GENERATING OF MELANOMA MOUSE MODELS

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ABSTRACT

Our ongoing project is to develop a line of transgenic mice with MutHbraf (mutated Human Braf) to generate melanoma that mimic melanoma in human patients. A line of transgenic mice have been developed and we are genotyping them to check for the presence of the MutHbraf gene using Polymerase Chain Reaction (PCR) method. We will cross breed MutHbraf transgenic mice with mice whose Ink4a gene (tumor suppressor gene) has been “Knocked Out”. New mice breed from the cross will be monitored to see if they develop melanoma.

The melanomas developed will be characterized and treated with the HGP75 optimized or HGP75 vaccine to suppress the melanoma.

HISTORY

DNA polymerase is an enzyme that exists naturally in living organisms, and its function is to duplicate DNA when cells divide. It works by binding to a single DNA strand and creating the complementary strand. The idea was to develop PCR (Polymerase Chain Reaction) a process by which DNA could be artificially multiplied through repeated cycles of duplication driven by the enzyme DNA polymerase. The technique of Polymerase Chain Reaction patented by Karry Mullis's original idea used enzyme in a controlled environment outside an organism. The double-stranded targeted DNA template or Amplicon was separated into two single strands by heating it to 96°C. At this temperature, however, DNA-Polymerase was destroyed so that the enzyme had to be replaced after the heating stage of each cycle. Mullis's original PCR process was inefficient since it required a great deal of time, large amounts of DNA-Polymerase, and continual attention throughout the PCR process. Later, this original PCR process was improved by the use of DNA-Polymerase taken from thermophilic (heat-loving) bacteria that grow in geysers at a temperature of over 110°C. Due to the simplified and automated PCR technique, amplifying a short segment of DNA to more than a million in a matter of hours involves the target DNA, two primers, deoxynucleotide triphosphates (dNTPs), reaction buffer and magnesium chloride ($MgCL_2$). Over the years the vast uses of PCR products such as DNA fingerprinting, detection of hereditary diseases, analyzing the functions of genes and DNA genotyping.

POLYMERASE CHAIN REACTION

PCR is composed of several components that enable the amplification of the DNA strand. They are listed as follows:

- **DNA TEMPLATE**: A DNA fragment containing the sequence which will be amplified. The template can be genomic DNA plasmid, cDNA from **RT PCR** (Type of PCR in which the amplification of cDNA is used as a template instead of DNA) or DNA from an earlier PCR product in such cases the PCR is called Nested PCR.

- **PRIMERS**: DNA strands--not more than fifty (usually 18-25 bp) nucleotides that exactly match the beginning and end of the DNA fragment to be amplified. They anneal to the DNA template at these starting and ending points, where the DNA-Polymerase binds and begins the synthesis of the new DNA strand. The choice of the length of the primers and their melting temperature (Tm) depends on a number of considerations. The melting temperature of a primer is defined as the temperature at which half of the primer binding sites are occupied. The melting temperature increases with the length of the primer. Primers that are too short would anneal at several positions on a long DNA template, which would result in non-specific copies. The optimum length of a primer is generally from twenty to forty nucleotides with a melting temperature between 60°C and 75°C. To have a good yield of PCR product considerations for constructing a primer include:

    1. GC-content should be between 40-60.
    2. Calculated **Tm** (temperature at which 50% of the primer molecules are annealed to the DNA template) for both primers used in reaction should not differ >5°C and Tm of the amplification product should not differ from primers by >10°C. To calculate the Tm for both primers the formula used depends on the length of the primers: Tm =2(A+T) +4(G+C) OR Tm= 81.5+ 0.41(%GC)-675/N *N is the primer length in bases.
    3. Annealing temperature usually is -5°C the calculated lower Tm. However it should be chosen carefully for individual conditions.
    4. Inner self-complementary hairpins of >4 and of dimers >8 should be avoided.
    5. 3' end is extremely case sensitive - it must not be complementary to any region of the other primer used in the reaction and must provide correct base matching to template.
    6. 3'-end Sequence- 3' position in PCR primers is essential for the control of mis-priming

(5').The inclusion of a G or C base at the 3' end of primers: This G/C helps to ensure correct binding at the 3' end due to the stronger hydrogen binding between G and C.

7. It also helps to improve the efficiency of the reaction by minimizing any error that might occur.

- DNA polymerase: One of the first thermostable DNA-Polymerases was obtained from *Thermus aquaticus* called *Taq.* Taq polymerase is widely used in current PCR practice A disadvantage of Taq is that it sometimes makes mistakes when copying DNA, leading to errors in the DNA sequence, since it lacks 3'→5' proofreading exonuclease activity. Polymerases such as *Pfu,* obtained from *Archaea,* have mechanisms that check for errors and can reduce the number of errors that occur in the copied DNA sequence. Combinations of both *Taq* and *Pfu* are able to provide both high fidelity and accurate amplification of DNA. Concentration of the enzyme is dependent on the type of PCR been conducted. Using more than the required amount of the enzyme does not increase the PCR yield, but results in smeared bands in an agarose gel.

- Deoxynucleotide triphosphates (dntp): Unit of nucleotides which form Nucleic Acids such as RNA and DNA.The base pair Adenine has its complementary nucleotide of Thymine in DNA, but in RNA Thymine is replaced by Uracil, and the complementary strand of Guanine is Cytosine. During Extension of the PCR cycle, base pairs adhere to the DNA template.

- 10x Buffer: Provides a suitable chemical solution for the DNA polymerase by controlling the pH of the reaction. Most buffers include KCL which increases the reaction rate of the DNA polymerase by at least 50-60%; a required concentration is 50mM.

- Magnesium Chloride ($MgCL_2$): The Magnesium is required for the activity of the DNA polymerase and also affects the stability of the primer-template reaction. For a given primer-template set, the optimal magnesium concentration must be determined, and usually lies between 1.0-3.0mM. Without the specific concentration of $MgCl_2$, it can either be, insufficient or in excess. If it's insufficient, Taq DNA polymerase becomes inactive, and an excess causes the enzymes to increase in its nonspecific amplification.

- Nuclease free **H_2O**: Serves as the primary solvent for the reagents. Regular or distilled water will change the pH of the reaction, ultimately degrading the DNA.

A PCR reaction is conducted in a thermal Cycler (FIGURE I) also known as a PCR Machine consists of 3 steps.

FIGURE I : Thermal Cycler (PCR Machine) picture is an example of the machine that has a heating block and an operating system. This allows programing the heat block and vary tempereture, following a cyclic program

THE PCR CYCLE

The PCR method consists of three steps:

1. DENATURING

Before the first cycle, the DNA is denatured for an extended time to ensure that both the template DNA and the primers have completely separated. The double stranded DNA has to be heated to 94-96°C in order to separate the strands. This step breaks apart the hydrogen bonds that connect the two, now single-strand DNA. Time: 1-2 minutes.

2. ANNEALING

After separating the DNA strands, the temperature is lowered so the pair of primers (Forward and Reverse) can attach themselves to the single DNA strands. The temperature of this stage depends on the primers and is usually 5°C below their melting temperature (45-60°C). A wrong temperature during the annealing step can result in primers not binding to the template DNA at all, or binding at random. Time: 1-2 minutes

3. EXTENSION:

The DNA-Polymerase begins to extend on the template. It starts at the annealed primer and works its way along the DNA strand. The extension temperature depends on the DNA-Polymerase. The time for this step depends both on the DNA-Polymerase itself and on the length of the DNA fragment to be amplified. Usually 1 min. for 1 kb (kilobase) DNA strand.

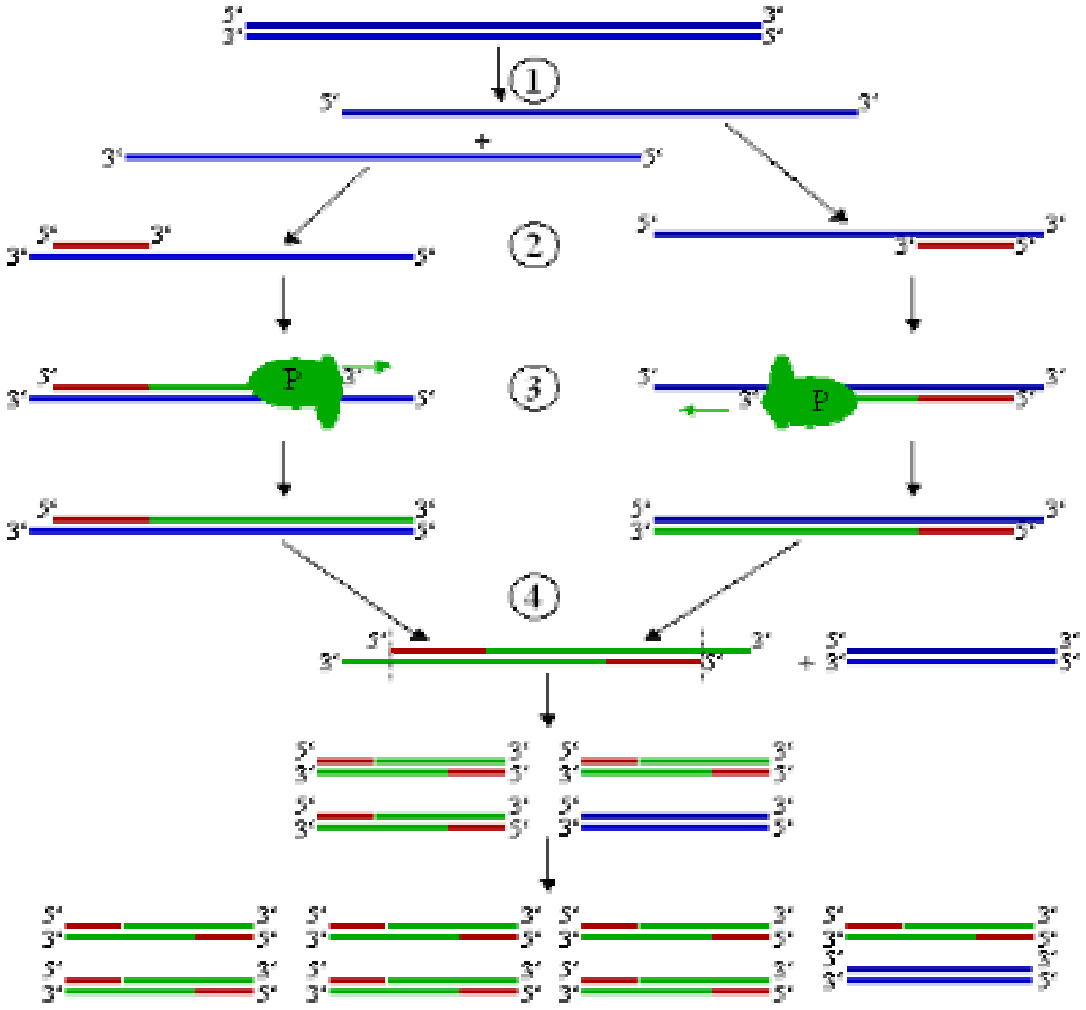


FIGURE II : A schematic version of the PCR Cycle shows the 3 steps of the PCR Cycle.
Step 1: Denaturing Stage, the double stranded DNA unwinds to single strands
Step 2 : Annealing Stage, the single stranded primers adhere to the complimentary nucleotide on the DNA template.
Step 3: Extension Stage, the primers begin extending to form a complimentary strand of the DNA template
Step 4: The result of the 3 steps is a double stranded DNA PCR product.

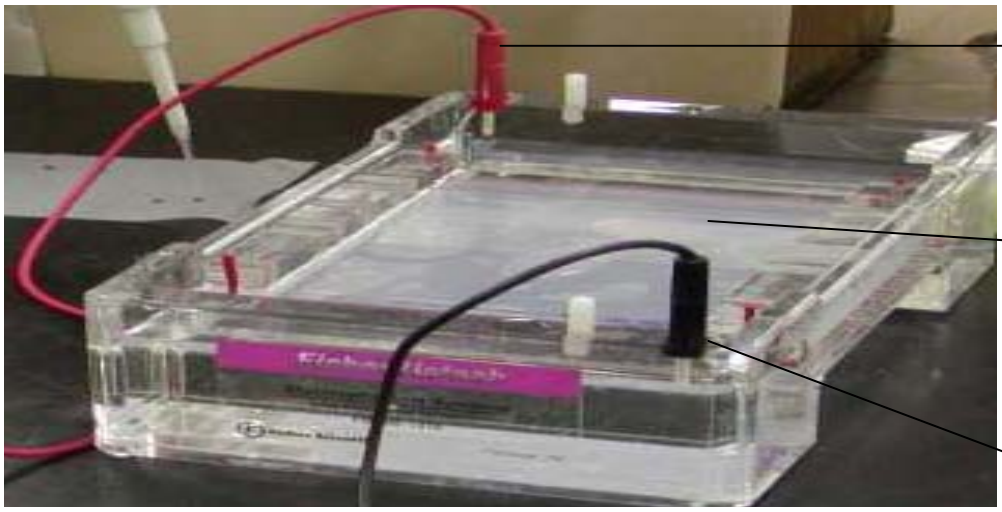
- As the cycle increases the number of DNA molecules increases exponentially. For example, if *N* is the cycle number, the DNA molecules generated will be to the $2^{(n)th}$ power.

CYCLE NUMBER (*N*)	DNA MOLECULE (2^n)
1	$2^{1} = 2$
2	$2^{2} = 4$
3	$2^{3} = 8$
4……..	$2^{4} = 16$
30	$2^{30} = 1073,741,824$

DETECTION AND ANALYSIS OF PCR PRODUCT

In order to check the PCR product of a PCR reaction, GEL ELECTROPHORESES is done. (Figure III shows pictures of the components in an example of a gel electrophoreses).It operates by allowing the flow of an electric field to drag negatively charged DNA molecules through agarose gel. The shorter DNA molecules move faster than the larger ones since they are able to slip through the gel more easily. The running of DNA in the agarose gel depends on its conformation. It is demonstrated in the three forms of a plasmid: superhelical, nicked, and linear. Each form will run differently, for instance ,the superhelical runs the fastest but the linear form runs the slowest. The presence of ethidium bromide (EtBr) in the gel causes DNA to run slower because it binds to the DNA, After a few minutes unbound ethidium bromide diffuses into the water. This will result in a clean, sharp photograph of the DNA bands. The voltage is a factor in migration. It cannot be too high because it would increase the ampers, that would lead to heating the gel, a hot gel will denature the PCR product. Loading dyes such as BLUE JUICE is added to the PCR product in order to visualize it and allow it to stay down in the gel well. There are a number of buffers used for the running of the agarose electrophoresis, tris acetate EDTA (TAE), and sodium boride (SB) are just two. On the other hand, the use of indicated markers or DNA ladder is also run alongside the PCR sample in order to confirm the size of the PCR product. As a final step, Polaroid photograph of the gel using ultraviolet light will clearly show the bands in the gel, and can be analyzed to see which sample worked during the PCR.

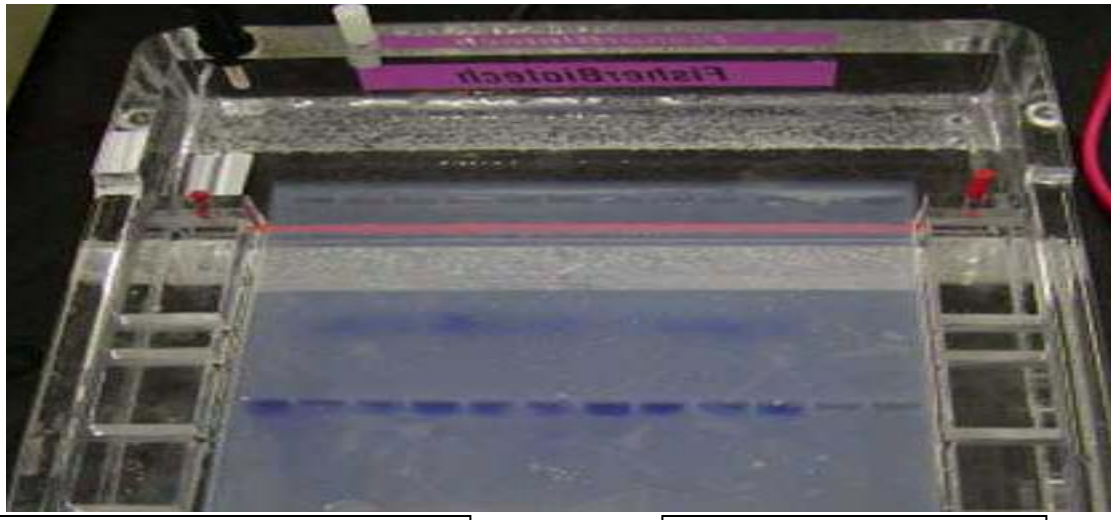
A



DNA runs from the negative to the positive end (+)

The type of buffer in use is the TAE Buffer

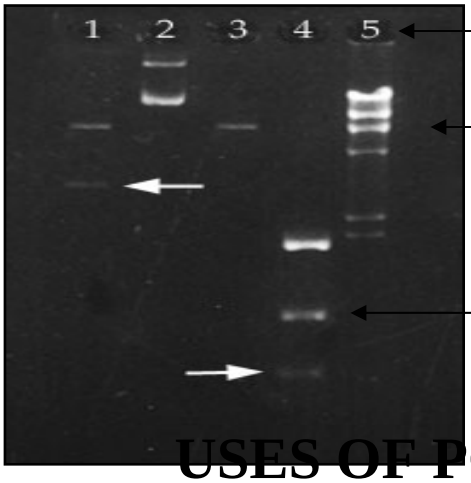
Positive end (-)



RUNNING A PCR SAMPLE IN AN AGAROSE GEL

The voltage used was 110 volts

B



The numbers indicate the wells in the gel

DNA marker

DNA bands

USES OF PCR PRODUCT

FIGURE III: Agarose gel electrophoresis. A. Shows the PCR product migrates from the negative end to the positive. B. the DNA bands are seen migrating from the gel wells and a marker run at the end determines the size of the DNA in a UV Polaroid picture.

The many uses of DNA PCR product are essential in the real world of today. Some of its uses include:

Genetic fingerprinting: is a forensic technique used to identify a person by comparing his or her DNA with a given sample, e.g., blood from a crime scene can be genetically compared to blood from a suspect. The sample may contain only a tiny amount of DNA, obtained from a source such as blood, semen, saliva, hair, etc.

Paternity testing: genetic relationships, such as parent-child or siblings, can be determined from two or more genetic fingerprints, which can be used for paternity tests.

Detection of hereditary diseases: Each gene in the human genome can easily be amplified through PCR by using the appropriate primers and then sequenced to detect mutations.Viral diseases, too, can be detected using PCR through amplification of the viral DNA. This analysis is possible right after infection, which can be from several days to several months before actual symptoms occur. Such early diagnoses enables early treatment.

Determine ancient DNA: PCR can be analyzed to determine DNA that is thousands of years old. PCR techniques have been successfully used on animals, such as a forty-thousand-year-old mammoth, and also on human DNA, in applications to identify Egyptian mummies.

STUDY FUNCTIONS OF GENES: PCR can also be used to introduce mutations into genes, called Site Mutagenesis which can be studied to understand the effects of the mutation and its function.

GENOTYPING TRANSGENIC MICE: PCR can be used to check for the presence of the wild-type, mutated gene or knocked out gene.

TROUBLE SHOOTING AND RECOMMENDATION

When performing PCR there can be many variables that can contribute to a failed PCR, precautions should be taken to ensure a good PCR yield.

- If the aliquot of DNA is not carefully placed into tubes, there wouldn't be any PCR products.
- DNA polymerase, dNTP's, Buffers and Magnesium Chloride, primers should have the correct concentration to eliminate any error during the PCR reaction.
- Tubes not clearly numbered can indicate the wrong sample to be placed in it.
- Agarose mixtures must be homogenous and bubble free.

RECOMMENDATIONS

- Wear new gloves before starting a PCR, to avoid contamination.
- Make a master mix before pipetting samples into tubes to eliminate inaccurate measurement.
- Carefully place, and correctly number tubes for future analysis.
- Carefully thaw reagents in order to pipette a uniformed solution.
- Do not leave PCR reagents or products at room temperature to avoid degradation.
- Loading PCR product with blue juice is expensive and the dye sometimes overlays the DNA bands. Instead try using **Glycerol** with **Xylene Cyanol,** it is inexpensive and prevents the dye from overlaying the DNA band depending on the size of the amplicon.

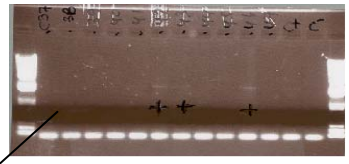
<u>To prepare a 10x solution</u>

A 6:4 ratio of H_20 and glycerol respectively in a tube; resuspend to mix

Add a tiny amount of Xylene Cyanol

FIGURE IV-A

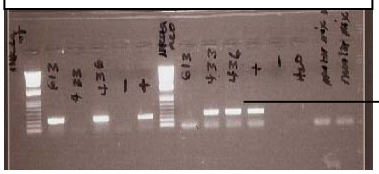
PCR product loaded with blue juice



Blue juice overlaying DNA bands

FIGURE IV-B

PCR product loaded with Glycerol +xylene Cyanol



The DNA bands are seen clearly because the Xylene dye is not overlaying the DNA bands

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REFERENCES

1. http://en.wikipedia.org/wiki/Polymerase_chain_reaction
2. http://oregonstate.edu/instruct/bb494/pcr.html
3. http://www.accessexcellence.org/LC/SS/PS/PCR/PCR_technology.html
4. www.invitrogen.com/pcr
5. http://www.eppendorfna.com/applications/pcr_appl_primer.asp?SX=NF&
6. Dieffenbach, C.W., Lowe, T.M.J., Dveksler, G.S., General Concepts for PCR Primer Design, in PCR Primer, A Laboratory Manual, Dieffenbach, C.W, and Dveksler, G.S., Ed., Cold Spring Harbor Laboratory Press, New York, 1995, 133-155.
7. Innis, M.A., and Gelfand, D.H., Optimization of PCRs, in PCR protocols, A Guide to Methods and Applications, Innis, M.A., Gelfand, D.H., Sninsky, J.J., and White, T.J., Ed., CRC Press, London, 1994, 5-11.
8. Sharrocks, A.D., The design of primers for PCR, in PCR Technology, Current Innovations, Griffin, H.G., and Griffin, A.M, Ed., CRC Press, London, 1994, 5-11.
9. Suggs, S.V., Hirose, T., Miyake, E.H., Kawashima, M.J., Johnson, K.I., and Wallace, R.B., Using Purified Genes, in ICN-UCLA Symp. Developmental Biology, Vol. 23, Brown, D.D. Ed., Academic Press, New York, 1981, 683.
10. Kwok, S., Kellog, D.E. McKinney, N., Spasic, D., Goda, L., Levenson, C., and Sninsky, J.J., Effects of primer-template mismatches on the polymerase chain reaction: Human Immunodeficiency Virus 1 model studies. Nucleic Acids Res. 18:999-1005, 1990.
11. http://www.life.uiuc.edu/molbio/geldigest/electro.html
12. http://www.promega.com/paguide/chap1a4.pdf
13. http://www.idtdna.com/Catalog/NucleaseFreeBuffers/Page1.aspx
14. Molecular Cloning, A laboratory Manuel Second Edition. Sambrook. Fritsch. Maniatis
15. http://www.bio.calpoly.edu/ubl/Alu.htm
16. http://images.google.com/imgres?imgurl=http://www.charlesghigna.com/pics/mice.jpg&imgrefurl=http://www.charlesghigna.com/books.html&h=257&w=180&sz=10&tbnid=q0M4w4P2WNoJ:&tbnh=107&tbnw=74&hl=en&start=15&prev=/images%3Fq%3Dmice%26svnum%3D10%26hl%3Den%26lr%3D%26sa%3DN