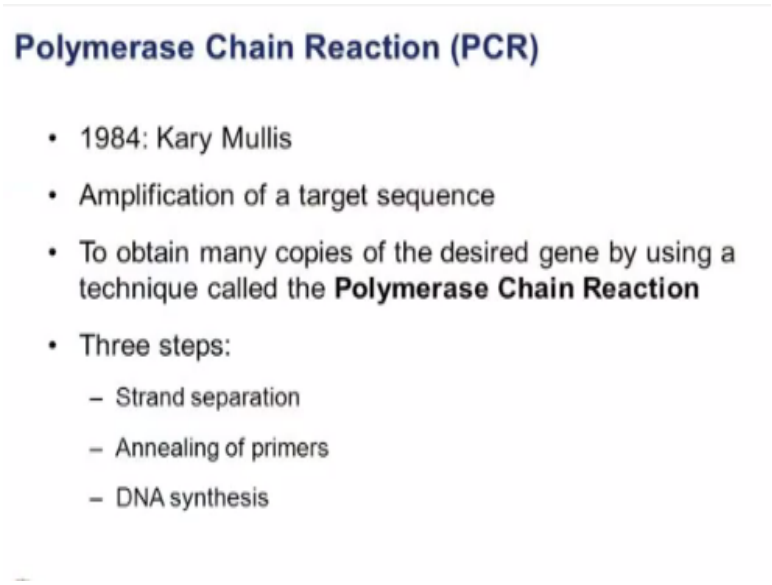
**Bioengineering: An Interface with Biology and Medicine**
**Prof. Sanjeeva Srivastava**
**Department of Biosciences and Bioengineering**
**Indian Institute of Technology- Bombay**

**Lecture – 09**
**DNA Tools & Biotechnology**

**(Refer Slide Time: 00:19)**


Let us switch here and talk about another revolutionary technique, which is polymerase chain reaction or PCR, something very similar to gene cloning but here you are using some chemicals to make multiple copies of a given gene.

**(Refer Slide Time: 00:39)**



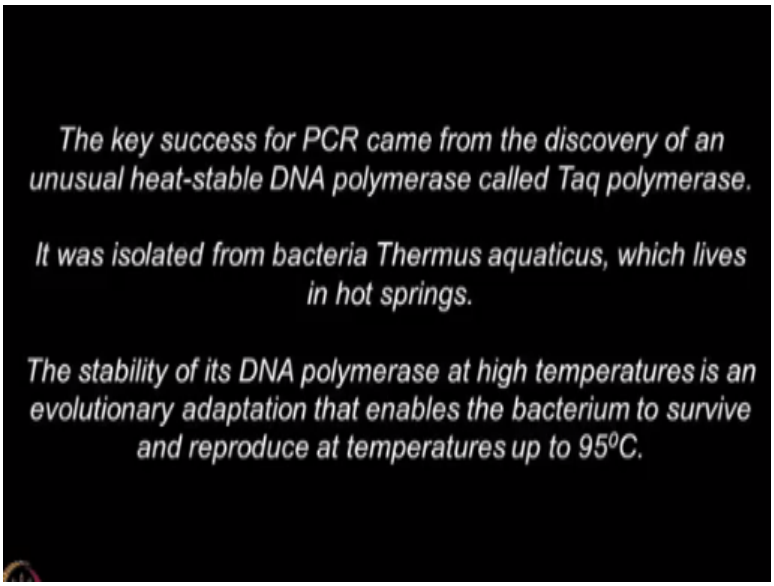

A scientist which is; we have, who has made, it has a mile contribution to this field, Kary Mulis in 1984, he first time discover this process polymerase chain reaction and some of these discoveries happen sometime accidentally, if you remember I were mentioning to you in you know old environment when we had these RK bacteria and some of the very you know, the organism living in the extreme conditions especially, you know the halophiles and some of the thermophiles.

Those who are living in the very extreme hot condition, if you observe those particular organism you might be able to derive certain properties from it and therefore, Kary Mulis was able to isolate some bacteria from a very hot spring of the sulphur hot springs from which he was able to get some bacteria, thermus aquaticus who can withstand and still reproduce at 95 degrees, 100 degrees temperature, so now these enzymes gives that bacteria that property, which can actually make it still live in those hot temperature.

So, he used isolated that enzyme, taq polymerase and that was actually better instrumental in developing this particular technique known as polymerase chain reaction. So, broadly in polymerase chain reaction, you have 3 processes; one is the DNA strands to separate, your double-stranded DNA, you will heat them, denature them, so that the double-stranded DNA become single standard, they get separated.

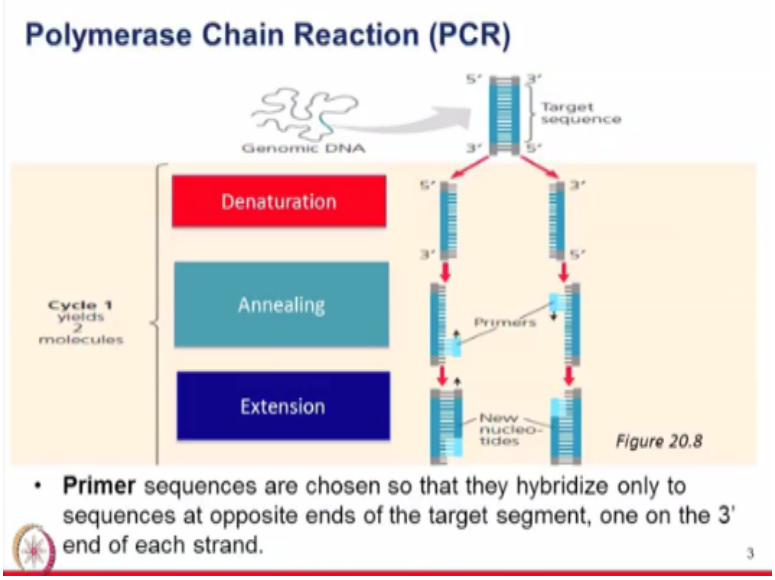
Then you want to have some primers or a short stretch of nucleotides, which will bind on both the opposite end of the pair from 5 degree; 5 primes to 3 primes and then you want to have a DNA synthesis using taq polymerase, all the nucleotides, which are required magnesium chloride etc. everything you add in the reaction mix.

**(Refer Slide Time: 02:19)**



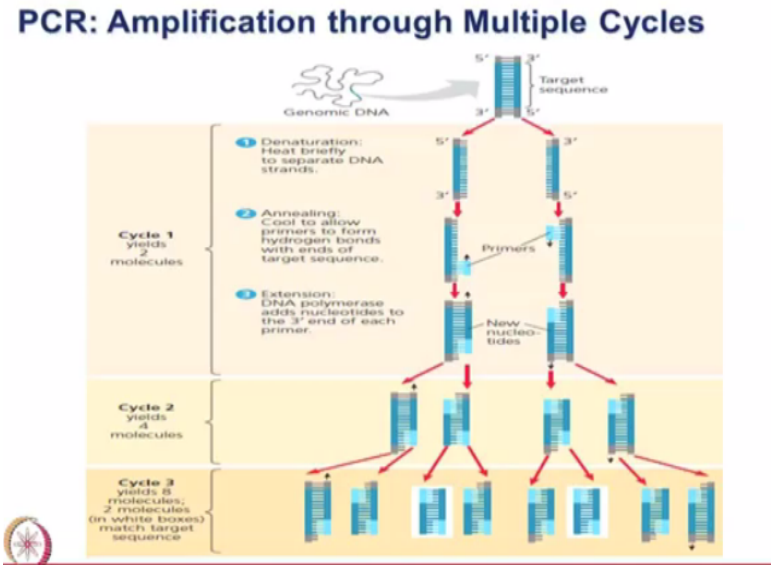

So, the success of polymerase chain reaction came from the key discovery of knowing about taq polymerase which was isolated from thermus aquaticus, living in the hot; hot springs. The stability of this DNA polymerase at very high temperature was very useful to derive this process of polymerase chain reaction because this bacteria was able to live and reproduce even at 95 degrees temperature.

**(Refer Slide Time: 02:44)**



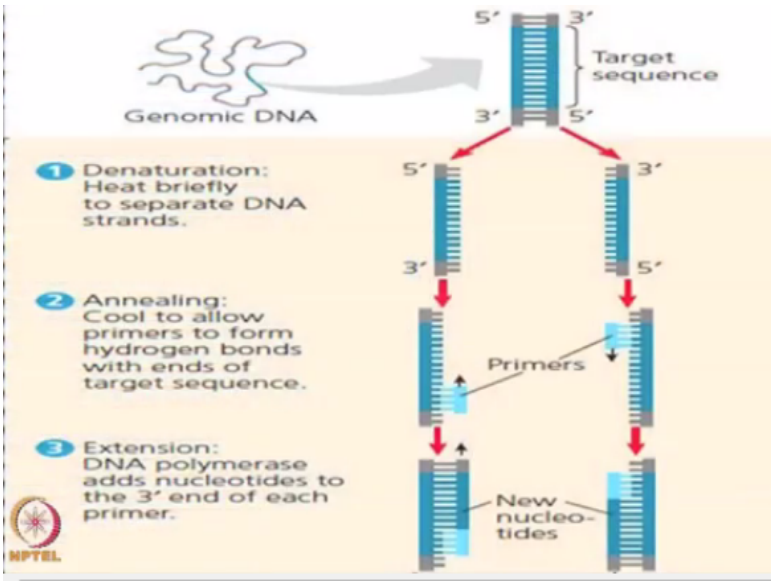

And therefore, the enzyme taq polymerase which was isolated from it became very useful for this process. So, broadly these are the 3 steps which happen in a polymerase chain reaction, first you have the double-stranded DNA which you are denaturing at very high temperature 95 degrees or 100 degrees and now, this becomes 2 single standard DNA. Then you are adding a short stretch of nucleotides, which can hybridised to the complimentary base pairing rule in the same manner.

**(Refer Slide Time: 03:22)**



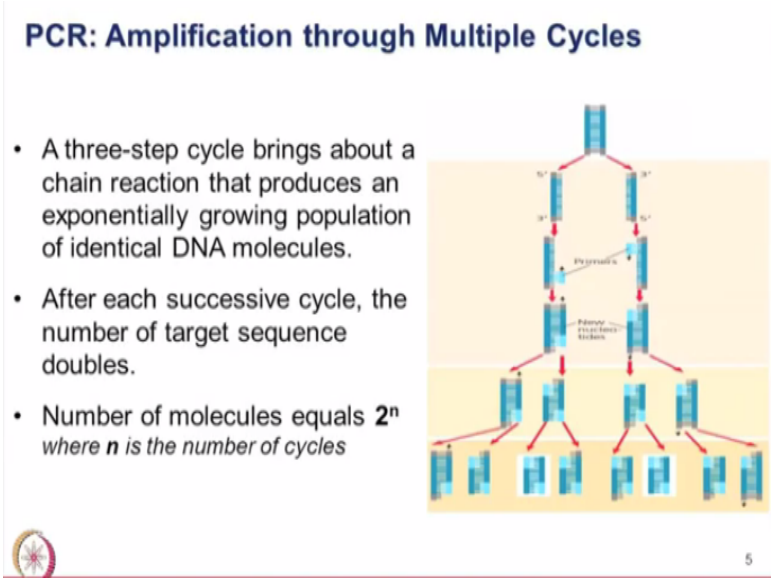

And now, from both the sides, you are giving you know that situation where now the second strand of the DNA can be synthesised, to do this part you are adding the taq polymerase.

**(Refer Slide Time: 03:37)**




You are also providing the right temperature here which is annealing temperature and you are adding all the dNTP's, or the nucleotides which are required for the synthesis.

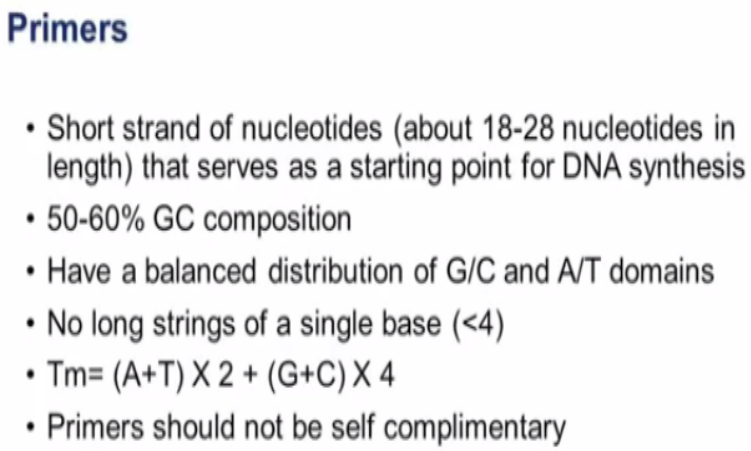
**(Refer Slide Time: 03:44)**




This thing is happening as the part of extension, when now a new a strand is being formed, so now the single-stranded DNA became double-stranded and one copy of DNA became 2 copies of DNA here. In this whole thing what is most important to understand is; primers, what are the primers; you have an idea or understanding for what are the primers? Alright, these are if you are a large gene sequence, within the large gene sequence, you do not amplify the full gene, you might want to amplify a, you know a large region of that.

So, you are finding some region which can be used to amplify that the gene and you are synthesising some nucleotides which are having some complimentary opposite base pairs.

**(Refer Slide Time: 04:30)**




And you are using those short stretch of nucleotides which could be 18 to 28 nucleotide length as a starting point for the DNA synthesis to happen, now these you are you know, in some way, you are just adding ATGC and putting that based on the complimentary sequence of DNA, so you want to ensure that you are picking up the sequence from a region which is not having too much GC contents or you are not having the same base pair in multiples present like you know, not A is continuous or G is continuous present.

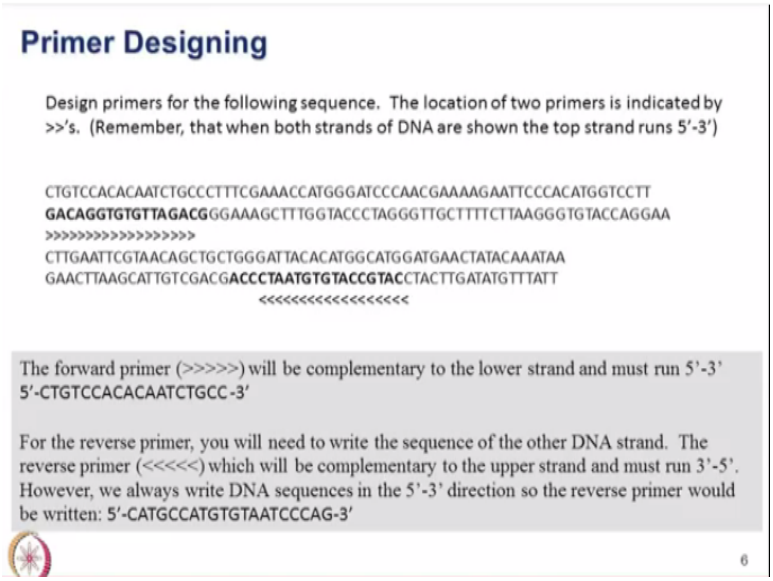
So and they should not be self-complimentary as well, one more important thing here it is known as the Tm or the melting temperature because if you go back, this process which is annealing, you are giving a specific temperature for this primer to bind to the DNA strands and this happens at a specific temperature which is known an annealing temperature, so you can actually calculate what can be possible annealing temperature by looking at calculating the AT and GC contents.

So, this is the formula for doing that you can have A + T times 2 + G and C contents times 4 that will give you the Tm value and that Tm value could be 65 degrees or 70 degrees, can be used as annealing temperature, so initially you used a very high temperature which was for denaturation,

then you are reducing the temperature down now, you are bringing a primer to bind to that particular DNA strands using annealing temperature.

And then you on to extends its further, again you are changing temperature around 72 degree for the taq polymerase to work.

**(Refer Slide Time: 06:06)**




So, let us look at one of the sequence and let us assume that you want to design the primers for this particular gene sequence and you want to amplify this you know, starting from this arrow region to this arrow region, you want to amplify their gene, so for both the DNA strands, we have mentioned here the, the full sequence here, so for the forward primer you have to have the complimentary opposite base pairs.

So, if A is here or the G is here, what will be the complimentary base pair? C, right, if you have A then, C then, what will be the complimentary, so can you start writing about what can be the forward primer sequence, please write that that will in the 5 prime to 3 prime direction, this is a gene sequence we have on to amplify the gene from this part here, to do this we are saying that you can start making a primer, you can synthesise a primer that will be; the forward primer will be complimentary to this particular segment which you want to amplify.

So, if you have just opposite sequence of that in 5 prime to 3 prime direction that can be your forward primer, now the reverse primer is much more is simpler and easy because we are deriving everything from 5 prime to 3 prime, you are synthesising in fact, chemically synthesising the primers, we now know how to synthesise chemically ATGC bases, so a primer nucleotide sequence can be synthesised, let comes in the powder form.

And then, you can actually you know add some water to make your primer mix, so now everything you want to have always in the 5 prime to 3 prime direction this is what you have used here for the forward primer. Now, if a opposite primer you want to design from this part here, it become much more simpler because you are just writing the sequence of the other DNA strand in this case for the reverse primer.

And therefore, your sequence for the reverse primer will become a starting from C, it will become CAT GCC, A and you can continue with that. Are you with me? So, you want to amplify a given gene segment and I have shown you the arrows from this part to this part you want to amplify that DNA, to do that you are adding a smallest stretch of nucleotides which you want to chemically synthesise along with those chemical synthesised primers, you will add the enzyme, you will add nucleotides, you will make the mixture everything provide the right temperature conditions inside the instrument.

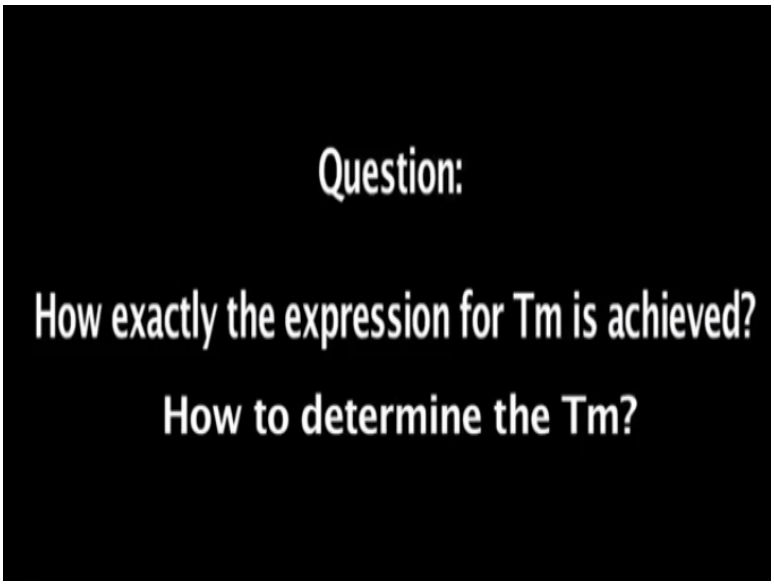
And perform polymerase chain reaction, so that your DNA can keep multiplying multiple copies that is the intention here. To do this, the forward primer you have taken from 5 prime to 3 prime directions, you have just use a complimentary sequence of it and you got this particular sequence derived for the forward primer. Reverse primer; the opposite strand of this which we have use for the DNA and because we have to derive in 5 prime to 3 prime in formation, we are just simply writing the sequence from the C 80 onwards.

So, this is where you can synthesise and design these primers now, if I am giving you this particular primer sequence which is from the 5 prime to 3 prime, what will be the melting temperature, given that you have this formula which will not be shown in the exam, what will be

the melting temperature Tm for this particular primer, a straight forward, just count A, T's, G's and C's.

So, if this was a temperature to be used for PCR, so the second condition which is annealing, you are going to use 64 degrees for annealing because you have some theoretical ideas that this is the right temperature how my base pairs will have the best annealing or the binding conditions, so you will use 64 degrees for the annealing condition to happen. Yes. **"Professor – student conversation starts".**

**(Refer Slide Time: 10:02)**




Alright, I think his question is right that you know let us say, you have derived some theoretical value of 64 degrees, how exactly it actually will help into amplifying that gene of interest, that is way very I think you know, practical question theoretically, you should see a ban amplified because of the Tm but usually, you know plus minus 2 degrees can happen, so sometime that you are deriving 64 degrees that it may happen 66 degree can be the right temperature for that gene to amplify, it is possible.

So, you have to play with certain temperature conditions to find out what is the best temperature for your gene of interest to bind. **"Professor – student conversation ends"**. So, now you had started from the double helix DNA, the double-stranded DNA, after doing the denaturation, you

got the single stranded once, you added this primers which are the short stretch of nucleotides which you have designed yourself.

Because you wanted to study their gene of lamin A for example and now you are amplifying their gene of interest now, you have added dNTP's and nucleotides and providing the right temperature conditions, so that the nucleotides are getting synthesised and new strands are being made, so now this became double-stranded DNA, this whole thing is only one cycle of PCR. Now, same way you are repeating the PCR cycle second time, third time and now you can do it n number of times.

Ideally, people go for at least 30 to 35 or 40 cycles to make multiple copies of the gene of interest, so just imagine that after each cycle of performing the polymerase chain reaction, you are generating n number of fragments and therefore, for many of the forensic applications, think about any kind of you know, the crime scene, when there is some heroine is fallen or some sort of blood spot is detected over there, you do not have too much DNA to do lot of investigation.

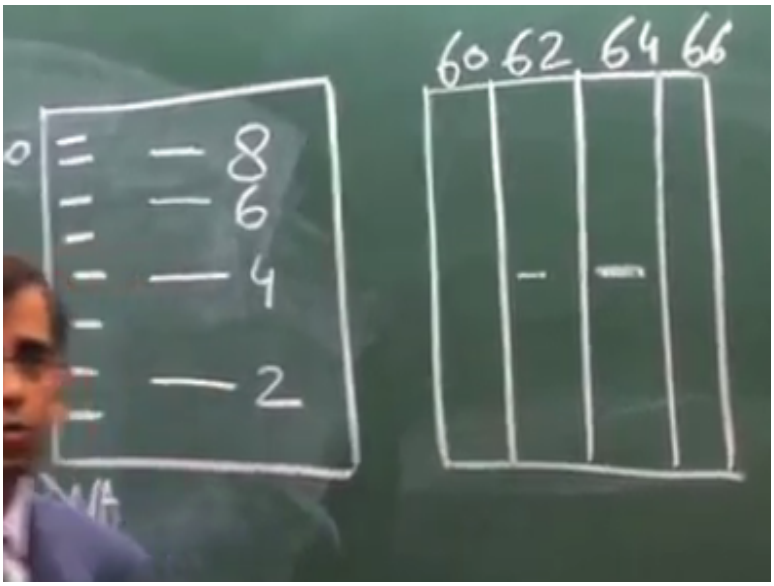
So, they use only the small part of those you know, the DNA extracted out of those bio specimens and then amplify those using these kind of conditions with the polymerase chain reaction, so that they have enough of the DNA, to then do further testing which can result into very accurate deduction. So, we are performing here multiple cycles in any of the polymerase chain reaction.

A 3 steps cycle brings about the chain reaction which produces the DNA chains in the exponential manner and after each successive cycle, you will have the target sequence which will double the numbers and these numbers will doubled; 2 to the power n, so if you have done 30 cycle or 40 cycle, ideally, it looks only 10 cycle difference but if you think about 2 to the power 30 or 2 to the power 40, there is a huge number of difference in how many copies you are producing for that gene of interest.

So, once you do the PCR, there are many things to be optimised, of course as somebody rightly mentioned, you have to look at the annealing temperature, what is the best temperature in which

your primers are going to bind and you may have to play within a range of temperatures from 60 to 65 or 70 to find out where your gene binds the best with the primers and then you have to see that at which cycle numbers, you can still see enough of the DNA being produced.

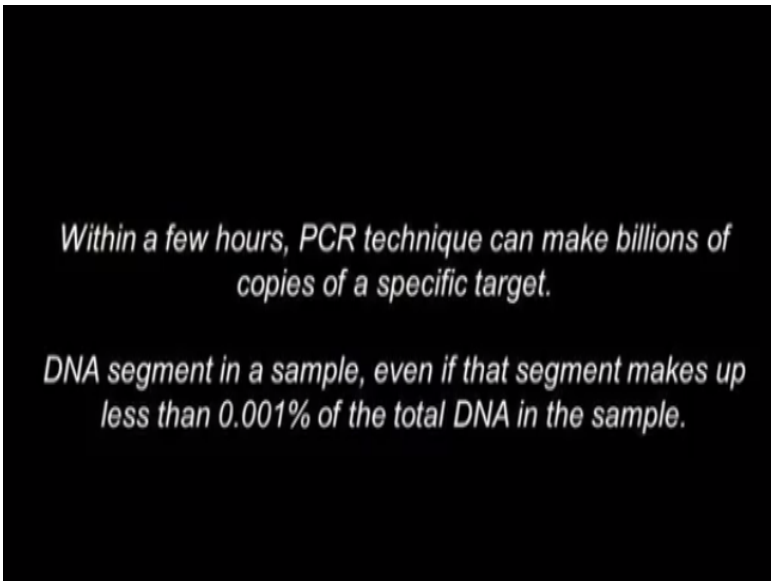
**(Refer Slide Time: 13:17)**




So, then what you can do; after doing the polymerase chain reaction, you can run your samples on the gel, so many time people do this gradient PCR, where they will use different gradient of temperature and now, they will run the each PCR condition, let us say starting here; 60, 62, 64 and 66 and these are my lanes and what I am finding it you know, the very fine band is appearing at 62 probably, you know a good band I can see at 64.

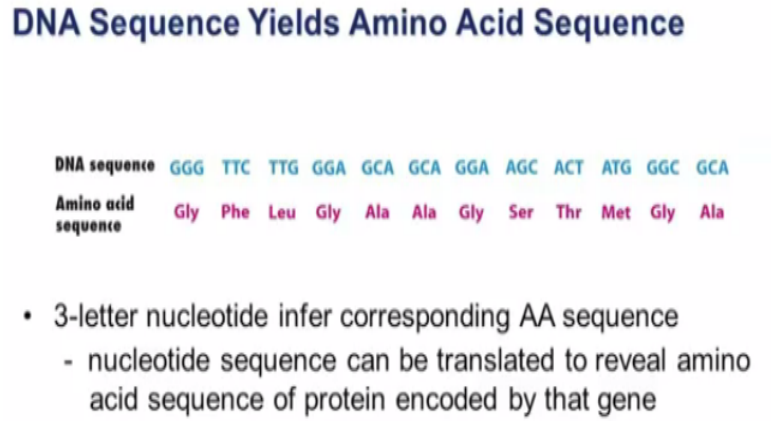
So, then probably this 64 is the right temperature for me to take my experiments forward, so this how people first tried to visualise where there you know the primers are going to get best bind to the, the gene of interest and now, once you have done that then, you will do 30 or 35 or 40 cycles to amplify and make enough number of copies of the gene of interest.

**(Refer Slide Time: 14:14)**




Alright, so within a few hours of doing this PCR or polymerase chain reaction, you can actually amplify your DNA sufficient, so that you can make multiple copies of that specific target and then you can do lot of gene testing based on the amplify DNA, which is present in the given sample.

**(Refer Slide Time: 14:23)**



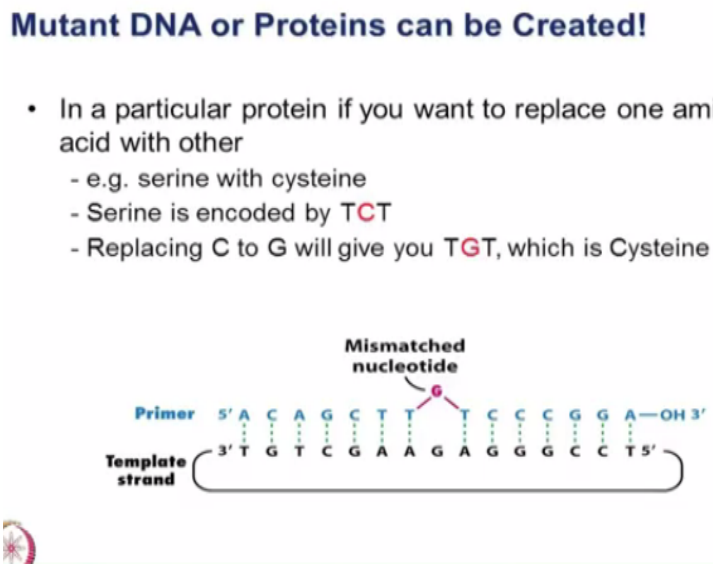

Now, just imagine that you know, so far we have been talking about all the things happening at the gene level, now let us think about you wanted to study in aberration or the change happening at the protein level especially, if you think about the context of progeria, there was a protein which was lamin A, which is defective, coming because of the defects from the lamin A gene, so

if you think about the, the DNA sequence, all this triplet codons are going to make one amino acid.

So, let us say we have glycine from triple G, phenyl alanine from TTC and like that we have you know multiple amino acids derived from this triplet codons sequence, so the 3 letter nucleotide, they are corresponding to a given amino acid sequence and these nucleotide sequences could be translated to give you amino acid sequence or looking at the polypeptide chain of that given protein.

So, if you want to study let us say you know, change happening at the protein level from the same kind of cloning experiment and the same idea of what we have discuss for the doing cloning, can you now think about, can you study those change at the protein level that something I think you have to now pay attention.

**(Refer Slide Time: 15:42)**




So, let us imagine that you have a particular protein which you want to study and because in that protein, there is some change happening at one amino acid level and that each amino acid is derived from the triplet codon of the gene sequence, so if you are looking at that gene sequence for example, TCT is the gene sequence for serine and you are just replacing a C with the G therefore, the TCT becomes TGT, which becomes another amino acid, which is 16.

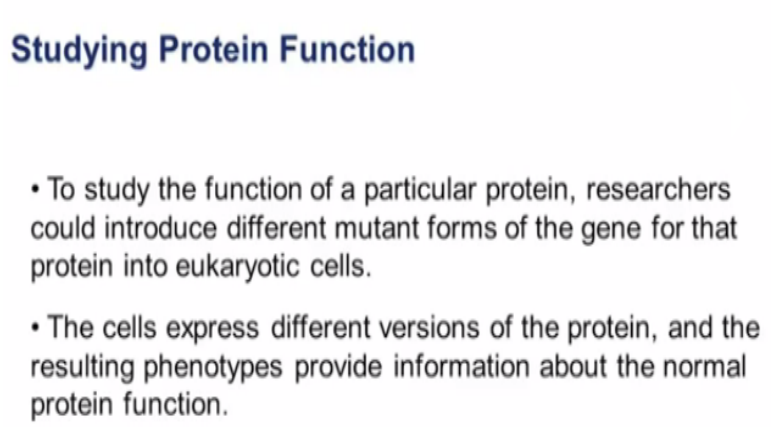
Just by changing one base pair, you have changed a triplet codon, you have changed from one amino acid to other amino acid, which will introduce so much change in the living system, right, so now if you think about even when you are designing the primer, even when you are studying the thing at the gene level, if you want to introduce the changes at the protein level, subsequently you can think about what changes you can make in the triplet codons, which may result into the changes at the protein level, right.

So, if you had this particular template is strand, if this is the strand, which is normal and now, you want to create a protein which is having slight difference only with one particular place, now this one nucleotide you have made a change and now, a proteins which is going to be derive from it or amino acid sequence going to be derived from it, will have mismatch, will have different as compared with the parental strand, right.

So, this is how if you, you are designing the primers at the site of primer designing itself, you can make some small changes and those may result into the variations which can be seen after doing the cloning, then you can see those changes happening even at the protein level, so to study the particular proteins, here still people have used bacterial as the system and just imagine it is very complex concept because think about we are you know, eukaryotes and very complex human system.

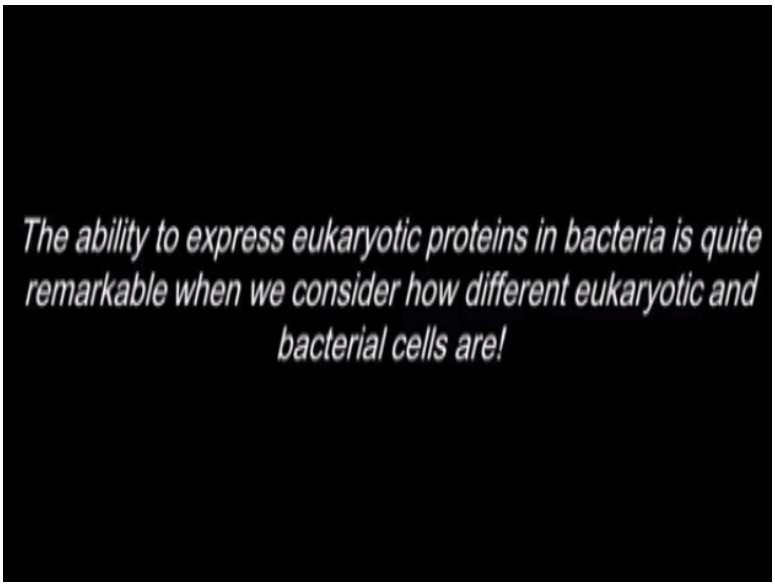
And now, for our human proteins to grow, we are still using bacterial system, which is prokaryotic system right, so but, but somehow with the genetic engineering, we have been able to overcome these barriers and we are still able to even grow, the proteins of our interest in the bacteria, so proteins of eukaryotic interest into prokaryotic origin.

**(Refer Slide Time: 18:04)**



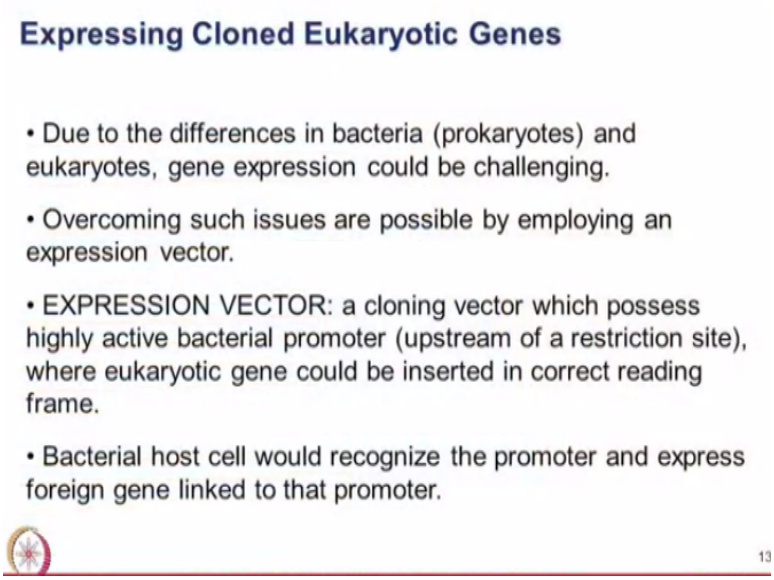

So, the cells express the different versions of the proteins and result into the phenotypes which can actually inform about the normal versus the abundant function of the given proteins.

**(Refer Slide Time: 18:16)**




And we can use those information to express eukaryotic proteins in bacterial system although, it is very challenging and is still a question that how different eukaryotic and bacterial cells are and how you can use the bacterial system to even make the human protein or the eukaryotic protein that is really a challenging question, so if you want to use the bacterial system to express a given protein of interest, you have to use certain promoters which are going to overcome this problem in the expression vector.

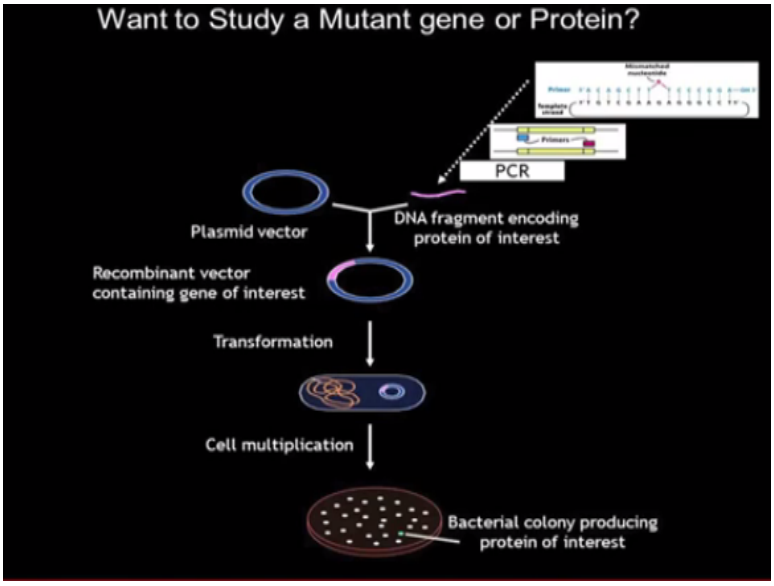
**(Refer Slide Time: 18:49)**




So, expression vector is a cloning vector which contains certain bacterial promoter which can provide the eukaryotic genes in the correct reading frame, so there are lot of difference in the prokaryotic and eukaryotic system and of course, you would not assume that your eukaryotic proteins are going to made in bacteria very correctly and going to be properly folded but somehow, you are still trying to use certain expression vectors say, some of the cloning vectors where you have some of the promoters inserted upstream of the restriction site which help you to at least put the things in the right frame.

So that the right amino acids can be synthesised based on the; that particular expression, so bacterial host cell would recognise a promoter and express a foreign genes or the eukaryotic genes which are linked to that promoter.

**(Refer Slide Time: 19:44)**




So, now let us take this particular situation which is really, really complex situation but we want to study a complex mutant protein, think about this part which is the cloning part which we have already pretty much familiar with now, right so, all the things which I have talked so far is now summarise in this particular image, let us pay attention to this image and see which are all things which we can discuss from here.

So, we have discussed, we had a plasmid vector where you wanted to insert a gene of your interest, you made this recombinant DNA and now you have done the transformation, you selected that and now you got the bacterial colony which are having your gene of interest right, this is what we discussed earlier. Now, this DNA which is a fragment, now this particular DNA you can also amplify using polymerase chain reaction, if you have very small copy of that DNA you can make multiple copies of it using PCR.

For doing PCR or polymerase chain reaction, you had use certain primers which are from both the sides right, you have anneal certain primers and those primers are the one which are going to amplify the gene of interest, now in the primer sequence itself, if you can introduce some variation, which can change your triplet codon, so that your protein which are going to be synthesised from it will have some changes.

You already know from which codon, what amino acid is going to be synthesised, so if you only make even one change in the base pair of the primer sequence, even that will result into mutation or different change, so therefore at the primer designing level itself, people can do lot of innovations, lot of ideas comes that you want to study a different form or different gene, now you can make those changes at the primary designing level.

Now, you do PCR, so your gene of interest will now contain certain added base pair or certain less base pairs, right that you can do using polymerase chain reaction and once you have done that then, the rest of the step of cloning remain same, now you can have rest of the step in the exactly same format, the way we have been discussing. Now, all of these things whatever we are talking for the DNA work everything you have to rely on your simple electrophoretic apparatus.

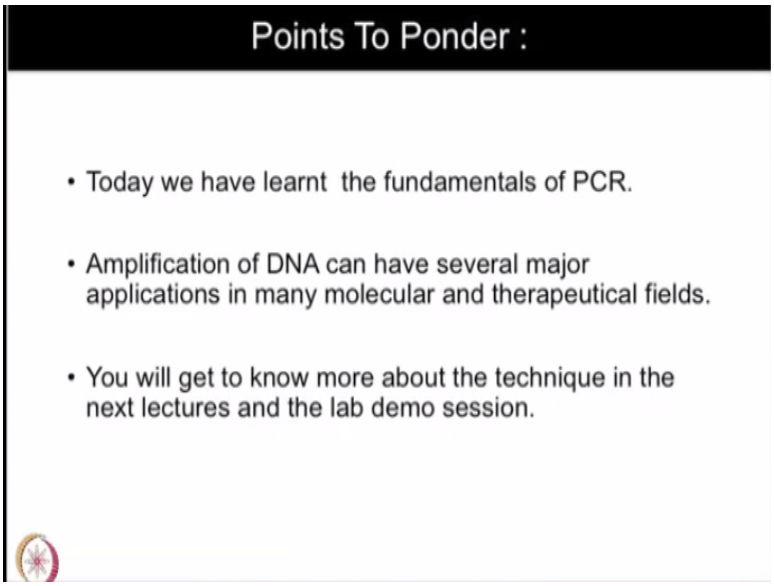
You have to amplify your gene, you have to run on the gel and you have to see that were, what the size of this particular band is; am I able to amplify by right gene, now let us say, if you have made a change in the gene because of the primer sequence which you have added, now is there some amplification you can see or some deletion you can see, a small base pair change, those you can against monitor on the agarose gels.

So, these are the kind of some certain you know technique which are very interesting for us to study, do you have a PCR; polymerase chain reaction, alright, so shortly, we are going to show you a thermo cycler, the instrument which is very simple you know, in general innovation, it is like 3 simple thermo states and in those thermo states, you are just very precisely changing the temperature, so while PCR looks like you know a big technique.

But you know shortly, we are going to show you the instrument, the polymerase chain reaction, it is very simple instrument thermo cycler, where we are just precisely regulating our temperature first initially, you are heating it at very high temperature, 100 degrees, then you are lowering the temperature based on your annealing temperature could be 55 or 60 degrees and then, you are again doing extension at 72 degrees.

So, by using these temperature changes, you are able to synthesise DNA using PCR, so it is again a very simple small instrument but with just works on a very much precision of the temperature and that has to be monitor.

**(Refer Slide Time: 23:27)**




**(Refer Slide Time: 23:41)**