EE 495

Engineering Physics Research Project:

DNA Analysis on a Chip

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April 23, 2001

Abstract

This paper summarizes work done in bringing Dr. Chris Backhouse's research group closer to the goal of being able to perform full DNA analysis on a single microfabricated chip. The work focuses on accurately and reliably measuring and controlling the temperature of the microfabricated chip. The result of the work is the successful execution of on-chip PCR, a vital step in performing full DNA analysis on a chip.

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Introduction

Genetic testing has become an extremely useful tool in medical diagnostics. The ability to identify the presence of specific genes allows physicians to say with high certainty that a patient has or will develop a specific disease. In some cases, early detection can alert the doctor and patient to the threat of future problems, leading to timely or preventative treatment that can greatly improve the health prospects of the patient.

Currently, genetic testing is relatively difficult and expensive. Unlike vaccination, there is no standard suite of genetic tests performed on the general public. Genetic tests are typically performed only on those who have a family history of a certain disease, or on those who already exhibit the symptoms of a disease. The result is that genetic testing has yet to reach its full potential in preventing disease and providing health benefits to the general public.

DNA analysis on a chip has many advantages compared to conventional DNA analysis. Thanks to its small size, an on-chip implementation is more portable and can complete tests more quickly. Its closed geometry makes it easier to sterilize and protect from contamination. Ultimately, it will require less user handling, and will be much simpler to operate. Most importantly, DNA analysis on a chip needs less expensive equipment and only minute volumes of expensive solutions, making it a potentially far cheaper alternative.

Background

Before any analysis of a patient's DNA can be performed, it must first be decided exactly what disease is being searched for. A genetic disease can be related to mutations in one or more genes, and to an extent, a gene simply corresponds to a specific length (or fragment) of DNA. So testing for a specific disease reduces to checking for mutations in the base pair sequences of specific fragments of DNA.

Selectivity and Amplification – Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) allows for replication of any specific gene of interest. PCR can be repeated many times over, resulting in a vast number of copies of a gene. The selective genetic amplification of PCR permits much greater ease of detection of specific mutations, and hence of the presence or absence of specific diseases.

PCR involves three steps, each triggered by temperature. The steps have a slightly different function in the first PCR cycle than they do in the subsequent cycles.

<u>Cycle 1</u>

1. At 94°C, the entire double helix of DNA denatures (splits apart) into two single free-floating strands. These strands extend indefinitely in both directions, and are referred to as primary template.

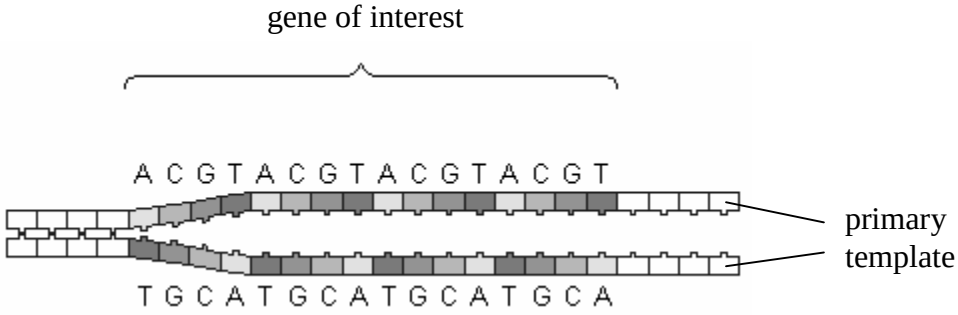

Figure 1: Cycle 1, Step 1 – Denaturing of double helix

2. At 63°C, start and stop primers in the solution anneal (bind) to the primary template, marking the beginning and end points of the gene of interest. These primers are tailor-made for each specific gene, making them expensive. The annealing temperature can vary, depending on the primers.

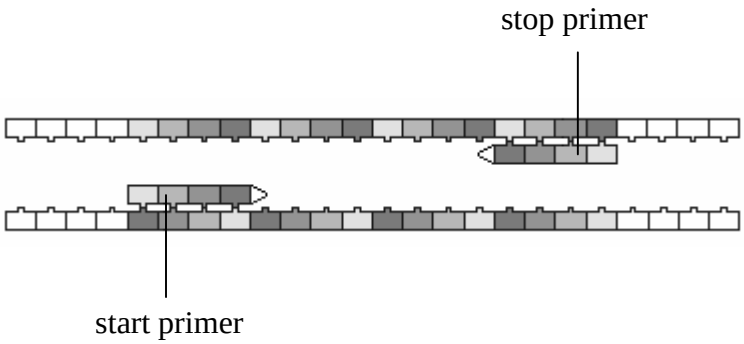

Figure 2: Cycle 1, Step 2 – Annealing of primers

3. At 72°C the polymerase enzyme in the solution uses free floating nucleotides as raw material to extend the primers, completing the base pairs of the single strands along the way. The polymerase extends the primers indefinitely in one direction only.

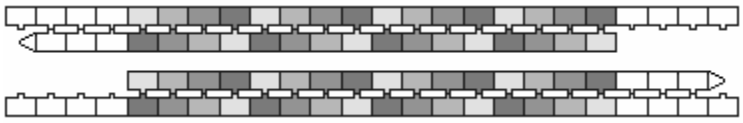

Figure 3: Cycle 1, Step 3 – Polymerase extension of primers

Cycle 2

1. Since the original DNA helix is already denatured, this time at 94°C the so-called primary and secondary templates are what split apart from each other.

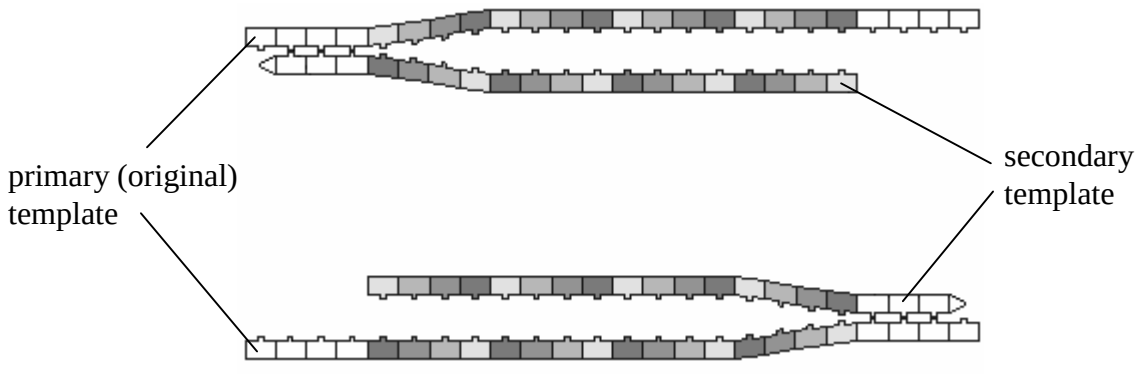

Figure 4: Cycle 2, Step 1 – Denaturing of primary and secondary templates

2. At 63°C, the start and stop primers anneal to both the primary and secondary template, again marking the beginning and end points of the gene of interest.

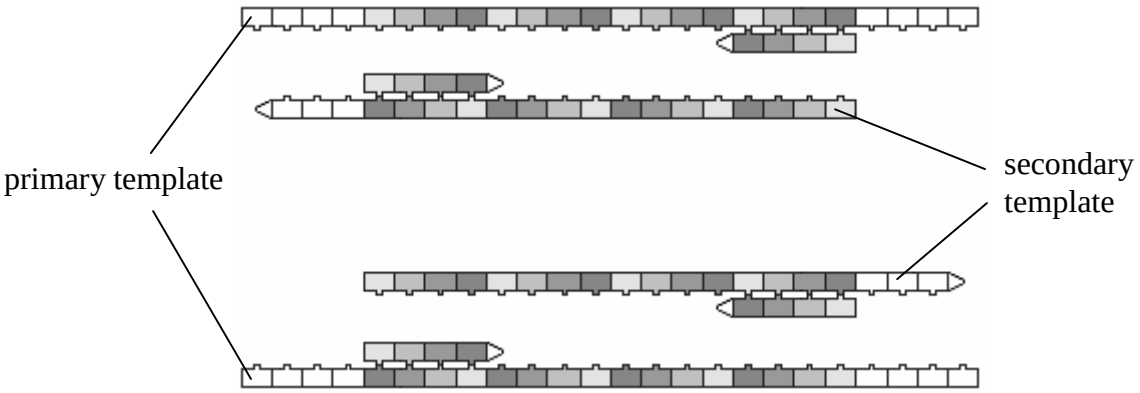

Figure 5: Cycle 2, Step 2 – Annealing of primers

3. This time at 72°C, the polymerase does two things. As in cycle 1, primers bound to primary template are extended indefinitely in one direction, creating new secondary template. However, those bound to secondary template are extended only to the terminating end of the secondary template, resulting in the desired product: copies of the gene of interest.

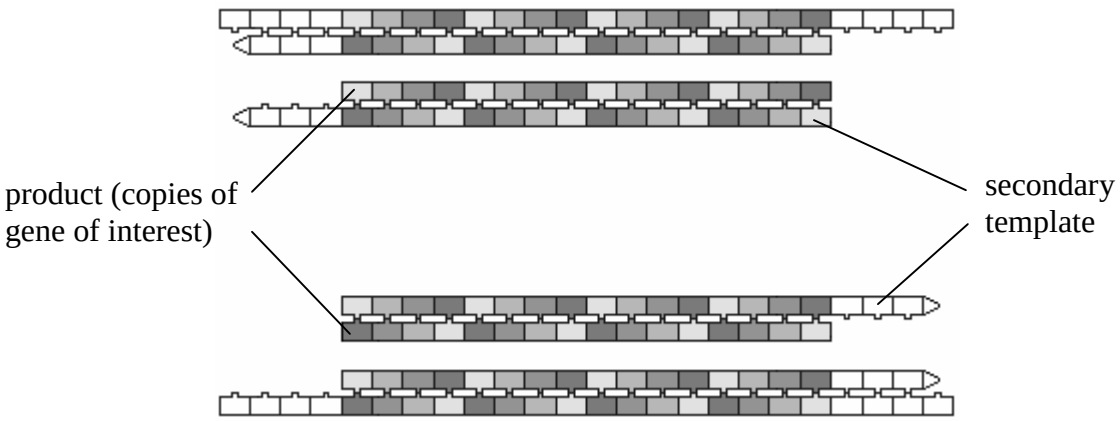

Figure 6: Cycle 2, Step 3 – Polymerase extension of primers

In all subsequent cycles, the product itself acts as template in creating more product, and quickly outnumbers the primary and secondary templates. Typically, the solution is cycled at least 30 times, creating on the order of 2^{30} (~1 billion) copies of the gene of interest.

Detection – Capillary Electrophoresis

The primers used in PCR can be made with tags that fluoresce when excited by a laser. This makes them optically detectable. Because the primers become integrated into the ends of the product, the product itself is also optically detectable. In capillary electrophoresis a thin capillary tube is filled with a polymer, and the solution of primers and product is inserted at one end. A voltage is applied across the ends of the tube, creating an electric field which, because of their slight polarity, pulls the components of the solution through the length of the tube. A laser illuminates a spot along the tube, and a detector at that spot looks for light at the wavelength at which the primers and product fluoresce.

The arrival of fluorescing dots, which correspond only to primers and product, is timed starting from when the voltage is first applied. This separation time depends on the size and geometry of the primers and product. Generally, longer fragments encounter greater friction as they are pulled through the polymer, resulting in longer separation times. Also, different base pair sequences result in different folding of the fragments, changing their cross sectional area and the amount of friction they encounter, and again influencing their separation time. The separation time of the gene being tested can be compared to that of the same gene known to have no mutations. Since mutations result in changes of gene length or base pair sequence, deviances in separation time can be used as an indicator of mutation.

Experimental Work and Results

The chip used in this project is made by Micralyne and consists of two micromachined pieces of glass sandwiched together. The chip has four wells, each 2mm in diameter, which are open from above. The wells are connected internally by an injection and separation channel, each 50μm wide and 20μm deep.

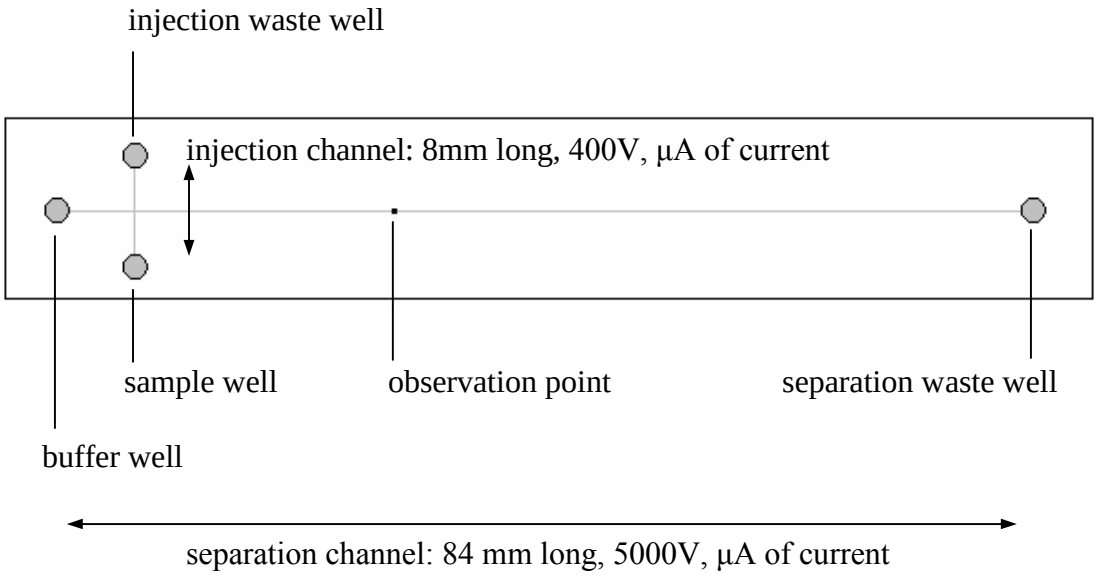

Figure 7: Micromachined glass chip

First, the solution of primary template, start and stop primers, polymerase, and other necessary ingredients are injected into the sample well. Next, PCR is executed by carefully cycling the temperature of the chip inside of a commercial thermal cycler. The solution is then extracted and reinjected into an identical chip loaded with polymer in its channels. A voltage is applied across the injection channel to electrostatically pull the solution components from the sample well through the injection channel to the intersection with the separation channel. Then, a voltage is applied across the separation channel, pulling the components at the intersection down the separation channel towards the separation waste well. At the observation point, a red laser illuminates the separation channel with light at $\lambda = 635$nm and a photomultiplier tube (PMT) is aimed through a microscope at the same point, watching for fluorescence at $\lambda = 670$nm.

To record the passage of fluorescing primers and product, the PMT voltage is recorded as a function of time.

The chip and detection equipment, both manufactured by Micralyne, existed prior to the beginning of this project and worked effectively in detecting and recording the passage of fluorescing primers and product. However, as shown in Figure 8, on-chip PCR was not creating any product.

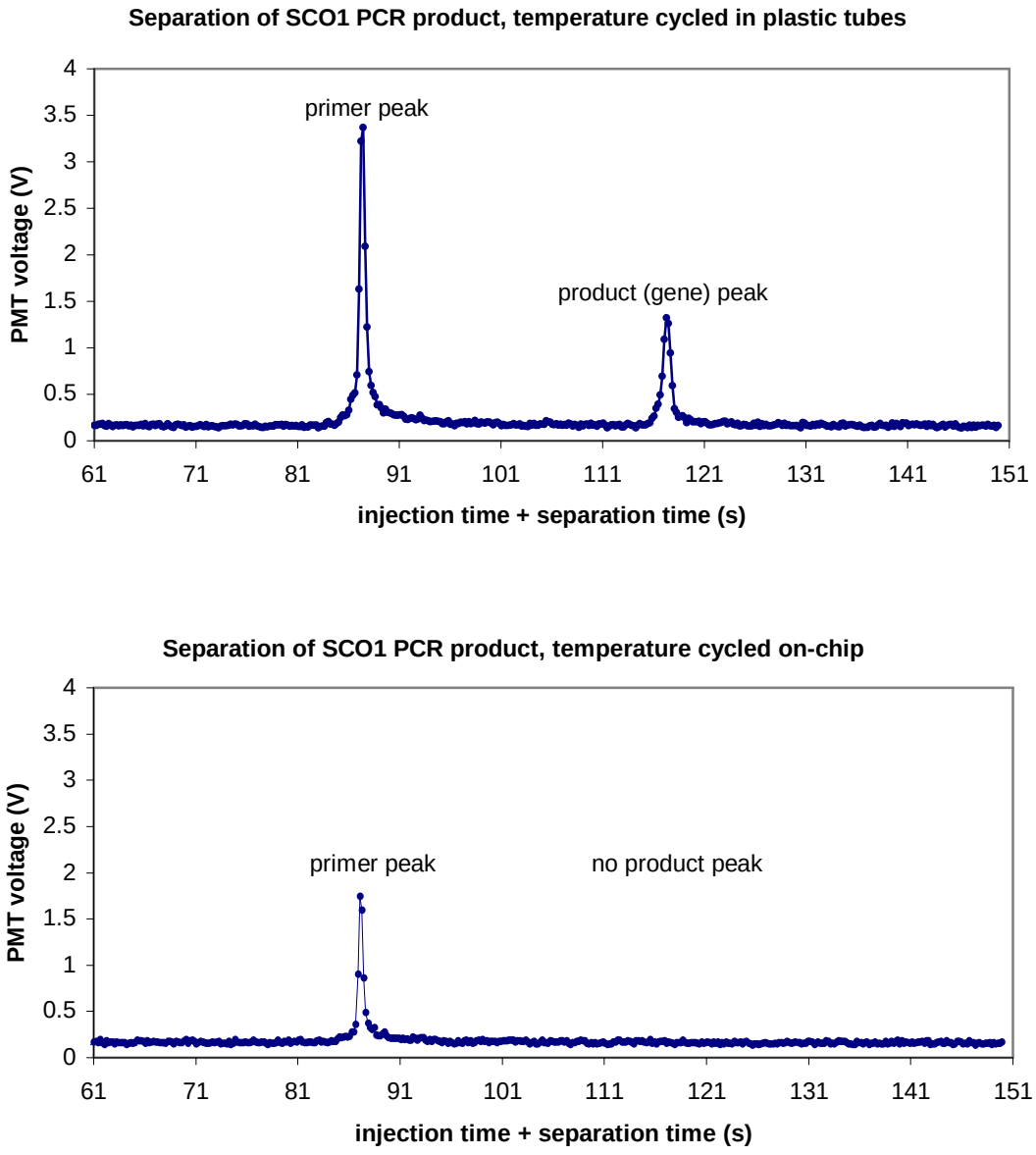

Figure 8: Initial results of conventional vs. on-chip PCR

PCR was working when executed in the conventional way in small plastic tubes inside the commercial thermal cycler, but not when executed in the sample well of the chip in the same commercial thermal cycler.

Some potential causes were investigated. One was a suspicion that perhaps glass was affecting the chemistry of the reaction. Glass tubes were made, similar in size and shape to the plastic ones typically used for PCR. It was found that PCR worked just as well in both glass and plastic tubes, reducing the chance that the composition of the glass chip might be the problem.

However, the likeliest and most obvious reason that on-chip PCR was not working was that perhaps the chip was not reaching the required temperatures and maintaining them for the required lengths of time. Investigating and solving this became the focus of the project.

During temperature cycling of the chip inside the thermal cycler, the chip sits clamped on top of a griddle, which itself sits on top a block:

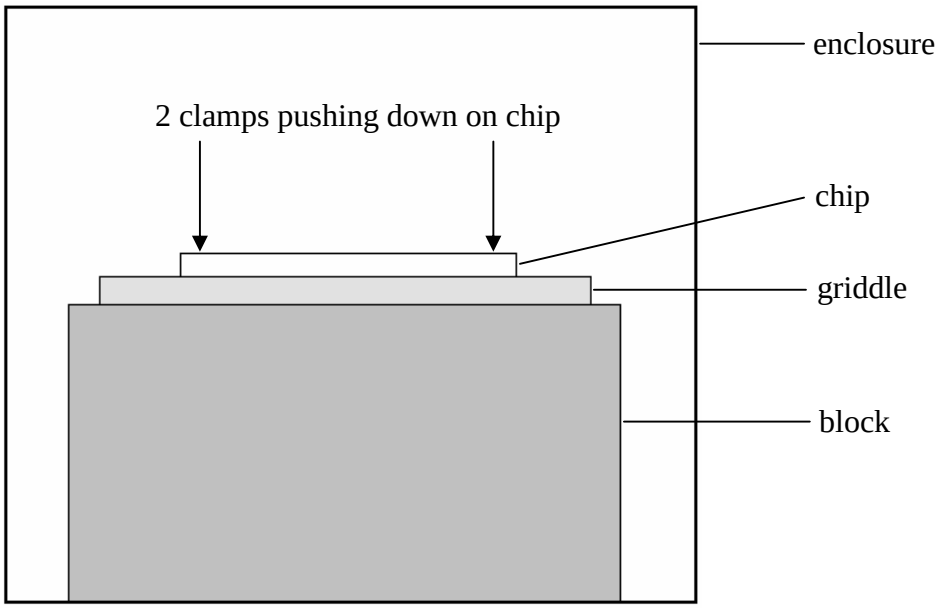

Figure 9: MJ Research PTC-200 commercial thermal cycler (side view)

The thermal cycler only monitors and controls the temperature of the block. Since perfect thermal contact between the chip, griddle and block is impossible to achieve, it is reasonable to assume that much of the time the chip is at a temperature different from that of the block. Thermal interfaces such as thermal tape were tried and discarded. It was found that they did more to hinder thermal contact between the chip and the griddle than help it. Instead, to ensure

the best possible thermal contact, both the bottom of the chip and the top of the griddle were thoroughly wiped down with alcohol prior to each clamping of the chip.

PCR is sensitive to temperature with a tolerance of about ± 0.5°C, so it was vital to find out exactly what temperature the chip was reaching during each step of the cycle. A temperature sensing device was assembled. It consists of two semiconductor temperature sensors sandwiched between glass slides with thermally conductive paste filling in the gaps. Similar to the glass chip in size, thickness, and composition, it was hoped that the temperature sensor would mimic the thermal properties of the glass chip closely enough to provide an accurate estimate of what temperatures the chip was reaching.

Before being put to use, the sensor was calibrated with a thermocouple in an oven capable of maintaining a constant temperature to within ± 0.1°C for long periods of time.

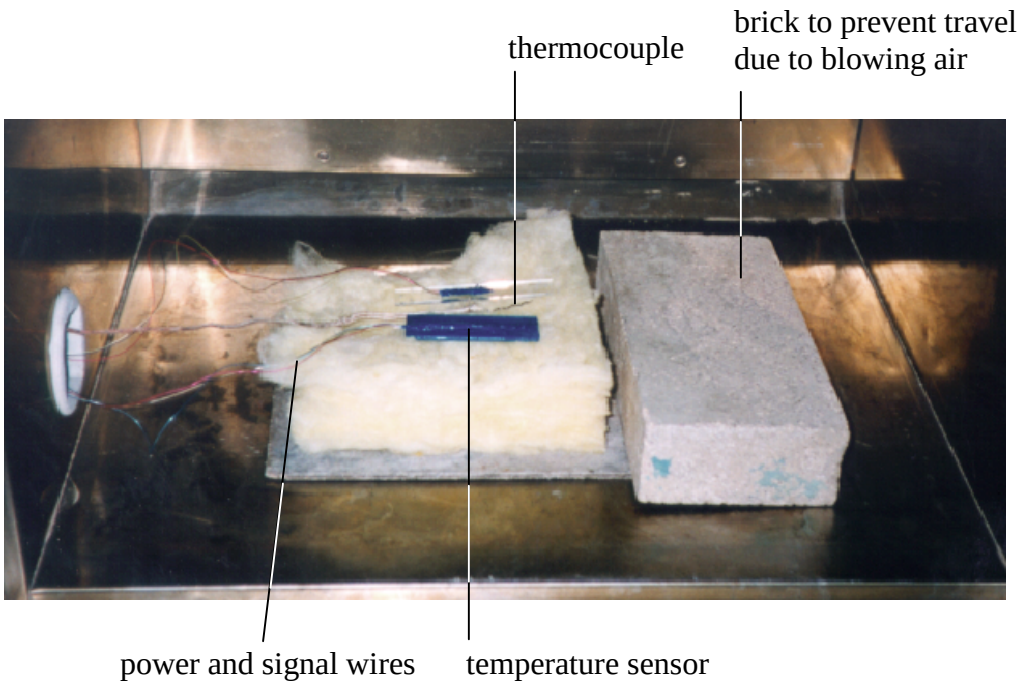

Figure 10: Calibration of temperature sensor inside an oven

The sensor was calibrated at 55°C, 72°C, and 94°C, temperatures in the range of interest for PCR. The sensor was kept at each temperature for approximately an hour to make sure that the oven and everything inside of it had reached thermal equilibrium. This was done a second time to ensure that the sensor readings were repeatable. The results were identical both times, providing a calibration accurate to within about ± 0.1°C:

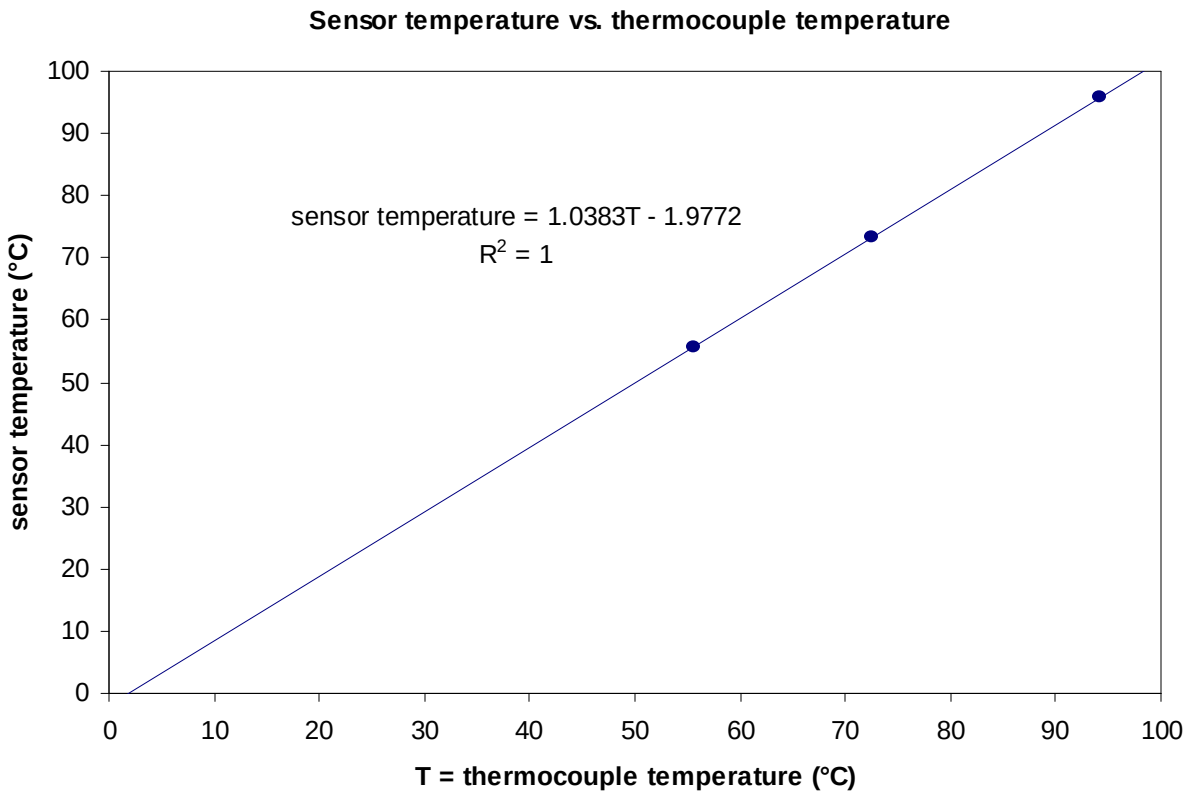

Figure 11: Temperature sensor calibration curve

In the meantime, on-chip PCR was tried with a different gene. Previous tests were done with SCO1, a yeast gene suitable for testing of equipment. Tim Footz, a researcher in the lab, tried the HFE3 human hemochromatosis gene. He found that when cycled under the same conditions as the SCO1 gene, a small amount of product formed:

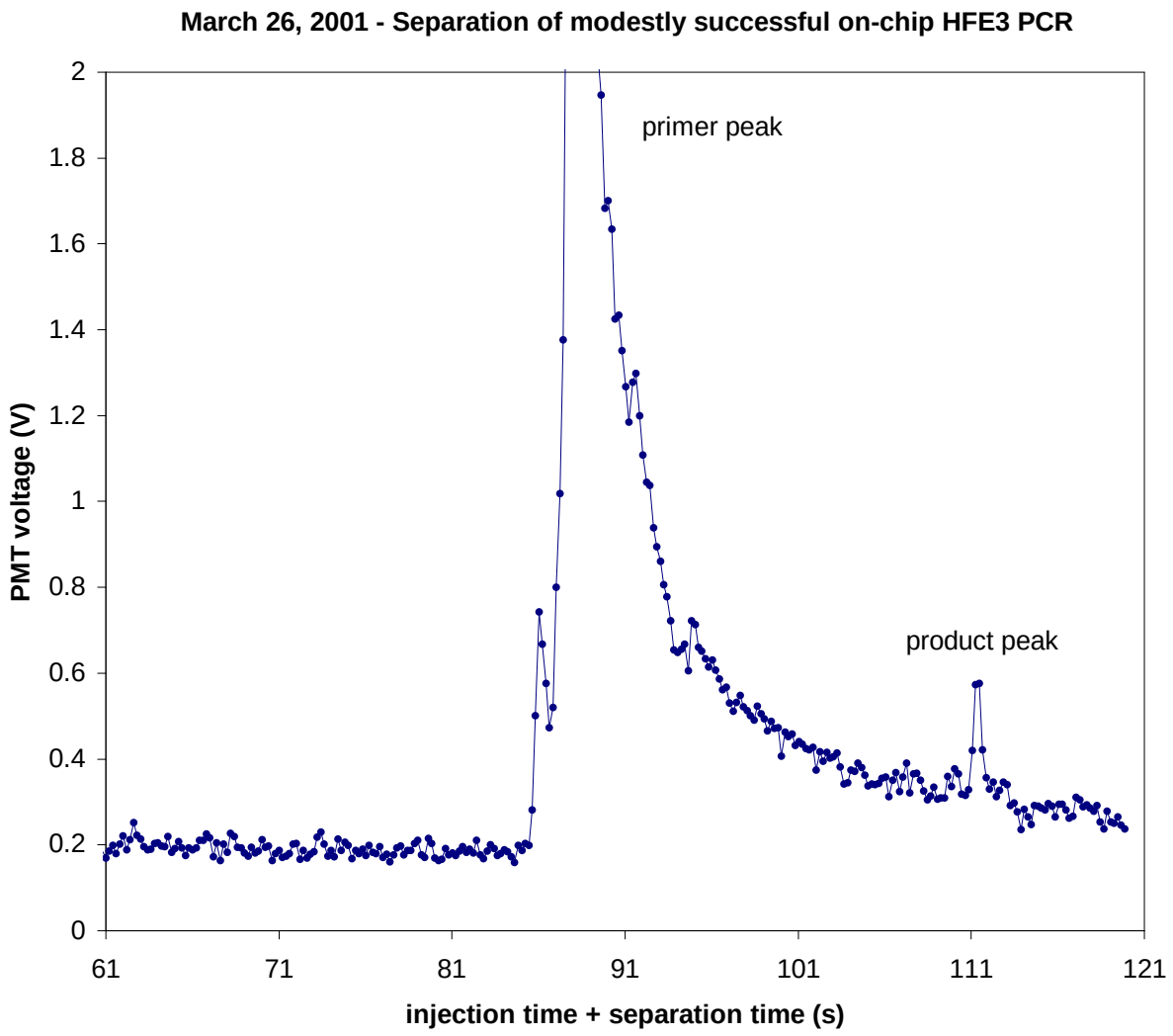

Figure 12: First detection of on-chip PCR product

The primer peak is enormous because few of the primers were used up in making product. The presence of a product peak, small as it may be, suggested that HFE3 is less sensitive to temperature than SCO1. SCO1 was abandoned in favour of HFE3 for testing on-chip PCR.

Before putting the calibrated temperature sensor to use, the clamping method was simplified. The two screw clamps used previously were discarded. In their place, the lid of the thermal cycler provides pressure, and a block of styrofoam transfers the pressure to the chip:

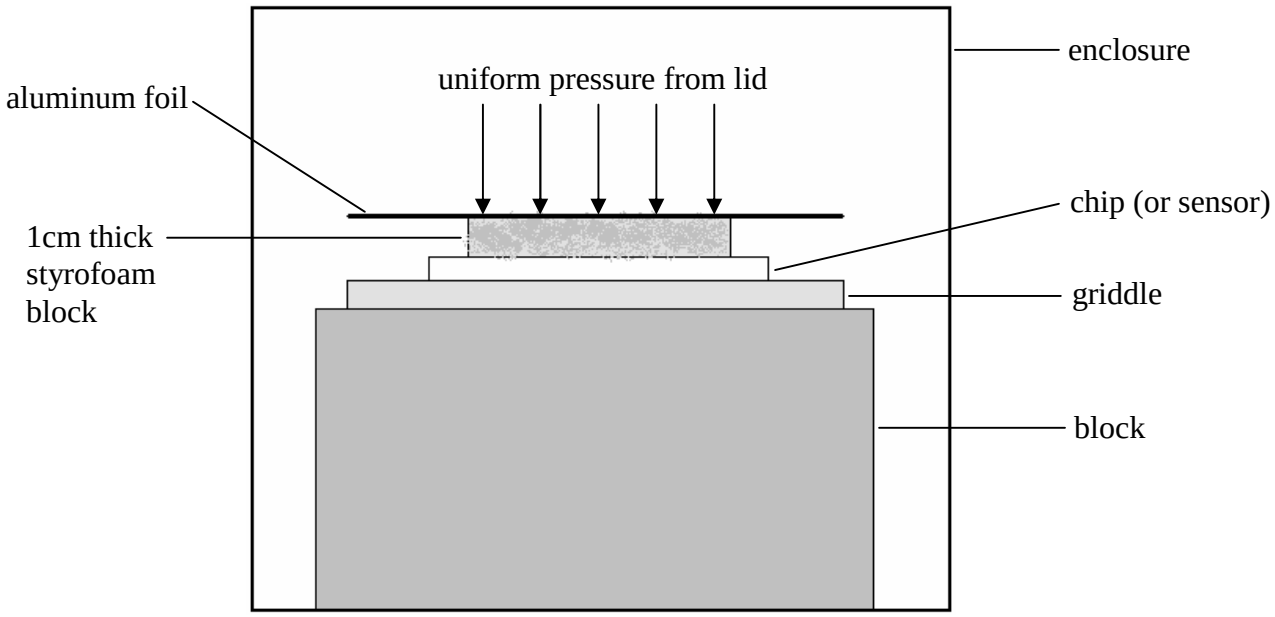

Figure 13: Revised clamping method

This method provides greater, more consistent pressure, ensuring more consistent thermal contact between the chip, griddle, and block. The aluminum foil helps to thermally isolate the chip from the lid of the thermal cycler, which has a relatively large thermal mass and was suspected of hindering temperature cycling of the chip.

The temperature sensor was mounted using the revised clamping method. It was found that with the temperature cycler's original block temperature protocol, the temperature sensor sitting on the griddle gave actual temperature readings up to 2° C or 3° C above or below the target values. A revised block temperature protocol was empirically devised.

Revised block temperature protocol

1. Begin at room temperature
2. 105° C for 59 seconds – overshoot
3. 96.1° C for 2 minutes – long initial denaturing of primary template, corresponds to ~94° C chip temperature
4. 96.1° C for 30 seconds – short denaturing of templates, corresponds to ~94° C chip temperature
5. 50° C for 25 seconds – undershoot
6. 63.8° C for 30 seconds – annealing of primers, corresponds to ~63° C chip temperature
7. 85° C for 12 seconds – overshoot
8. 73.5° C for 30 seconds – polymerase extension of primers, corresponds to ~72° C chip temperature
9. 105° C for 36 seconds – overshoot step
10. Goto "4" 34 times

The purpose of the overshoot and undershoot steps is to get the chip to the target temperatures as quickly as possible. As shown above (steps 4, 6, and 8), for the chip to reach a target temperature, the block temperature has to be set somewhat higher than the target temperature of the chip. This revised block temperature protocol resulted in the temperature trace shown in Figure 14.

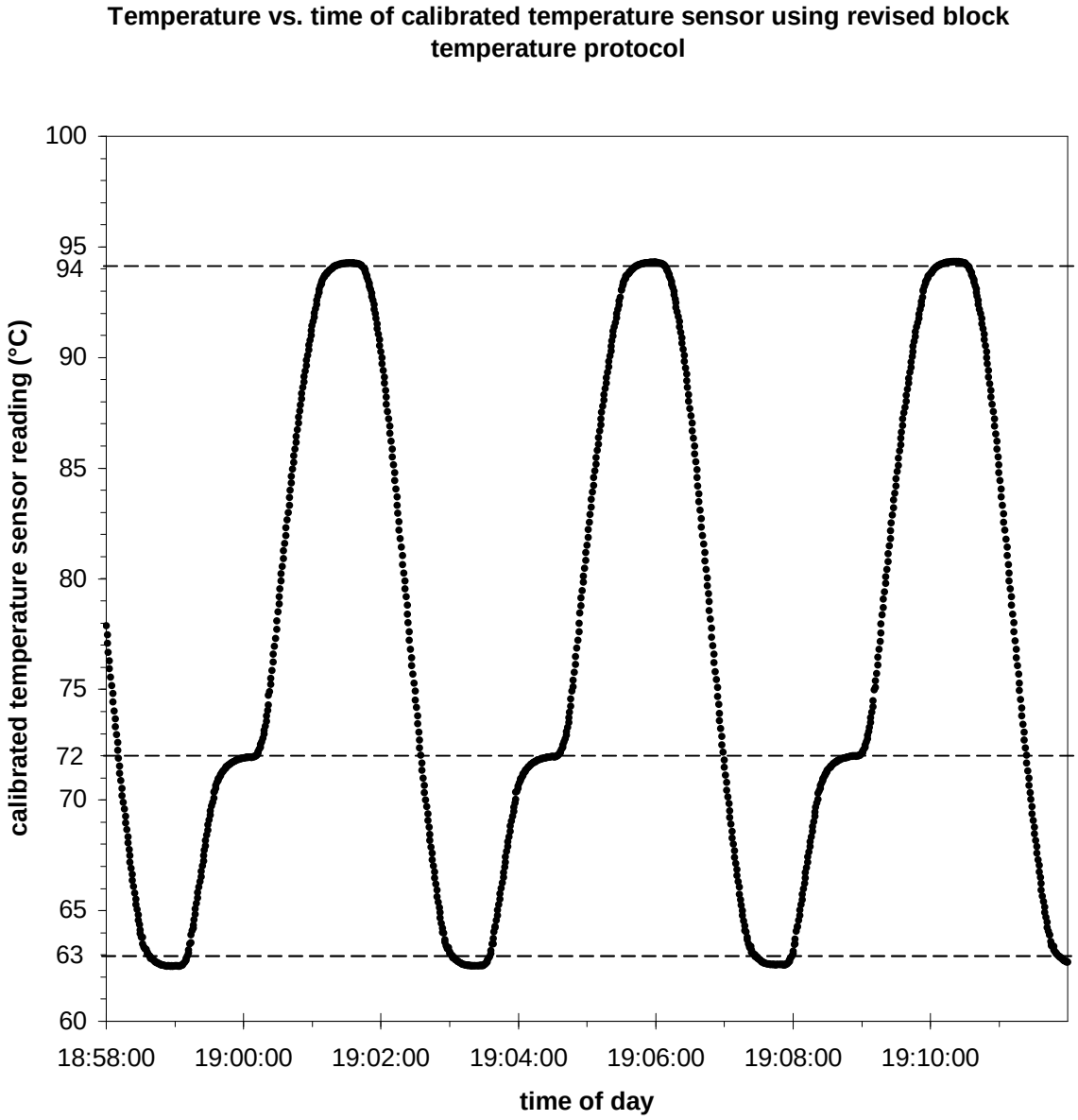

Figure 14: Temperature trace using revised block temperature protocol

The revised protocol results in a temperature trace whose maximum, minimum, and inflection points correspond closely to the three target temperatures of 94° C, 63° C, and 72° C.

Finally, on-chip PCR was tested using HFE3, the revised clamping method, and the revised block temperature protocol, all at the same time. The results are shown in Figure 15.

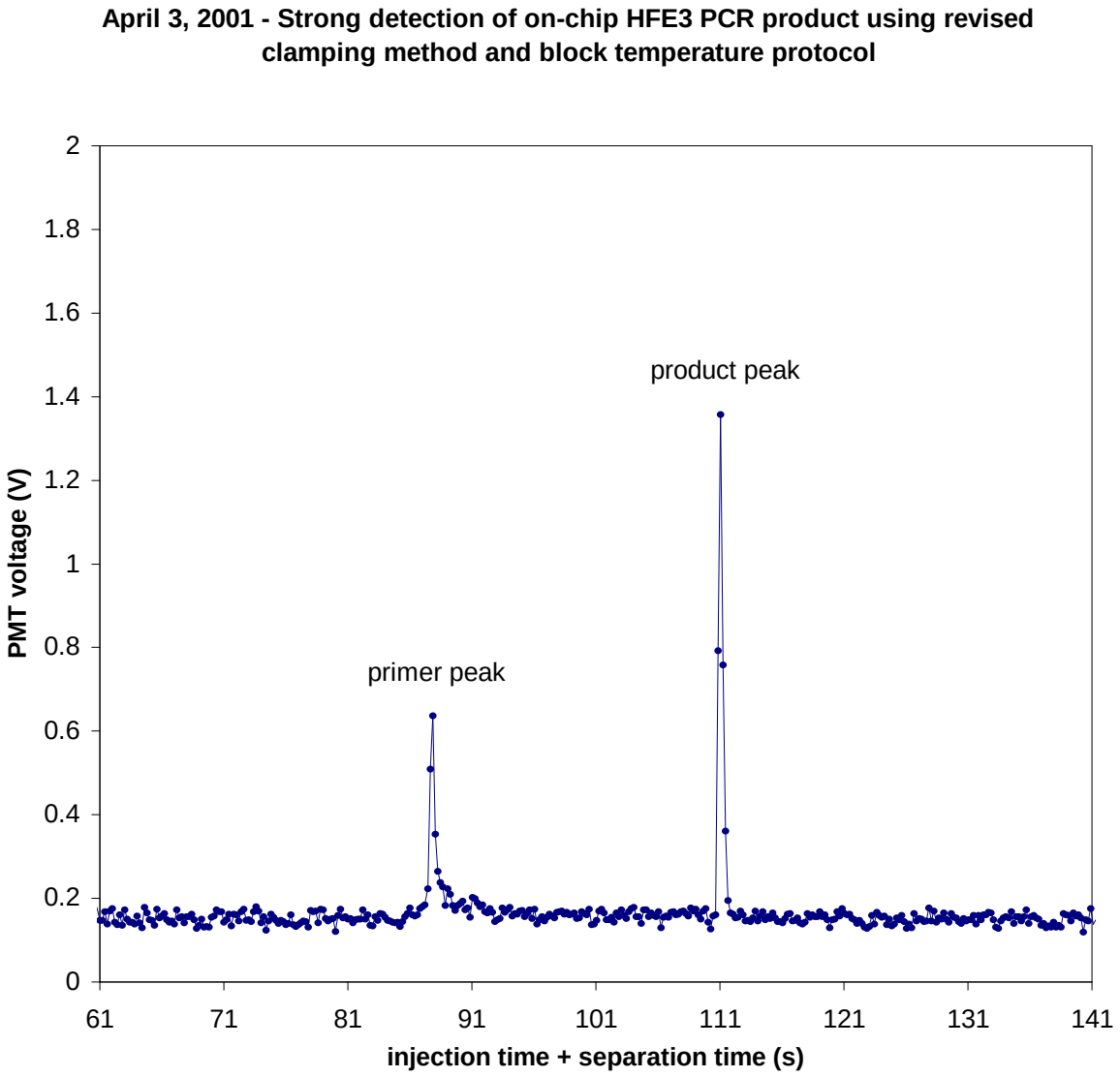

Figure 15: Fully successful on-chip PCR

The results show a large product peak and a relatively small primer peak, indicating that most of the primers during the reaction were depleted and incorporated into the product. Surprisingly, the results seem to surpass those of conventional PCR done in plastic tubes.

Future Work

There is much further work to be done before on-chip DNA analysis reaches its potential as a fast, cheap, and portable method of genetic testing. The results need to be verified, and the procedures need to be tested with other genes. Currently, the biggest problem is that solution stored in any of the wells on the chip tends to be blown out during temperature cycling, often resulting in dried out wells. Also, PCR and separation are currently performed in two separate chips. This is because the polymer in the injection and separation wells, although required for separation of primer and product, seems to be interfering with PCR. Future chip designs may solve both of these problems by embedding magnetically actuated valves within the chip to easily seal off sections and control the motion of solution throughout the chip.

In its current incarnation, on-chip PCR takes about 3 hours to complete. Future work might involve tests to see if, thanks to the small volumes used, it is possible to reduce the length of time the solution needs to linger at each set temperature, and even reduce the number of cycles required to get acceptable amounts of product. Finally, the commercial thermal cycler will ultimately be replaced with a smaller, cheaper version custom-built specifically for quick temperature cycling of chips.

Conclusions

A vital part of DNA analysis, PCR has been shown to work in a micromachined glass chip. Temperature is critical for PCR. Calibration and thermal contact must be accurate and reliable enough such that the temperature of the chip is controlled to within about ± 0.5° C of target values. Preliminary results indicate that on-chip PCR may have the potential to surpass the performance of conventional methods.

Acknowledgements

I would like to thank the following people for their support during the course of this project:

Dr. Chris Backhouse, Tim Footz, Ben Bathgate, Steve Kulak, Jim Fern for help with the oven, Keith Brown for providing the basis for the PCR figures, and the MAD Lab for the use of some of their instrumentation.