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Optimizing Rapid Cycle DNA Amplification Reactions

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The complete optimization of a DNA amplification reaction unfortunately requires some trial and error. Optimizing an amplification requires finding conditions such that:

1. the template melts at the denaturation temperature,
2. the primers pair with their complement at the annealing temperature, but not with non-specific sequences.
3. Temperature and time conditions are adequate for the complete extension of the product.

Failure to meet any of these conditions will cause failure of the amplification reaction. You may notice that two of these conditions involve DNA duplex stability, so it's not surprising that two of the most important variables in DNA amplification, annealing temperature and salt concentration, both affect DNA duplex stability.

What follows is a short discussion of each of the components of an amplification reaction and then an outline of a systematic optimization protocol. This protocol has allowed the successful amplification of both DNA and RNA (via cDNA) using many different primer pairs.

A. Components of an Amplification Reaction.

1. Primers

Primer selection can greatly influence amplification success. Sometimes there is little or no latitude in the selection of primer position, in which case the following discussion is moot. Since the amplification reaction is quite robust, the chances are good that any primer pair can be made to work. However, with forethought, the optimization time can be minimized.

Given flexibility in primer selection, an intelligent choice of primers can simplify the optimization process and maximize both product yield and specificity. There are several commercially available programs for selection of primer pairs and we have found them helpful. These programs can help you avoid cross hybridization with other parts of your sequence, internal primer complementarity and the like.

If you are picking primers by eye you should try to make them similar in length (20 - 30 nucleotides) and GC content (30 -70%) as balanced primers are easier to optimize. We have found that using longer primers (25 - 30 nt) and relatively GC rich primers (50-60%) increases product yield with rapid cycling. There are reports of primer dimer formation when the last two 3' bases are complementary, but they are seldom seen in rapid cycling reactions.

Primer selection and DNA sequence analysis programs will provide a Tm value or even an "annealing temperature" for a given primer sequence. All of these numbers should be viewed with healthy skepticism. Different programs can give Tm values for the same primer that differ by as much as 20° C. In fact, the actual Tm value of primers under DNA amplification conditions is controversial because of the unknown effects of buffer constituents and changing DNA concentrations. We use primers at a final concentration of 0.5 μM. We make a 10X 5 μM stock solution containing one or both primers.

2. Template DNA

DNA amplifications are normally done on one of two types of template, genomic DNA or plasmid DNA. We usually put about 104 to 105 copies of the ta get sequence into a 10 μl reaction. For human genomic DNA that is about 50 ng of DNA, for Escherichia coli genomic DNA it is about 50 pg and for plasmid DNA it is about 100 fg.

The template DNA should be denatured before the cycling reaction begins. We link a two minute hold at 94° to the beginning of the cycling program. Alternately, genomic DNA that has been denatured by boiling and then stored at -20° never reanneals.

3. Mg Concentrations

We use 3 different reaction buffers in our amplification reactions and they differ only in Mg2+ concentration. The low, medium and high Mg2+ buffers contribute 1.0 mM Mg2+, 2.0 mM Mg2+, and 3.0 mM Mg2+ to the final reaction respectively.

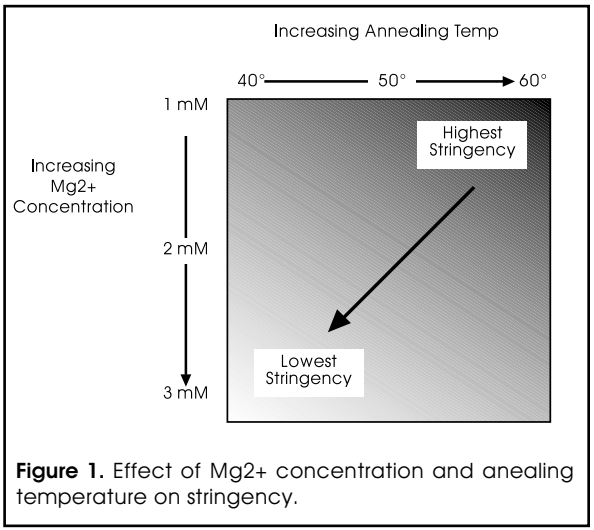

Figure 1. Effect of Mg2+ concentration and anealing temperature on stringency.

The optimal Mg2+ concentration is different for every template-primer set and must be determined experimentally. Magnesium ions stabilize DNA duplexes. Therefore lowering Mg2+ concentration increases stringency while raising Mg2+ concentration lowers stringency (Figure 1).

It has been reported that high Mg2+ leads to an increased rate of nucleotide misincorporation so if you are cloning your DNA products you may wish to avoid the high Mg2+ buffer.

4. dNTP's

Our usual final dNTP concentration is 200 μM of each dNTP. Increasing this concentration does not effect the yield of the reaction. If you are using low Mg2+ concentrations remember that each dNTP chelates a magnesium ion, so you need at least 0.8 mM Mg2+ to have any free Mg2+ at all.

5. Reaction Buffer

Our standard reaction buffer is a 10X buffer containing 500 mM Tris pH 8.3, 2.5 mg/ml crystalline BSA and MgCl2 at 10, 20, or 30 mM. The BSA is critical for preventing denaturation of the polymerase on the glass surface of the capillary.

If you plan to run your finished reaction on an agarose gel you can add 5% Ficoll 400 and 10 mM tartrazine to your 10X buffer. This allows you to add the reaction directly from the capillary tube to an agarose gel well. We use tartrazine instead of bromphenol blue or xylene cyanol because it does not affect the amplification reaction. Tartrazine runs faster than bromphenol blue.

If you wish, you may use commercially available reaction buffers for rapid cycling but you must add BSA. Failure to add BSA will cause denaturation of the polymerase and therefore failure of the reaction.

6. Enzymes

Most heat stable enzymes come at a concentration of 5 U/μl. We make a 1:12.5 dilution in a enzyme dilution buffer that consists of 10 mM Tris pH 8.3 and 2.5 mg/ml crystalline BSA. This gives a 10X stock solution. This dilute enzyme solution is stable for at least 2 days at 4°C. We have found little significant difference between the enzymes from different suppliers. However, different heat stable enzymes may differ in their reaction rates, and temperature and time paramters may need to be adjusted accordingly .

7. Reaction Volume

Our standard reaction volume is 10 μl. This produces enough DNA product for most applications. If you do need more DNA, multiple 10 μl capillaries can be filled from the same master mix or you can use larger 25 or 50 μl capillaries. The larger capillaries require a short (5-20 second) hold at denaturation and annealing to allow the larger sample to reach temperature. Because the temperature is not as well defined, we prefer to use multiple small capillaries.

8. Cycling Times and Temperatures

A cycling protocol requires setting three temperatures: denaturation, annealing, and elongation temperatures.

Denaturation should be set at as high a temperature as possible without killing the enzyme. We routinely use 94° C. Altering this temperature has not been helpful.

A rapid air cycler (Idaho Technology, Salt Lake City, Utah) can hold the denaturation time for as long as desired but we have not found any advantage in holding denaturation. We recommend a denaturation time of 0 seconds when using the standard 10 μl capillary tubes. Because of a larger thermal mass, samples in the 25 μl tubes require a hold time of 5-10 sec, and samples in 50 μl tubes require 10-20 sec.

We use 70° C as our standard elongation temperature. The extension rate vs. temperature curve (Figure 2) for Taq polymerase activity shows a broad

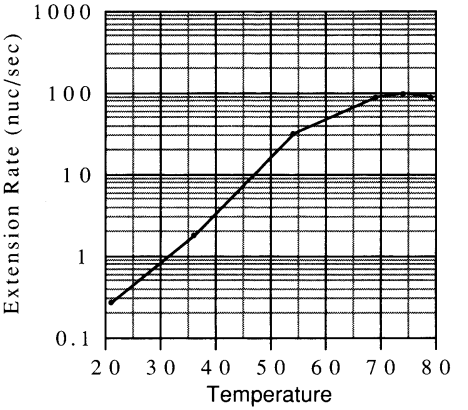

Figure 2. Extension rate vs temperature for Taq polymerase.

peak of about 100 nucleotides per second between 70 and 80° C.

The amount of time at elongation should be varied with product length. Taq polymerase catalyzes the addition of about 100 nucleotides per second at 70°C. For very small DNA products (target<100 bp) no elongation time at all is required. These products will elongate in transit. For medium length targets (100 - 500 bp) 5 to 15 seconds elongation is sufficient. Longer products must use proportionally longer times, approximately 15 and 30 seconds per kilobase of product.

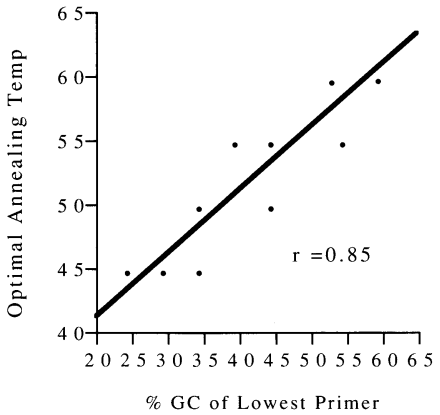

Figure 3. Correlation of optimum annealing temperature of GC content of lowest primer.

The annealing temperature is the most important variable in a DNA amplification. As mentioned above, calculated values of Tm should not be taken too seriously, but the consistent use of a single program can be helpful in predicting effective relative annealing temperatures for different primer sets.

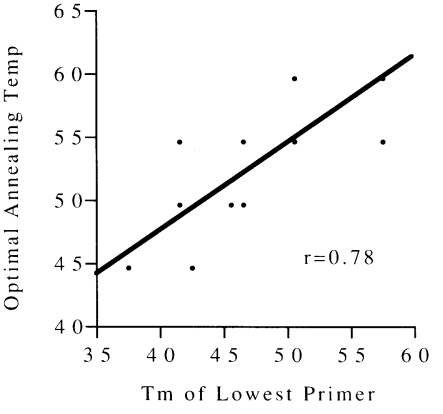

Figure 4. Correlation of optimum annealing temperature to Tm of lowest primer.

In a group of 15 pairs of twenty nucleotide long primers we correlated the percentage GC and the Tm calculated by a commercially available program to the final optimized annealing temperature. We found that the best predictor of annealing temperature was the GC percentage of the lowest GC primer (Figure 3). The Tm of the least stable primer was almost as good at predicting annealing temperatures (Figure 4).

The amount of time spent at annealing has a direct effect on the specificity of the amplification reaction (Figure 5). The longer you spend at the annealing temperature the more non-specific priming you see. You will notice in Figure 1

that the polymerase has significant activity at temperatures that are commonly used for annealing. As you spend more time at the annealing temperature there is a greater chance of non-specific priming and extension of undesirable product. We recommend that when using the standard 10 μl capillary tubes, you set your annealing time at 0 seconds to maximize specificity. As with the denaturation temperature, the 25 μl tubes require a hold time of 5-10 sec., and the 50 μl tubes require 10-20 sec. Some amplifications, especially those with low Tm's, may also require longer annealing times (5 to 15 sec).

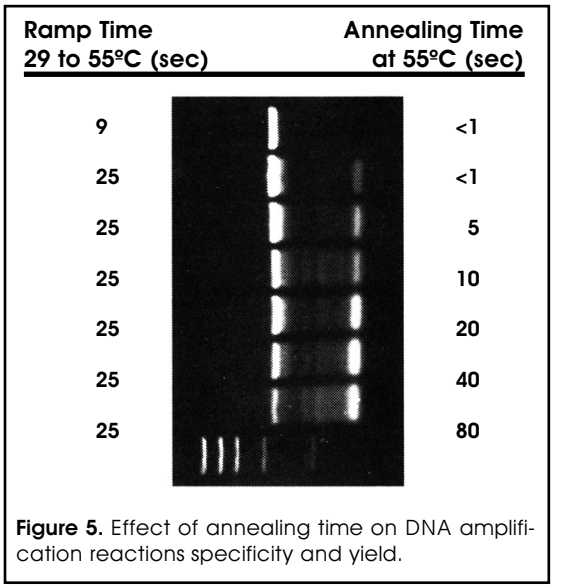

Figure 5. Effect of annealing time on DNA amplification reactions specificity and yield.

B. Systematic Optimization B. B. Protocol for Rapid Cycling

When trying to optimize a new primer pair we run test reactions at annealing temperatures of 40°, 50° and 60° C. At each of these temperatures we run high, medium and low Mg2+ concentrations (1.0, 2.0 and 3.0 mM MgCl2). This gives nine different reaction conditions which cover a wide range of DNA hybridization stringencies. The low Mg2+ buffer at 60° gives the highest stringency while the high Mg2+ buffer at 40° gives very low stringency (Figure 1). Usually one or more of these conditions will provide good specificity and yield. If needed, intermediate temperatures or Mg2+ concentrations can be tried in a second experiment.

If you are running a large number of primer pairs then nine reactions per pair can get a little out of hand. About 80% of primer pairs can be successfully amplified in the medium Mg2+ buffer at 40°, 50°, or 60°C. Even if none of these three conditions is ideal you will often get a clue as to what conditions to try next. If you have no band at 50° or 60° and a weak band at 40° then you will want to try the high Mg2+ buffer next. If 60° is giving non-specific amplification you will want to try the low Mg2+ buffer.

Conclusion

To paraphrase Robert Pirsig, "optimization of new primer pairs requires great peace of mind." Our lack of understanding of the amplification reaction prevents the formation of a set of rules for predicting conditions that will be successful. This lack of simple rules can make the optimization process frustrating. Fortunately there are usually only two variables to worry about, and the reasonable range of these variables is limited. Annealing temperatures are rarely less than 37° or more that 70°. Magnesium concentration is never less than about 1 mM and rarely more than 5 mM. It doesn't take many experiments to cover this range. With perseverance you can eventually get any primer pair to work.

Good Luck

Buffers and Reaction Components for Rapid Cycling

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Many different buffers and reactant concentrations have been reported for DNA amplification. Rapid cycle DNA amplification was originally optimized with the following buffer (1):

20 mM KCl
50 mM Tris, pH 8.5
3 mM MgCl2 (with 500 μM each dNTP)
500 ug/ml BSA

BSA is required when capillary tubes are used; gelatin is a poor substitute and greatly reduces amplification yield. Although we have tried conventional buffers that contain 50 mM KCl (2), a greater number of amplifications with various primers were successful at lower KCl concentrations. In sequencing reactions, the best extensions are reportedly obtained when no KCl is included (3). We now routinely use a buffer system without KCl:

50 mM Tris, pH 8.3
2 mM MgCl2 (with 200 μM each dNTP)
250 ug/ml BSA

Some primer pairs have only amplified with this "no KCl" buffer; conventional high KCl and our original buffer were not effective. Although it is probably true that no single buffer is best for all amplifications, we have successfully amplified about 80% of untested primer pairs with this buffer. Most of the remainder can be amplified by varying the Mg concentration from 1-3 mM.

DNA amplification reactions are very resilient, and many additives appear to have little or no effect on the reaction. Ficoll 400 (0.5 - 1%) and tartrazine (1 mM) are convenient to add to a reaction mixture before amplification if the products are going to be analyzed by gel electrophoresis. This allows direct transfer of the solution into a gel well from the capillary tube after amplification, without intermediate mixing (1). If you run many reactions and are looking for quick results, this is very convenient. By running parallel reactions with and without Ficoll/tartrazine, no significant differences in specificity or yield have been observed.

Whether certain buffers are more amenable to rapid vs conventional cycling has not been adequately studied. Reaction kinetics and equilibrium constants will change with different buffers, but the effects on amplification are poorly understood. Buffers other than those suggested here can be used for rapid cycle amplification, but BSA must be included in the reaction. It is convenient to add the BSA with the enzyme. A 10X enzyme solution of 0.4U/μl can be obtained from a 5U/μl enzyme stock by diluting in an "enzyme diluent" as follows:

11.5 μl enzyme diluent
(10 mM Tris, pH 8.3, 2.5 mg/ml BSA)
+ 1.0 μl enzyme

This is enough to run about 12 reactions. When the 10X enzyme solution is diluted, enough BSA is included for efficient amplification, even if no additional BSA is added with the buffer. The 50% glycerol storage media of most, enzyme preparations makes pipetting 1 μl very difficult. If accurate volumes are desired, microcapillary pipets (1 μl Microcaps, available from Sigma) can be used. The other components of a master mix can also be stored as 10X stocks. A 10X solution of human genomic DNA (50 μg/ml) conveniently has an absorbance of 1.0 at 260 nm. One μl of this 10X solution provides about 15,000 template copies per 10 μl reaction.

Table 1.

Rapid Cycle Reactant Concentrations

Component	[10X Stock]	[1X Reaction]	Volume/10µl
Buffer	500 mM Tris, pH 8.3	50 mM Tris	1 µl
	2.5 mg/mL BSA	250 µg/mL BSA	
	5-10%Ficoll	0.5-1.0% Ficoll	
	10 mM Tartrazine	1 mM Tartrazine	
Low Mg	10 mM MgCl2	1 mM MgCl2	
Med Mg	20 mM MgCl2	2 mM MgCl2	
High Mg	30 mM MgCl2	3 mM MgCl2	
dNTPs	2 mM each dNTP	200 uM dNTP	1 µl
Left Primer	5 µM	0.5 µM	1 µl
Right Primer	5 µM	0.5 µM	1 µl
DNA			1 µl
Genomic DNA (mammalian)	50 ng/µL or A (260)=1.0	5ng/µL	
Bacterial DNA	5-50 pg/µl	0.5-5 pg/µl	
Plasmid DNA	0.1-1.0 pg/µl	10-100 fg/µl	
Diluted Enzyme	0.4 U/µL	0.4 U/10µL	1 µl
	2.5 mg/ml BSA	250 µg/ml	
dH2O			4 µl

Note: BSA is present in both the 10X buffer and the enzyme diluent for a final concentration in the reaction of 500 ug/ml.

We usually use a 5 μM 10X solution of each primer. The concentration of primer stocks should be determined spectrophotometrically at 260 nm. The extinction coefficient of an oligonucleotide is affected by base sequence and is best estimated by considering neighboring pairs (4). Commercially available computer programs, such as Oligo 4.0 (National Biosciences, Hamel, MN) automatically perform the calculation.

A summary of reactant concentrations is given in Table 1. Suggested volumes of reaction components for a master mix for 4, 8, 16, 10 μ l samples are listed for convenience in Table 2 (below). Kits supplying the components of this system (exclusive of primers, template DNA, and enzyme) are commercially available (Idaho Technology, Salt Lake City, Utah).

Table 2

Component(10X)	Number of 10μl Reaction Tubes		
	4	8	16
Buffer	5 μl	9 μl	17 μl
dNTP's	5 μl	9 μl	17 μl
Left Primer	5 μl	9 μl	17 μl
Right Primer	5 μl	9 μl	17 μl
Template DNA	5 μl	9 μl	17 μl
Taq (0.4 U/μl)	5 μl	9 μl	17 μl
dH2O	20 μl	36 μl	68 μl
Total Volume	50 μl	90 μl	170 μl

References

1.Wittwer, CT and DJ Garling. Rapid cycle DNA amplification: time and temperature optimization. BioTechniques 10:76-83, 1991.

2.Wittwer, CT and JL Cherry. Momentary denaturation and annealing for DNA amplification, submitted.

3.Innis, MA, KB Myambo, DH Gelfand and MAD Brow. DNA sequencing with Thermus aquaticus DNA polymerase and direct sequencing of polymerase chain reaction-amplified DNA. PNAS 85:9436-9440, 1988.

4.Fasman GD, ed., Handbook of Biochemistry and Molecular Biology, 3rd ed., Nucleic Acids - Vol. 1, pp 589, CRC Press, Cleveland, OH, 1975.

A RAPD Protocol for the Air Thermo-Cycler

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Idaho Technology's Air Thermo-Cycler is unique among commercial thermal cyclers in the speed with which PCR reactions can be performed. The RAPD reaction, a PCR technique for generating useful genetic markers (Williams et. al. 1990. Nucleic Acids Research. 18: 6531-6535), also runs much faster in the ATC. Published RAPD protocols are based on the use of metal block machines and require relatively long total cycling times. The RAPD reaction can be economically performed in the Idaho Technology ATC with a total cycling time of less than 90 minutes.

Template DNA should be clean and relatively free of RNA. Treatment with RNase followed by an alcohol precipitation is sufficient to remove most of the RNA. Clean DNA may give good RAPD products even when it is significantly degraded. 5X solutions of template and primer are prepared in a TE buffer that is 1mM Tris (pH=7.5) and .1mM EDTA (pH=8.0). A 25X dNTP and MgCl2 solution is prepared in distilled H20. 5X reaction buffer contains 250 mM Tris (pH=8.5), 5mM MgCl2, 100 mM KCL, 2.5 mg/ml BSA, 12.5% Ficoll 400 and .1% xylene cyanole. For a set of 100 reactions a master mix containing dNTP's, MgCL2, Taq DNA polymerase (Promega Corp.), and reaction buffer is prepared by mixing 200 μl 5X

buffer, 40 µl 25X MgCl2- dNTP's, 12 µl Taq polymerase (5 units/µl), and 348 µl distilled H20. Reactions are then prepared in 10 µl volumes by combining master mix, 5X template, and 5X primer in the ratio 3:1:1. The final concentrations in volumes of 10 µl should be 2 ng/µl template DNA, .4 µM primer, 100 µM each dNTP, 2 mM MgCl2, .06 U/µl polymerase and 1X reaction buffer.

Amplification is divided into two steps. For the first two cycles the thermal profile is 1 minute at 92° C, 7 seconds at 42° C, and 70 seconds at 72° C. Subsequently, an additional 38 cycles are performed with denaturation for 1 second at 92°C, annealing at 42° C for 7 seconds, and elongation at 72° C for 70 seconds. Following these forty cycles the temperature should be held constant at 72° C for 4 minutes.

Some Comments on Reaction Optimization

A particular RAPD product generated from a unique primer and template combination will require a specific set of optimum conditions. Our goal was to get good bands for a large number of primers making our protocol as general as possible. Also, we wanted to be able to run the reactions in a short amount of time to maximize throughput. Interactions between concentrations, times, and temperatures are important. Changing the value of any parameter may change some reaction results. However, reaction parameters near those given here will give good results. For example, annealing for 7 instead of 8 seconds is somewhat arbitrary. Attempts to improve the efficiency of the reaction by significantly lowering or raising the polymerase concentration has not worked, in general. Using the protocol described above, we have obtained satisfactory reaction products from thousands of RAPD reactions.

Results

The following figures show some results from our lab using the above protocol.

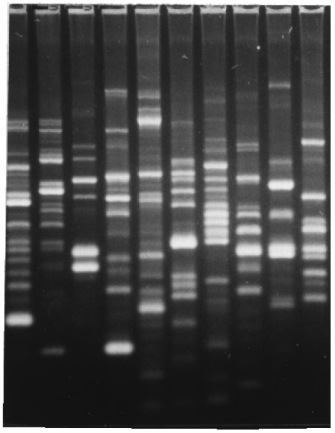

Figure 1. The gel shows RAPD amplification products from 10 different primers with a single Phaseolus vulgaris (bean) DNA preparation. The number of bands amplified for a given primer can vary from 1 to about 16.

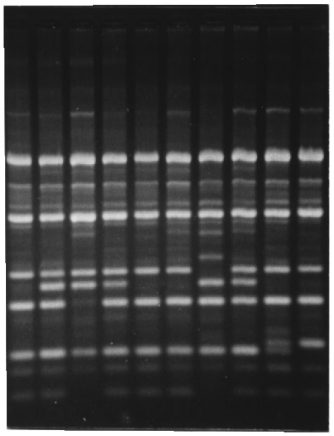

Figure 2. The gel compares 10 different P. vulgaris genotypes amplified with a single primer.

Research In Progress at Idaho Technology

Kirk M. Ririe
Idaho Technology

Idaho Technology is committed to improve the state-of-the-art in rapid cycling, instrumentation and accessories. Several recent developments are worth noting.

Linear Actuator Tests:

We recently tested a prototype instrument outfitted with a linear actuator in place of the solenoid on the 1605 ATC. Our primary objectives are to eliminate the noise produced by the solenoid and to gain better control of the temperature/ time curve. While we have done our best to make the 1605 ATC a flexible instrument, there are some restrictions imposed by the original design. Since the solenoid is either fully open or fully closed, the machine is limited to a single cool down rate. This rate is factory set by adjusting the solenoid to cool down from 94° C to 55° C in about 8 sec. Cool down rates as low as three secs are possible with this design, however, product yield is decreased with rapid cooling. This is due to the temperature rebound which occurs when the door closes. Apparently, there

is not enough time for the primers to anneal and the enzyme to function before the increasing temperature causes denaturation. A linear actuator can in theory produce cool down rates in the range of 3-4 secs and then hold the lower temperature for precisely the required time. This would allow a slight decrease in cycle time, yet substantially increase product yield. This work is especially important for low annealing temperature protocols such as RAPD.

We are committed to developing a more flexible instrument, while retaining the simplicity of the ATC. The linear actuator will allow much finer control of the temperature/time curve. Two temperature cycling and three temperature cycles with unusually high annealing temperatures will be facilitated. Upgrades to a linear actuator system should be available by the summer of 1992.

Sample Handling Advances

We recently received sample quantities of a 25 μ l plastic capillary tube. Initial tests confirm that the reaction runs at slightly reduced speeds compared to glass capillary tubes. Using the 25 μ l plastic capillary tubes, the sample comes to temperature in 5-10 secs, which is comparable to sample response when using 25 μ l glass capillary tubes. We intend to continue our tests and make a positive displacement sample handling system available soon.

We have recently tested a microscope slide rack using 24 x 60 mm cover slips (Fisher Scientific). Early test results look promising. The slide rack will be available for purchase in May.