ASIC200 PERSONAL GENOMICS LECTURE (PART 2/PCR LAB).

Essentially covering the Polymerase Chain Reaction and Gel Electrophoresis. As mentioned earlier, Sanger sequencing, and Next Gen sequencing will not be tested for on the final exam.

First, a primer on the polymerase chain reaction.

The polymerase chain reaction, or PCR, is a powerful genetic technique that allows researchers to amplify DNA sequences of interest. This amplification allows better and easier genetic characterization and can be used for a variety of tasks including those in the context of genetic disease diagnosis, maternity/paternity tests, DNA forensics, or simply in the production of large amounts of DNA fragments for further study.

In nature, cells make more DNA by undergoing a process known as replication. This is where the amount of DNA is essentially doubled so that a cell dividing into two new cells can give each of these two cells a complete copy of the DNA. However, replication is a relatively complex process **(see replication reading and also slides 12 to 22)**, requiring the regulated actions of at least half a dozen different enzymes – technically challenging to pull off in the laboratory. PCR is therefore an experiment that simplifies the process of replication for greater efficiency in test tube settings.

A bit of history :Kary Mullis is the principle investigator behind the techniques. Interesting (slightly eccentric fella) who won a Nobel Prize for Chemistry in 1993 for the technique (actually shared the Nobel that year with our very own Michael Smith).

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The Gist and how it more or less works:

So how does PCR work? And how, exactly do you simplify something like replication? (again: make sure you do the reading – this also refers to **slides 25 to 28**). Think of it like this. **Hacking (or simplifying) replication…**

POINT ONE: replication requires open or single stranded DNA. Usually acomplished by many enzymes that unwind DNA (such as helicases). Screw these enzymes – Let's use HEAT to open up our DNA. Bye bye Helicase!

POINT TWO: replication also needs a primase enzyme to make primer for the polymerase (remember one of the rules for polymerases). BUT, you can make your own! You can literally buy your own short piece of nucleic acid (the process is called "oligonucleotide synthesis", your own short piece of single stranded DNA which can act as your primer (it's oligonucleotide synthesis if you want to look deeper). Therefore bye bye primase!

POINT THREE: Remember DNA pol I (the fish in the lecture)? In nature, the primer that is made by that primase enzyme is composed of RNA. This is what DNA pol I was all about (replacing RNA with DNA). Since we're adding our own primer (and it happens to be made of DNA), DNA pol I is now redundant. Bye bye DNA pol I!

POINT FOUR: with a forward and reverse primer system, don't need LIGASE. Ligase was all about dealing with those small Okazaki fragments, a result of only being able to open your helical DNA at very localized spots (with the helicase). Since we're opening up the DNA with heat, and since that is a total global opening, you can also do away with LIGASE. Bye bye ligase!

POINT FIVE: with heat globally opening your DNA, you don't have to worry about things like structural stress. Don't need TOPOISOMERASES either.

POINT SIX: And so, what this all boils down to: is the fact that you can get replication to work with only one enzyme – a workshorse DNA polymerase (i.e. DNA pol III).

HOWEVER, we do have one problem: the high temperature used to open up the helical DNA molecule will basically knacker out any protein structures,including our polymerase. TO get around this, let's use a polymerase from a bug that grows in high temperatures (thermophile). i.e. this will be a heat stable polymerase.

TAKE HOME MESSAGE is that we can simplify the process of replication so that it works in a test tube environment. Ultimately this will entails the collection of a few components needed for a successful DNA amplification reaction. This is what was in your tiny PCR reaction tubes. Let's quickly summarize what they are. They include:

1. A sample of DNA to start with: there are many ways to get this, but you can use the cheek cell procedure outlined in this post (see below).

2. Two primers: These are short pieces of DNA that have been specifically designed to complement the region of your DNA that you wish to make more of. As mentioned before, they can be ordered from places that do something called oligonucleotide synthesis (fancy talk for making small pieces of DNA synthetically). Our primers were designed to look at the region previously discussed (chromosome 8, around the TPA-25 loci)

3. A solution containing the 4 different nucleotides (the A's,T's, C's, and G's): necessary for use as the building blocks for DNA replication.

4. A heat stable DNA polymerase: This is an enzyme that is actually responsible for making a complementary DNA copy (i.e. replicating). The cheapest ones are usually those derived from the *Thermophilus Aquaticus* bacteria, and are called Taq polymerases. These polymerases will often come with a buffer solution which is a specific recipe that allows the polymerase to function at its best.

5. Equipment setup to allow a PCR reaction to cycle through three different temperatures for certain lengths of time, over and over again. These are often called thermal cyclers.

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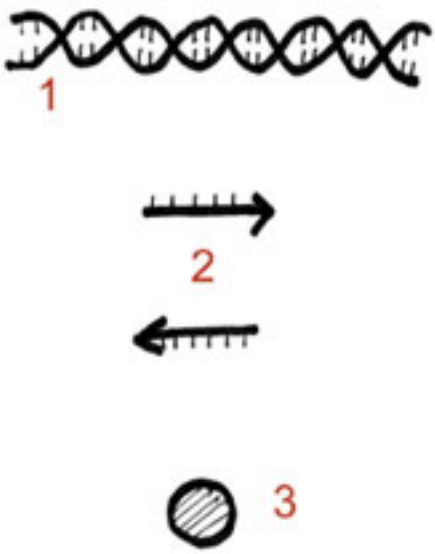
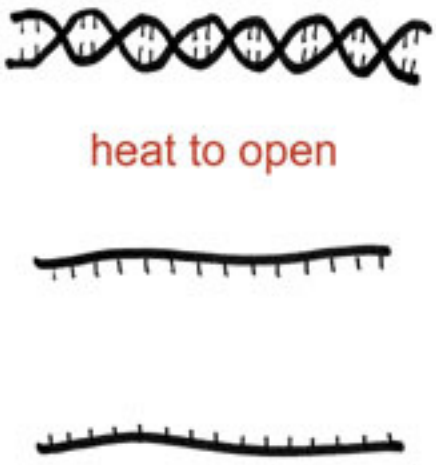
Let's take a look at what's going on inside that PCR reaction tube

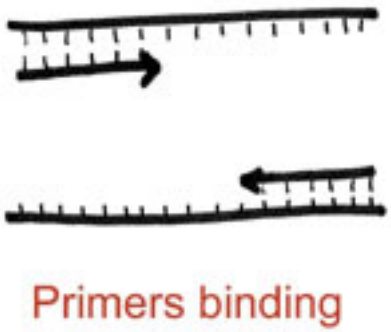
To start the PCR reaction, you'll need to mix in a plastic tube, your DNA sample, the primers, the nucleotides, and the heat stable DNA polymerase. In our class experiment, this is where you left off your samples. i.e. you added #1 (your DNA) to a small reaction tube containing all of the other constituents (#2 are your primers, and #3 is the heat stable TAQ pol). Your sample was then taken to a thermal cycler.



First up, is a high temperature step usually at about 95C for about 1 minute, and often called the "denaturation" step. This is all about allowing your DNA to open up, since it naturally exists as a tight helical coil. The high temperature step will effectively melt the double stranded DNA strands into single strands. This step whilst elegant in its simplicity is the reason for the need of heat stable DNA polymerases, since the enzyme needs to be able to withstand the heat.

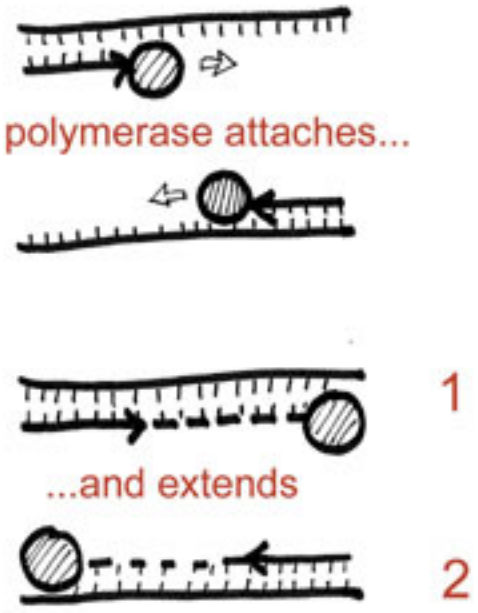


With the DNA open, we move to the second temperature step, referred to as the "annealing" step. Here, the primers, which have been designed to interact with the desired region of amplification, can now "get in." This binding is mediated by choosing a second temperature which ensures a specific interaction, usually around 60C or so, and



usually for about a minute.

With the primers bound, we can now move on to the third and final temperature step – the elongation step. By switching to a temperature where our enzyme works best, the heat stable DNA polymerase can extend the production of DNA from the two primers. This is the actual "replication" part, and consequently also involves uptake of the nucleotides. Note that at the end of this step you have two copies of the original piece of DNA of interest.



And there you have it! You've essentially made more of your DNA of interest (doubled it actually). However, what is cool is that you can now repeat the process over and over in the same experiment. In other words, take the two copies you've produced and subject them to the same three temperature steps. You'll hopefully see that this allow your reaction to go from two copies to four. In essense, going through these three temperatures (which is called a single cycle) will result in the doubling of your DNA of interest. The thermal cycler simply allows you to do this automatically and in a programmable fashion. Most PCR experiments are done with a total of 40 or more cycles (or 40 rounds of doubling), so that you can effectively increase your DNA interest by trillion times or more.

Factor in the fact that a single cycle is often less than 4 minutes, and what you have is an incredibly powerful, yet simple, procedure to amplify out your DNA in a relatively short period of time.

Here's a good animation showing multiple cycles (click on the "Amplification" button). URL = http://www.dnalc.org/resources/animations/pcr.html

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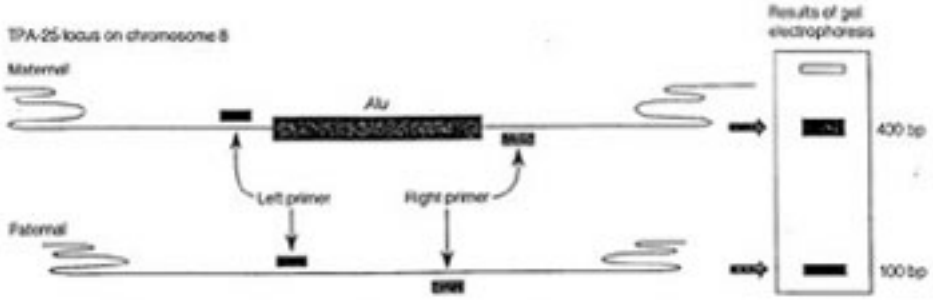
Your Turn… Looking for an Alu Insertion (slides 1, 2, 11, 29)

Our class experiment will allow an individual to look within their own genome for a genetic element called an Alu insertion. Basically, the Alu insertion is a small 300 base pair DNA sequence that is often classified as junk DNA. A person will actually have approximately 500,000 copies of them scattered throughout their own DNA code, but this experiment will focus on looking for the presence or absence of one Alu insertion in

particular – specifically at the TPA 25 loci on chromosome 8 (address lingo for being around a gene called Tissue Plasminogen Activator). Furthermore, since humans received genetic instructions from both their mother and father, each individual will actually have two different chromosome 8's to query. Therefore, possible genotypic outcomes may be +/+, -/-, or +/- for this Alu insertion.

It's also very important to note that this Alu insertion in found within a junk region of the TPA 25 loci, so in effect represents junk DNA within junk DNA. Consequently, the results obtained will be phenotypically neutral, meaning that they do not mean anything and have no consequences (positive or negative) on your wellbeing. However, this experiment done on multiple family members can possibly result in paternity/maternity discrepancy, so it is advised not to perform this particular DNA fingerprint without first contemplating this issue.

Two primers, called the Alu Forward and the Alu Reverse, have been designed to flank the point of insertion where this Alu insertion may or may not exist. This means that they will be amplifying the region of DNA where your Alu insertion may exist. Consequently, data readout will be based on whether this amplified DNA has the extra 300 base pairs of your Alu insertion or not. Since the readout is dependant on observing a discrepancy in the size of the amplified product, you will visualize your data by doing a gel electrophoresis. The figure below summarizes this:



Here, the TPA 25 locus contains a dimorphism reflecting the presence or absence of a 300bp Alu sequence. When the Alu sequence is absent, amplification by PCR yields a 100bp product. If the Alu sequence is present, the size of the PCR product is increased to 400bp.

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The Gel: How does it work? (Slides 31 to 37)

Basically, the gel is used for a procedure known as "gel electrophoresis." This is an experiment that allows us to characterize molecules on the basis of size (i.e. we have a way of determining whether something is bigger or smaller, etc). How it works is relatively simple. In our case, the gel is composed of a sugar known as agarose. This

can be envisioned as a long filament type molecule, which means that if you were to view the gel as microscope man or woman, it would look very much like a mesh (imagine the game pick-up sticks and what that initial pile sort of looks like – sort of a mesh of sticks).

Here, you have to imagine your DNA (in our case 100bp or 400bp fragments) as having to work its way through this mesh in the gel. The main idea here, is that the smaller the molecule, the easier time it will have moving through the mesh simply because it has an easier time going through the holes, and hence have a faster mobility rate.

In our case, you can kind of imagine the following:

We all loaded our samples in the wells at the top of the gel. Then, we connected the gel to an electric circuit. Here we can force our DNA (which is negatively charged) to run through the gel in a specific direction – towards the bottom of the gel. In a way, you can think of it as a race: the 100bp fragment (being smaller) should move down the gel faster than the 400bp fragment. Therefore, at a certain point in time (dictated by the size of the gel equipment, how high you crank the powerpack, etc), you should be able to stop the gel, and take a peek at where your bands are. The relative positions of where your DNA bands represent their respective mobilities, and consequently represent their respective sizes.

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The Experimental Details (Slides 3 to 10)

1. Pour 5ml of saline solution into your mouth and swish for 30 seconds.
2. Spit the “spit” into a paper cup.
3. Pour the sample solution from the paper cup back into the tube that contained the saline solution and close cap tightly (label tube or note number).
4. Spin sample in a centrifuge at medium speed (1500rpm) for 10 minutes.
5. Assuming you have a tight (and not flocculent) pellet, you can carefully pour off supernatant (liquid on top) into sink.
6. Using the plastic pipette, transfer as much of your cheek cell pellet as you can to a tube that contains about 500ul of chelex beads. (make sure you label your chelex beads tube)
7. Mix cells and Chelex by vortexing for about 10 or so seconds.
8. Add a lid lock to your tube, and place your tube in a boiling water bath for 10 minutes.
9. Carefully remove your tube from the boiling water bath.
10. After boiling, centrifuge your sample at maximum speed for 5 minutes.
11. Use p20 pipetteman and a fresh yellow pipet tip to transfer 5 μl of supernatant (clear solution on top) to your PCR reaction tube.
12. Your sample will then be placed in a PCR machine which has been programmed to cycle 30 times (2^{30} amplification). The reagents in the PCR tube are there to seek out the area where our Alu insertion of interest happens to be.
13. After the PCR reaction, the sample will then have a “loading buffer” added, and then loaded on an agarose gel for visualization.