parts and in the middle the taq polymerase is attached. Polymerase enzymes amplify sequence between two primers.

First step of PCR :

DNA is a double stranded genetic material, the two strands are attached to each other(C-G) (T-A), so you have to denature it to separate the strands from each other. We treat the DNA with temperature, the first temperature is 94 centigrades for 1 min and a detergent, this will separate the two strands.

Second Step of PCR:

Before step 1 it was difficult for the primers to attach because both strands were attached to each other, but now the primers will have the chance to find its complementary sequence and attach to it. So they will hybridize with the homologous nucleotide stretchs on each strand of the target viral DNA genome.

DNA polymerase, taq polymerase (it is the open square in the figure below), this enzyme has the capability to act at high temperature because it's extracted from the thermophillus aqueticus bacterium which lives in hot springs, so it has the property of tolerating the high temperature. When we exposed the DNA to 94˚C, it wasn't affected, however, its *optimum* temperature is around 72-74˚C.

So, firstly we have to separate the two strands, this is done by raising the temperature to 94˚C, and after one minute it is reduced to 52˚C for 20 seconds in order to reach the perfect temperature for the primers to attach to the complementary sequence on the single stranded DNA to allow the annealing of primers. And after that DNA polymerase (taq polymerase) can work when the temperature is raised to 72˚C which is the third step.

Third Step of PCR:

Here the temperature is raised to 72˚C for 5 mins.

Why? In order to allow the enzyme – taq polymerase- to work and DNA polymerization to occur.

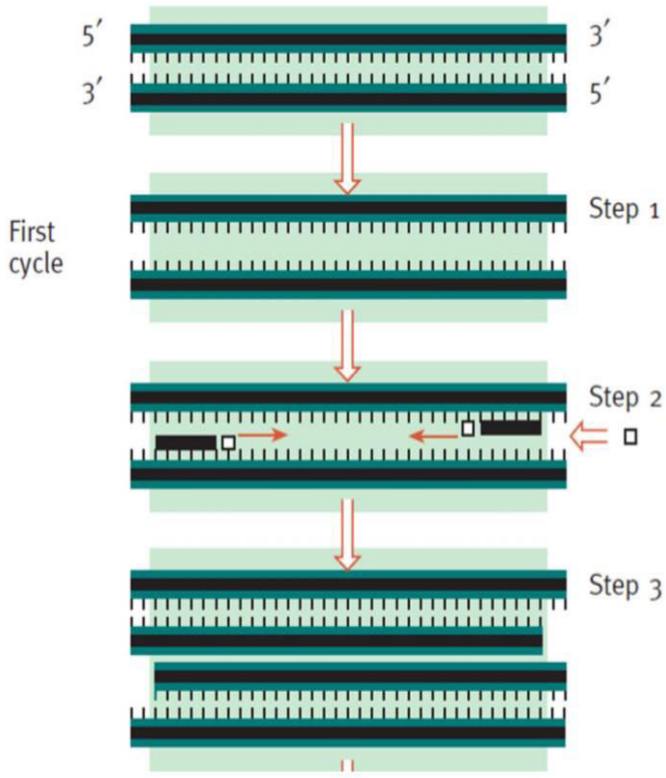
This enzyme synthesizes a complementary strand for each single strand of the viral DNA which work as templates for DNA polymerase. Of course, this needs adding nucleotides; remember that the prepared mixture/buffer has *oligonucleotide bases* that are used for this purpose (they are added to a preexisting chain; which is the primer).

Then multiple copies will be amplified from each strand including this enzyme and they do this in a multiple cycles.

Therefore, one copy of viral DNA can be amplified for million copies.

Then, because the amplified DNA cannot seen by eye, You take these copies and run it on a gel with pores that allow the DNA to migrate (detection material). It's used to separate the genetic material based on the size. We expose/put it in an electrical field (with positive and negative poles). The genetic material will migrate towards the positive pole (since the DNA is negatively charged). The DNA is separated, and the smaller the size, the more easy for DNA to migrate through pores and the more close it will be to the positive pole. (since pores are small)

And detecting/visualising is done using a stain called ethidium bromide, this stain will intercalate with the genetic material and makes it visible.



Looking at the figure:

- These are the two strands 1 and 2, You separate them using the temperature.
- There's a space for the primer to come.
- In step 2 the primers attach as you can see to these strands. (forward and reverse primers)
- Step3, Now the DNA polymerase (open square) will do its job by amplifying the strand.

So you will have from each strand 2 strands, as you see they were originally two strands we separated them primers and taq polymerase attached, they amplify each strand, so you will end up with 2 copies of double stranded genetic materials.

And the cycle goes on.. So each cycle will give you extra copies of the DNA.

To get things more clear :

Step 1 >> ↑Temperature (94˚C for 1 min), Denature the DNA, the strands are separated, space is available for primers.

Step 2>> ↓ Temperature (52˚C for 20 sec), Primers forward and reverse) can attach, Taq polymerases are added.

Step 3>> ↑Temperature (72˚C for 5 min), Polymerases start working, 2 copies of Double stranded genetic material. This Figure is not in the book, only for understanding. But I recommend checking it from slides to see the colors.

Here, we separated strands then primers annealed to the DNA strand. We have forward primer and reverse primer. Then taq polymerase will amplify.

- From where the polymerase will get the C G T A ?

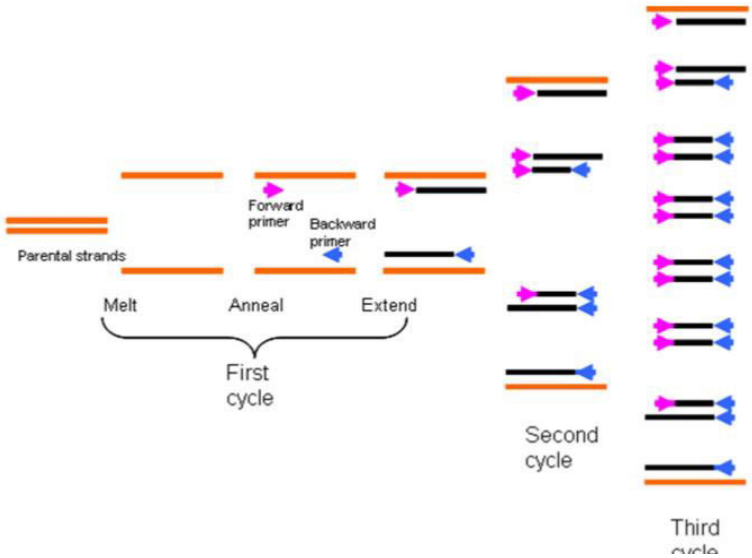
From the solution we already prepared with oligonucleotide bases. The solution has everything; nucleotides and enzymes.

So taq polymerase will choose based on what it's attached to.

So what we will get from each strand?

we will have a dsDNA from the first one and another dsDNA from the other one.

And the cycle will goes on them both, separate them and give other copies, so we will have million copies.



*NOTICE:

After one cycle, 2 copies are produced.
After 2 cycles, 4 copies are produced.
After 3 cycles, 8 copies are produced,
and so on.

And now we're done with the conventional PCR. ☺

http://www.youtube.com/watch?v=iQsu3Kz9NYo

Nested PCR:

-If you understand the principles of polymerase chain reaction this will be easily understood-.

Nested PCR is very sensitive. The idea comes after the initial amplification of a unique stretch of the viral DNA.

after we separate the two strands and add primers 1 and 2, in the next cycle when you separate it again you add another pair of primers, so a further set of internal primers is added and will anneal to the DNA within the original fragment, leading to allow smaller stretches to be amplified and get more sensitive amplification.

From slide:

> Following initial amplification of a unique stretch of viral DNA, a further set of 'internal' primers is added that anneal to DNA within the original fragment allowing a smaller stretch to be amplified.

Imagine that the space between the first conventional primer and the second primer one kpb and taq polymerase should work on these 1000 bp, it will give you accurate results but not 100%, BUT if we give him a break in the middle of the way by putting other primers this will decrease the probability of making mistakes, so the result will be more accurate and correct.

http://www.youtube.com/watch?v=nW9CSNpETHg

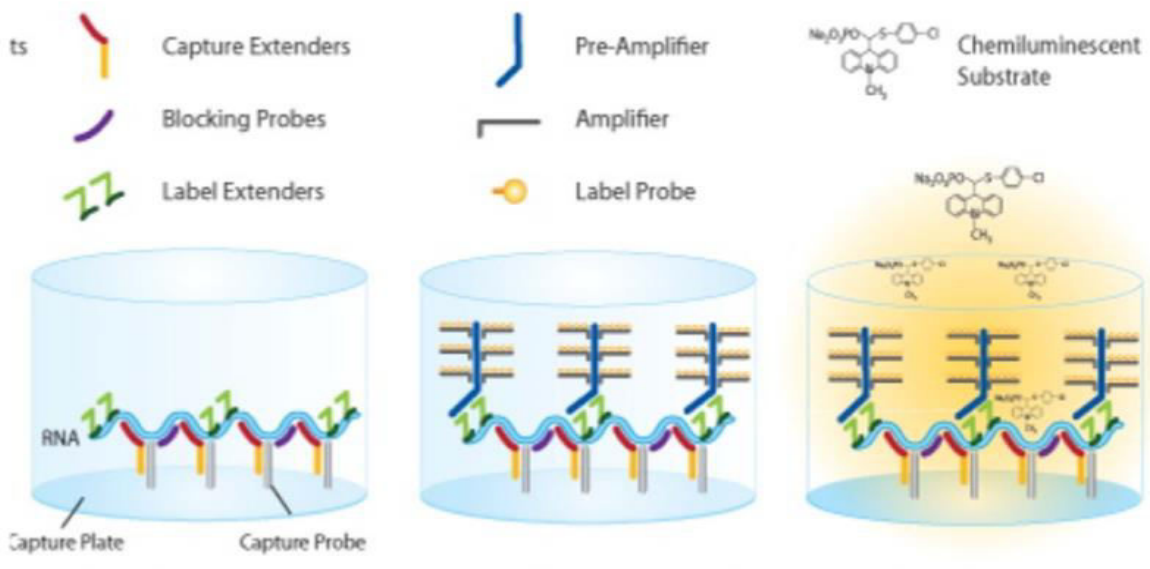
Branched Chain Technique:

Molecular technique which is used in detecting and quantifying the genetic material (**RNA only**).

It is highly sensitive, it is what we call **signal amplification method** compared to the PCR which is a **target amplification**, because you amplify a target part in the genetic material, but in this method we amplify a signal.-we'll know what does signal mean-.

- It is faster, less laborious and expensive and requires less technical ability than PCR.

This figure is not in the book.



This is a capture plate; with this capture plate we have a **capture probe**. It will capture any complementary genetic material, so it is **negative**.

-Similar to PCR, When the primers were positive and the genetic material was negative-.

1- You have your own sample, and you lyse it, you breakdown the proteinatous material in the virus which is the capsid, using some buffers so the genetic material will be available free now.

Notice that this technique is used only for RNA viruses.

2- You add your sample to the capture plate.

The helical structure is the RNA, when you add it consider it's the positive

3- The probe will capture it –negative- , because there will be complementation, and the bases will attach to each other. So it will capture it.

Till now there's no any indicator whether it's captured or not

4- Now we will add the amplifier (Z shaped) or the branched DNA material, why branched? Because it has two pieces –branch like- the upper part of the Z and the lower one.

Lower piece attach to a specific complementary piece of the viral RNA – if it's there-, the other part of the Z is available.

Until now we have 3 parts :

- The capture probe
- The RNA
- The bDNA (Z-shaped), part of it is complementary for the RNA and there is also a free part

In this level we still can't see anything.

5- Now we add a pre-amplifier, or it's called alkaline phosphatase linked probe. At the end of it, there is a complementary sequence for the remaining (free part) of the branched DNA (Z) . This will attach to the free complementary part of the branched DNA.

However this probe –alkaline phosphatase linked probe- is an enzyme and has a place for a substrate.

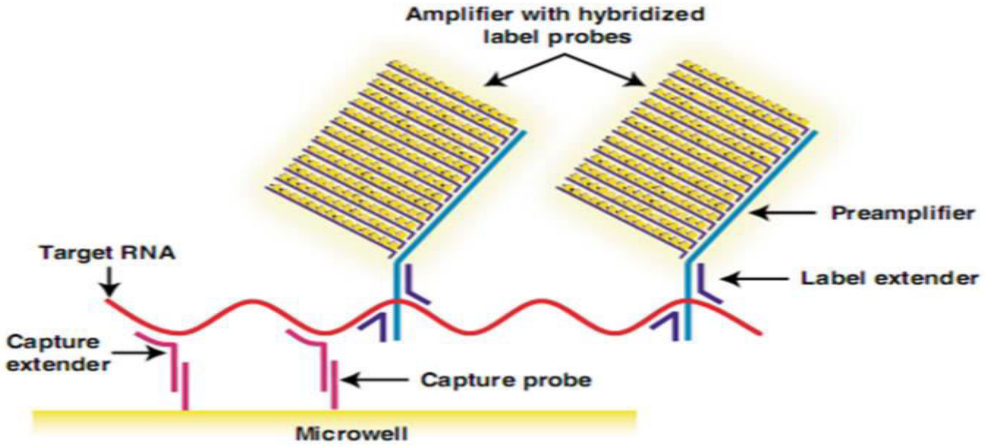
6- At the end, we need to detect if there's a viral RNA or not, and that happens by adding chemical substrate that is catalyzed by the alkaline phosphatase (AP) if it's there, if it's not that means there's no genetic material. Because all the previous things depend on the reaction between the RNA (genetic material) and the capture probe.

REMEMBER that after each step we do WASHING, so:

- If we add RNA that binds to its specific capture probe then we do washing, it will still bound. After that we add the branched DNA and part of it will bind to the RNA then wash. Alkaline phosphatase (AP) is added will attach to the free part of the branched DNA, and when the substrate is added, it will be catalyzed by it producing a colour change.
- The substrate originally is colorless, but when it's catalyzed it will turn into colorful substrate.
- So we will see change in the color which indicates that there's a genetic material.
- The intensity of the color is related to the quantity of the genetic material. (أحمر غامق أو فاتح أو فاهي)

And then we measure it by spectrophotometer.

Another figure for Branched chain technique:



Nucleic Acid Sequence-Based Amplification (NASBA):

- The target is the RNA viruses or the mRNA. (Same target of the branched DNA technique)
- It uses 3 enzymes and 2 primers and can be quantitated

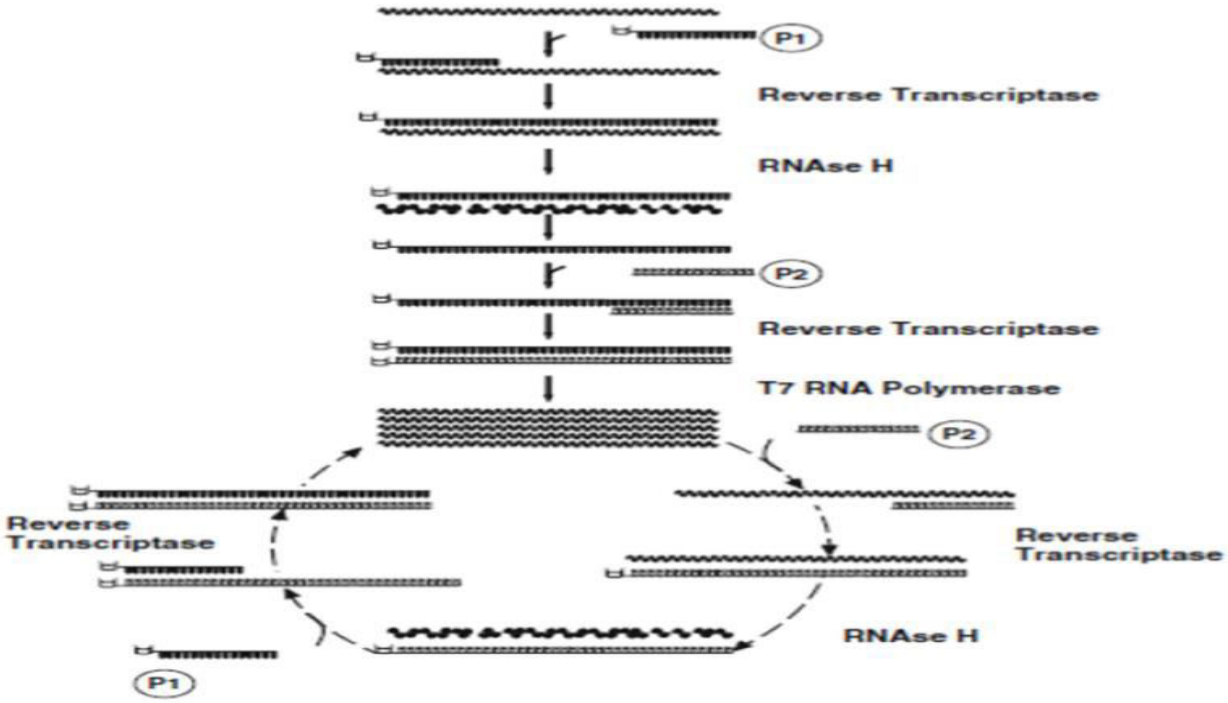
The 3 enzymes are Reverse Transcriptase (RT), T7 DNA-Dependent RNA Polymerase, RNase H.

- It is isothermatic, it needs a **unified temperature 41°C** , not like the PCR 94°C then 52°C then 72°C.

What do we mean by DNA dependent RNA polymerase?

- DNA dependent: means it needs DNA to work on as a template
- RNA polymerase: The function is to polymerize RNA.

Figure also not in the book –only for understanding- .



- This is a chain of RNA and this is the primer (P1)
- The primer is attached to the RNA genetic material
- RT will make a complimentary copy of the RNA so we'll have *double stranded structure consisting of the original RNA and the complementary DNA*.
- RNase H: Where there is *RNA in a double strand* , It degrades it (the RNA strand) and we're left with the complementary DNA.
- Primer 2 will attach to The complementary DNA, and then reverse transcriptase (RT) will make another copy producing dsDNA.

Now there's no RNA, only DNA. BUT I'm trying to detect and quantify the RNA? In other words, What's the benefit from degrading the RNA although I'm trying to detect it?? :/

- Here it comes the role of the RNA polymerase which is a DNA-dependent RNA polymerase, its function is to transcribe the DNA strands and use them to synthesize RNA strands

And the process is repeated as long as the enzyme is there and the balance is towards the polymerase so we will have a lot of RNA transcripts.

My target is to have a DNA at the end of the day to use it to transcribe a lot of RNA copies from it.

- To Sum up

I started with RNA and ended up with multiple RNAs. And made a DNA intermediate because the enzyme needs DNA to work on!

** A student in section 1 asks: Why doesn't "RNase H" break the new formed RNAs? Simply, because they are ssRNA, and this enzyme degrades RNA in a double stranded structure not the single ones.

Real time PCR

Is the same like PCR, and the distinguish thing is that you will get results faster and it's very sensitive, you will get the results as you amplify the genetic material.

We have 2 copies you will get a color, more copies denser color. You DON'T have to wait till you have millions of copies and run it on a gel and then stain it with ethidium bromide. This technique based on a dye that intercalates into any produced DNA strand.

If there's a copy of the dsDNA produced then the dye will bind to it. And it gets denser as you're having more copies. The device will quantify it step by step.

http://www.youtube.com/watch?v=EaGH1eKfvC0

Uses:

1- Monitoring the effect of the antiviral as we said before.

You're treating an **HIV patient** with HAART (combination therapy, Highly Active Anti-Retroviral Therapy).

- ✓ Always in HIV you have to treat with a combination of antivirals, so you are treating HIV patient, you need to monitor how efficient is your treatment. How? Using the molecular techniques which one of them is PCR.
- ✓ You have to monitor the patient every three months.
- ✓ Your target is not to cure, your target to have like 5o or less genome copies (old number) but the new might be 10.

- How can you detect how many viruses in the plasma? By the molecular techniques mainly the polymerase chain reaction (PCR).
  - ✓ In an untreated patient, 10000 copies will be detected.

Chronic Hepatitis: a thousand fold decrease of the copy number after you start the treatment hepatitis B, which is one of the viral infections that you have to monitor when you start the treatment using lamivudine, famciclovir and adefovir. You will hear about treatment more in the antiviral.

- Same with the **hepatitis C** when we use the interferon and ribavirin.

The molecular techniques here will help you to detect which **phenotype** of hepatitis C virus your patient is having.

Hepatitis C virus has 5-6 types. In the book it's said 5 types but actually they're 6 because one of them is divided to A and B.

There's a common type in South America and Middle America, and other common type in the Middle East.

When you know which type is in your patient, you will know what anti-viral and medication they perfectly responds to. So you have to keep that in mind and you might stop the medication at earlier stages. Instead of giving him medication for 2 years it's 12 months now, because you will see a big drop in the copy numbers of the hepatitis C of this phenotype.

2- Analysis for Drug Resistance Mutation:

Mutation development which might lead to resistance mutation is very important which you can detect using molecular techniques.

If you are treating a patient and the patient is not responding anymore, then think that there might be some resistance developing, and to detect that resistance which is basically based on point mutation, as an example HIV the medication that you use as we said before is a combination of antivirals, one works on the RT and the other one works on proteases. So you have to detect if you have a point mutation in the target and you detect that using molecular techniques like

- **Point mutation assay**
- **Sequencing**: very common and very accurate. If you have 1000bp, you can detect each base of them by this technique. No much details mentioned about it in the textbook so no much details are required. But you need to know that it's an automated method and the method of choice and you can outline each base pair in the sequence.
- **Chip technology.**

Next lecture we'll talk about what remained of the diagnosis.

Eventually, it's your choice to fail or success. Good Luck ☺