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Capillary Tube Handling with the Rapidcycler

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One of the biggest concerns for new users of air-cyclers is the handling and sealing of glass capillary tubes. While they are a bit more difficult to use than the traditional microcentrifuge tube, the rapid cycle times and temperature homogeneity made possible by the capillaries makes them more than worth the extra trouble. After a little practice, you may wonder why you ever worried.

Single Tube Handling

Mixing the Sample

You can mix your reaction in any sort of container, I use low protein absorbing microtiter dishes (IT#2590). Take care at the mixing step as one of the most common causes of reaction failure is forgetting a component of the reaction (see "The 10 most common mistakes", Rapid Cyclist 2:11-12). The chances of leaving something out can be reduced by making up "master mixes" that contain everything but primer and template. The mix can be stored at 4° C for up to 3 months (see "Reaction mixes and buffer recipes", Rapid Cyclist 2:9).

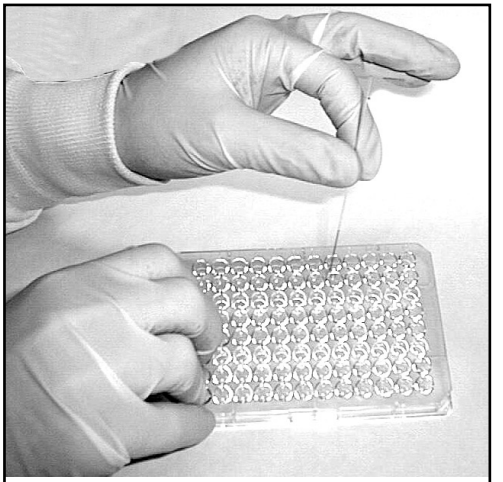

Figure 1. Tipping the capillary tube sideways to increase the rate of liquid uptake.

Loading the capillary

Glass capillary tubes are easily loaded by capillary action. You can increase the rate of liquid uptake by tipping the capillary tube sideways (figure 1). You can also load the capillaries using a Drummond microaspirator (IT#1690) to draw the reaction mix up into the tube, or you can use a pipetman to directly inject sample into the tube (Figure 2). The 10 ul size tubes hold 2.2 ul/cm and can be used for reaction volumes from 5 to 15 ul. The 10 ul capillaries come to temperature so quickly that they require no holds at denaturation or annealing. The 50 ul tubes hold 9 ul/cm and are useful for reaction volumes from 15 to 70 ul. These tubes require a 15 second hold at the denaturation and annealing temperature.

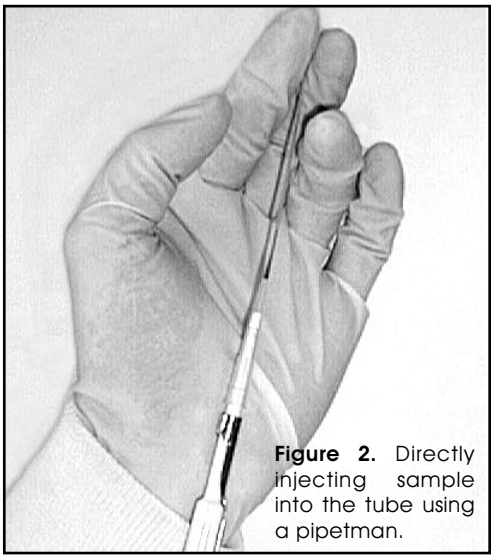

Figure 2. Directly injecting sample into the tube using a pipetman.

Sealing the capillary

The glass capillaries sold by Idaho Technology are made out of a high sodium, low melting temperature glass. This makes them very easy to flame seal with just about any flame, They can be sealed with a Bic lighter (Figure 3), a Bunsen burner, a candle, or, my favorite, a Blazer mini propane torch (IT#2721).

After the capillary is loaded, tip the tube to center the liquid. Hold the tube in the center and place the end just into the flame. Rotate the tube in the flame by rolling it between your thumb and index finger. You should be able to see the glass slowly close in on itself. Try to avoid leaving the tube in the flame too long, as you can end up with a big ugly glob of glass which will not fit into the holder . This is more

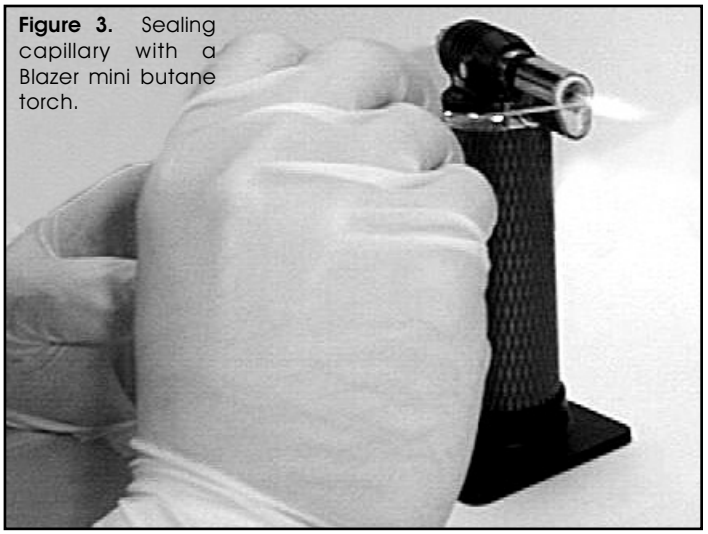

Figure 3. Sealing capillary with a Blazer mini butane torch.

likely in very hot flames. Cutting down the air to the flame will cool these burners down and make the capillaries easier to seal.

You can confirm that the end is sealed by looking carefully at the end for a continuous wall of glass around the end. You can also confirm sealing by blowing on the hot end of the capillary and watching to see if the liquid moves toward the end of the capillary as the glass cools (This is more dramatic for the first seal than the second).

Repeat the sealing process on the other end and then insert the tube into the capillary holding module. A module rack (IT#1735) makes these manipulations easier.

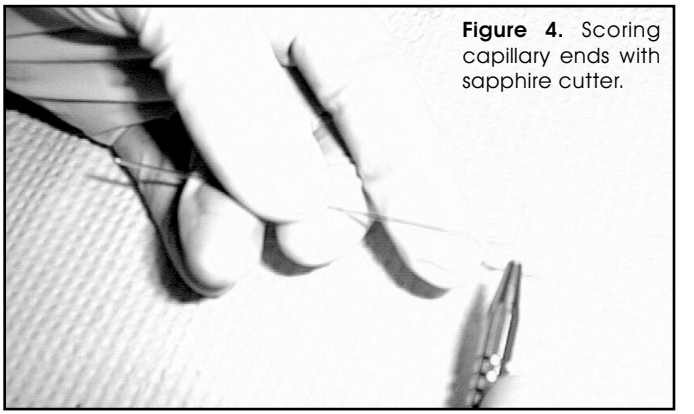

Figure 4. Scoring capillary ends with sapphire cutter.

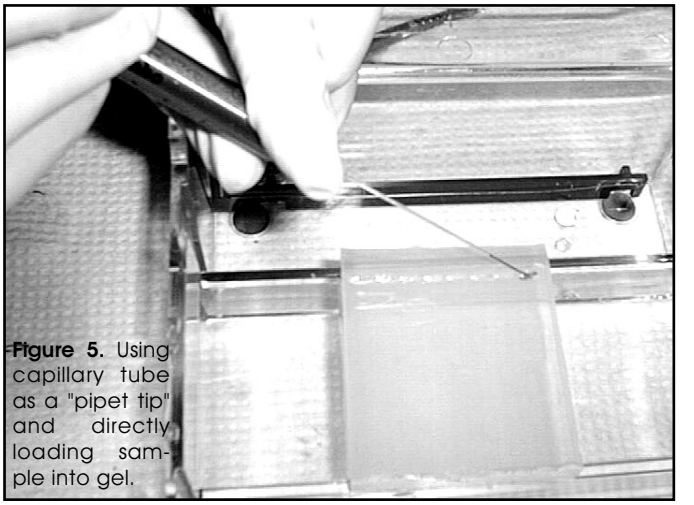

Figure 5. Using capillary tube as a "pipet tip" and directly loading sample into gel.

Sample Recovery

After your reaction is done you pull the tube from the module, lightly score the two ends with a sapphire knife (Figure 4, IT#1691) and break off the ends. The capillary tube then becomes a 3pipet tip2 for the Drummond microaspirator (IT#1690) and can be used to directly load your sample into a gel (Figure 5), or into a storage tube.

Beware, the pressure caused by sliding the capillary into the microaspirator can cause your sample to be blown out of the tube. This is easily prevented by dialing the microaspirator back a bit as you insert the capillary tube. The silicon tips of the microaspirator wear out quite quickly, so if your microaspirator stops working try replacing the tip (IT#1870).

Multiple Tube Handling

Once you get single sample handling down, you may want to try some of these "advanced" multiple sample handling tricks.

Eight Sample Handling

When sample modules are made with microtiter spacing it is possible to mix up eight samples at a time in a microtiter dish and draw them up simultaneously by capillary action (Figure 6). All eight samples can be centered by tilting the module and then the tubes can be sealed by passing the tubes through a flame one at a time (Figure 7). Once the reaction is done you can score all eight tubes at once by lightly drawing the sapphire knife across the top of the module (Figure 8) and then breaking off each tube top (Figure 9). Press the module down to the other end of the capillary tubes and repeat the scoring and breaking.

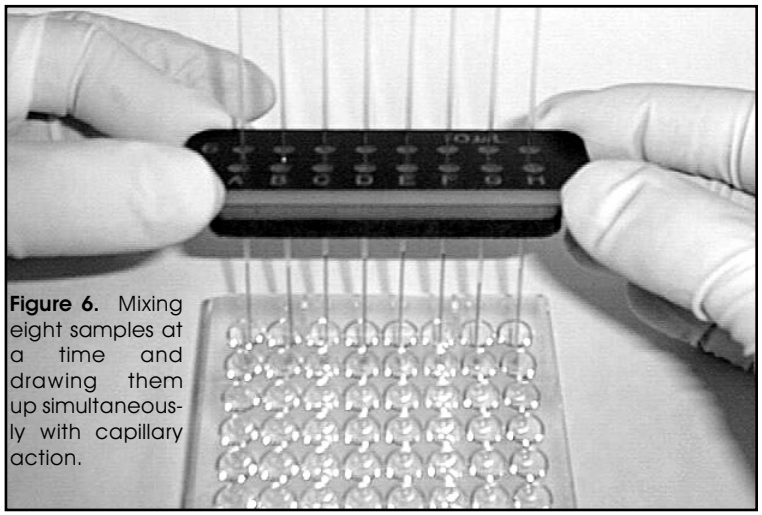

Figure 6. Mixing eight samples at a time and drawing them up simultaneously with capillary action.

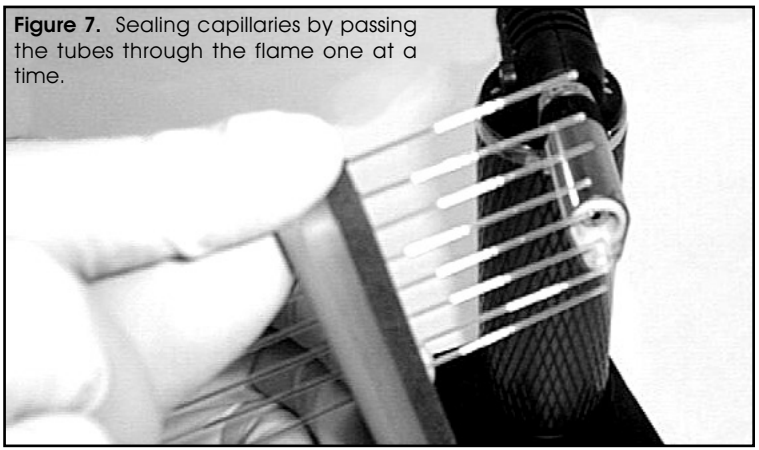

Figure 7. Sealing capillaries by passing the tubes through the flame one at a time.

Sixteen Sample Handling

After mastering the eight sample tricks, you may want to try 16 at a time. All sixteen tubes in the module can be filled simultaneously by capillary action. After centering the samples the two rows of eight tubes can then be staggered off from each other by pressing the tubes down on a bench top. The bottom of the first row of eight tubes, and the top row of the second row of eight can then be sealed one at a time by passing through the flame. The staggered rows can then be switched and the remaining two ends can be sealed. After the reaction is done the ends can be scored as in the eight sample example.

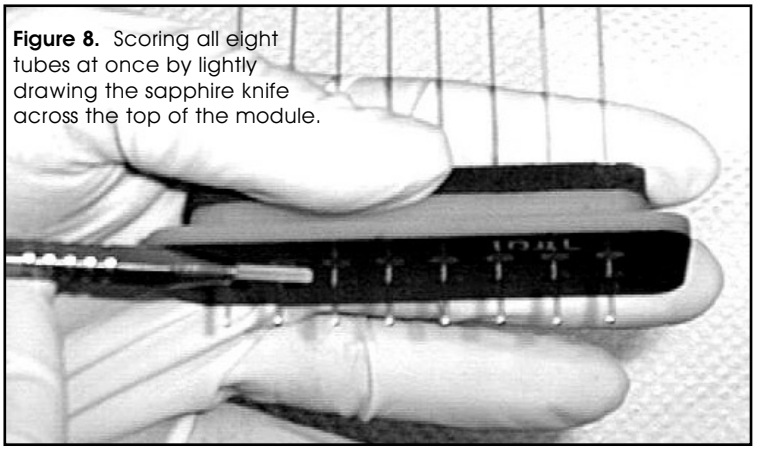

Figure 8. Scoring all eight tubes at once by lightly drawing the sapphire knife across the top of the module.

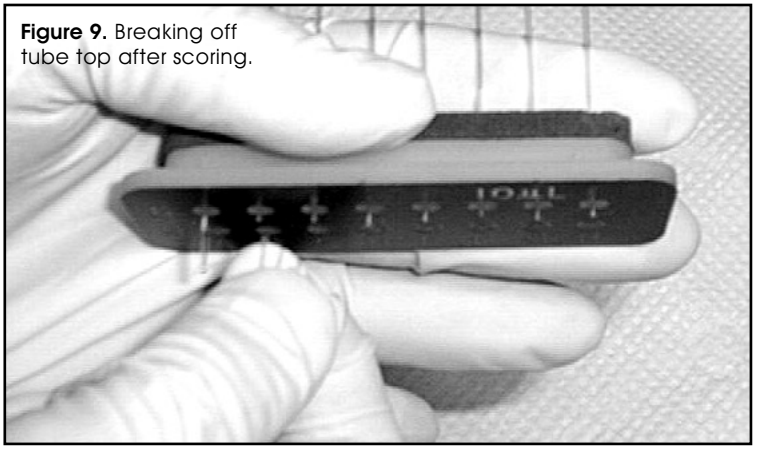

Figure 9. Breaking off tube top after scoring.

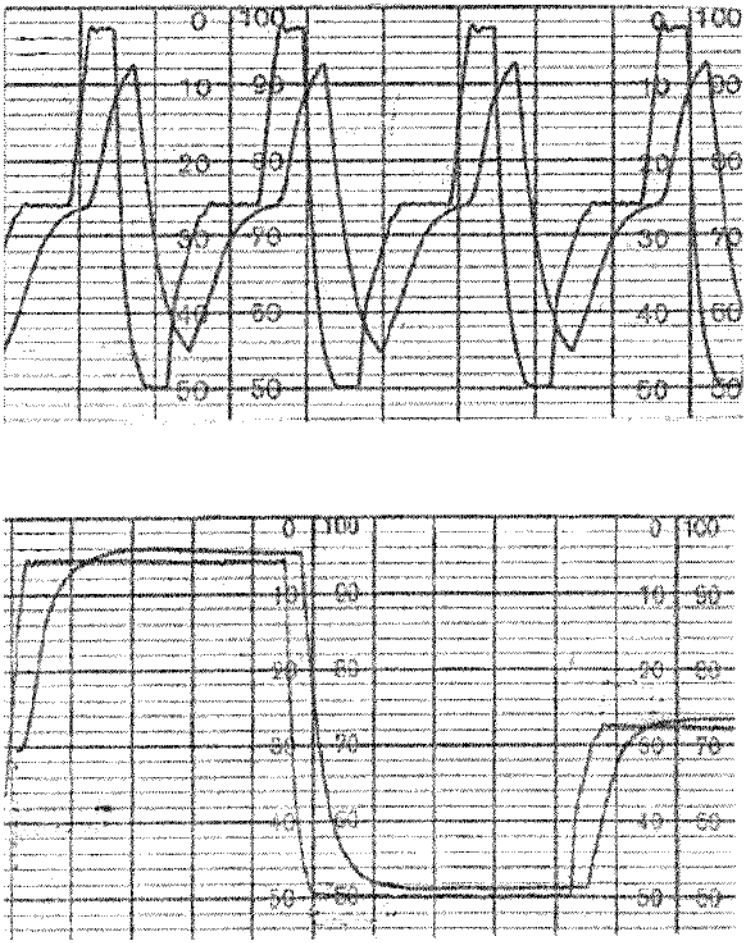

Figure 2. Temperature traces of the hold method (2B) versus the over heat and under heat method (2A). Traces are of air temperature and actual sample temperature. Notice how the sample temperature always lags behind the air temperature, and how the over/under heat method brings the sample to temperature more quickly.

Use of Thin Walled Microcentrifuge Tubes with the RapidCycler

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The development of thin walled micro test tubes makes it possible to combine the speed of the air cycling with the convenience of that "universal vessel" of molecular biology, the microcentrifuge tube. While the Rapidcycler was developed for use with glass capillaries, it provides excellent results with thin walled microcentrifuge tubes. Using modified sample modules, the Rapidcycler can hold up to 48 micro test tubes Figure 1 shows that all 48 positions give a clean, bright, 500 bp product in a DNA amplification from Human genomic DNA.

Thin walled micro test tubes have many advantages over capillary tubes. First, handling of the sample tube is much simpler; reactions can be made up in the micro test tube, no heat sealing is required, concern about breaking the tubes is eliminated. Second, there is no need to adjust buffers or protocols. The buffers that manufacturers provide with their thermostable polymerases work in these tubes without modification. Published protocols developed in heat block instruments seem to transfer more readily to the Rapidcycler when micro test tubes are used.

The thermal properties of thin walled microcentrifuge tubes are much better than their thick walled ancestors, but they are still no match for a capillary tube. Using thin walled microcentrifuge tubes requires a sacrifice in speed and in sample temperature uniformity. A 10 μl reaction that would take 15 minutes in a capillary tube, takes 35 minutes in a thin walled microcentrifuge tube, a 50 μl reaction that would take 20 minutes in a capillary, takes 50 minutes in a microcentrifuge tube.

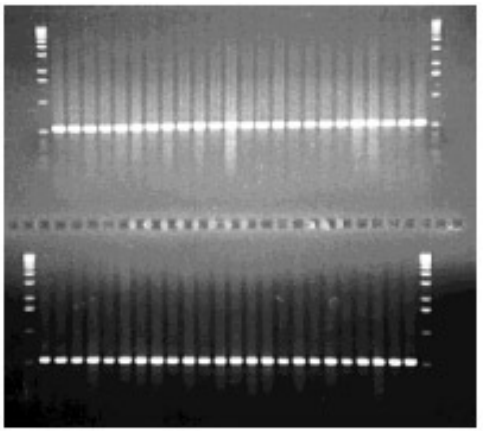

Figure 1. Amplification of a 500 bp target from human genomic DNA in all 48 sample positions of the Air Thermo-Cycler. Reactions volume was 50 μl, no oil overlay. Reactions contained Idaho Technology medium buffer, 200 μM each dNTP, 5 μM each primer (RS/KM), 50 ng human genomic DNA. Cycling parameters were 96° for 30 seconds, then 30 cycles of 96° for 30 seconds, 55° for 30 seconds, 75° for 20 seconds.

Because the Rapidcycler was developed for capillary tubes the temperature values that you program into the machine, and the temperatures displayed during cycling, reflect what the temperature would be in a 10 μl capillary. When using microcentrifuge tubes you must modify the program parameters to compensate for the thermal differences between capillaries and microcentrifuge tubes.

Thin Walled Microcentrifuge Tube Cycling protocols for the Rapidcycler

There are two possible approaches when using microcentrifuge tubes. You can set the machine to the temperature you want, and wait for the microcentrifuge tube to get to that temperature (Figure 2B) This is what the slower heat block cyclers do). This method is slow, but it assures you that no part of your sample is ever over the target temperature. A faster approach is to overheat and under heat the air. This brings the sample to temperature more quickly (Figure 2A). The faster heat block instruments do this), but some parts of your sample may be slightly above or below the target temperatures.

I have had good success with the faster overheat and under heat approach. The following protocols have been successful with a variety of primers and DNA sources. If you prefer the sit and wait approach 10 μl samples require 40 second holds at denaturation and annealing, 50 μl samples 60 second holds at denaturation and annealing. Elongation requires 25 nucleotides per second plus about 15 seconds.

10 μl Reactions

Predenature: 98°C for 10 seconds

Cycle:	Denature	98°C for 10 seconds
	Anneal	40°C to 60°C for 10 seconds (as appropriate for your primers)
	Extend	74°C for 25 nucleotides per seconds

50 μl Reactions

Predenature: 96°C for 30 seconds

Cycle:	Denature	96°C for 30 seconds
	Anneal	40°C to 60°C for 30 seconds (as appropriate for your primers)
	Extend	74°C for 25 nucleotides per seconds

Optimization of Reactions in Thin Walled Microcentrifuge Tubes

The same optimization protocol that has been recommended in capillaries (Optimizing Rapid Cycle DNA Amplification Reactions, Rapid Cyclist 1:1-5,1992) has provided excellent results in thin walled microcentrifuge tubes.

Optimal reaction conditions are found by running amplifications at 40°C, 50°C and 60°C with 2 mM, 3 mM and 4 mM MgCl2 at each temperature. This allows you to test 9 different stringencies, while only requiring you make up three different reaction mixes.

I have used this optimization protocol successfully with Idaho Technology buffers (low, medium and high MgCl buffers), Promega 10X Taq buffer and Stratagene 10X Pfu buffer.

Are Mineral Oil Overlays Required?

The thin walled microcentrifuge tube holders for the Rapidcycler put the entire tube inside the reaction chamber. This keeps the whole tube at the same temperature and thus reduces condensation. A small amount of condensation occurs on the leeward side of the tubes, but I have not found this to be a practical problem, even for 10 μl reactions. While a little mineral oil does stop this condensation, in general, oil is not needed for 10 μl reactions.

50 μl reactions show minimal condensation, but will occasionally pop open during reactions if no oil is used. The frequency with which this occurs seems to vary with reaction buffer and with tube manufacturer, so you may wish to experiment with your particular combination.

Real Versus Set Temperatures

The actual sample annealing temperature may not be important to you if you optimize the reaction experimentally as recommended above. If you do need a particular annealing temperature, the value you should set can be calculated using the equations in figure 4. I have provided graphs for 10 μl reactions with a 10 second hold (Figure 4A) and for 50 μl reactions with a 30 second hold (Figure 4B).

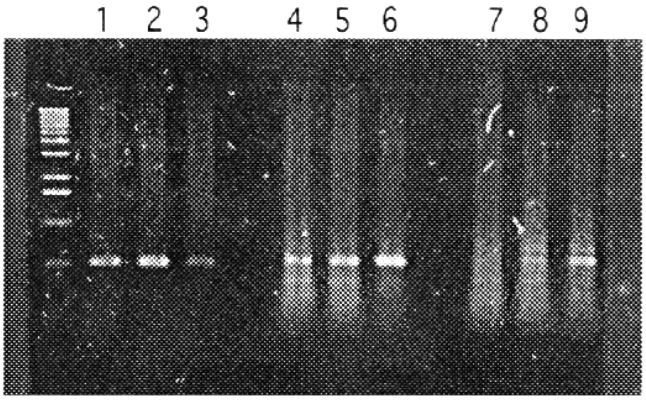

Figure 3. Optimization of RS/KM primer pair in microfuge tubes. Lanes 1-3: 60°C annealing, 4, 3 and 2 mM MgCl. Lanes 4-6: 50°C annealing, 4, 3 and 2 mM MgCl. Lanes 7-9: 40°C annealing, 4,3 and 2 mM MgCl. 10 μl reaction volume, no oil, 10 sec. holds at annealing and denaturation.

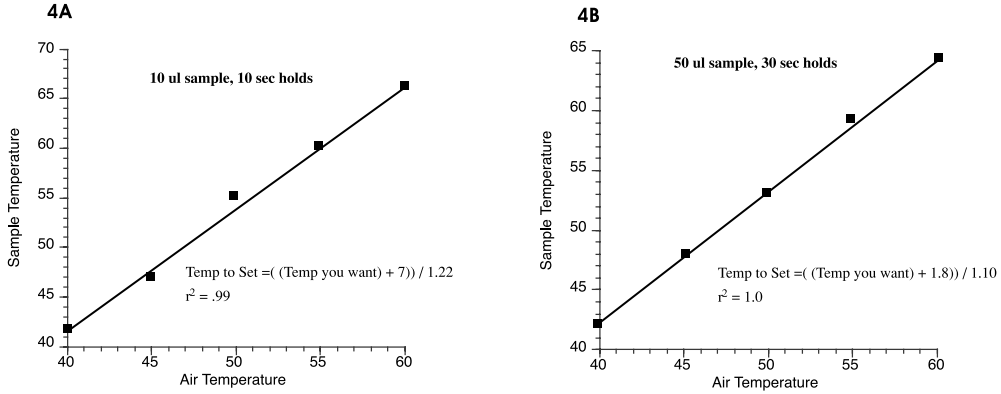

Figure 4. Linear relationship between the temperature programmed into the air cycler and the actual sample temperature for thin walled capillary tubes. 4A: 10 μl samples, 10 second holds, no oil overlay. 4B: 50 μl samples, 30 second holds, no oil overlay.

Direct Sequencing of Long PCR Products

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Introduction

Vitamin B12 is an essential cofactor of many non-photosynthetic eukaryotes. It is synthesized by prokaryotes and archebacteria both aerobically and anaerobically. In Salmonella typhimurium the anaerobic pathway is dependent on at least 30 genes. Several of these genes also occur in Escherichia coli, allowing synthesis from intermediates.

In spite of the fact that over 1% of the Salmonella genome is dedicated to B12 synthesis, cells unable to make the cofactor do well anaerobically under laboratory conditions. Only a few B12 dependent pathways are known and none seem essential. For example, the eut enzymes enable growth on ethanolamine as a source of carbon or nitrogen; the pdu regulon allows utilization of propanediol as a carbon source; and the MetH protein provides an alternate route for the final step in methionine biosynthesis.

Knowing the sequence of the eut operon forms a fundamental part of our strategy in characterizing the synthesis and importance of B12 in Salmonella. I have not been able to clone eut using standard techniques, suggesting that minor variations in its expression levels may have dramatic effects on the well-being of the cell. Instead, I have chosen to amplify portions of the operon from the genome and to sequence these PCR products directly.

Sequencing Strategy

Direct PCR sequencing has the advantage of blending Taq polymerase-induced errors into the background. However, linearized double-stranded template yields short, dirty "reads" with many premature stops. The technique is usually avoided in favor of sequencing cloned amplified DNA. Such inserts often contain polymerization mutations. Two and sometimes three independent clones must be sequenced to determine the primary structure with confidence. This, together with the overhead of plasmid preparation, increases the time required.

Recently, a fast and efficient method for DNA strand separation based on magnetic bead technology became commercially available (Dynal; 5 Delaware Drive, Lake Success, NY 11042; 800 638-9416). This allows exceptionally clean direct PCR sequencing using single-stranded templates. In addition, by optimizing for long amplification products, less time is spent preparing DNA.

Mispriming & Parasites

I routinely amplify product in the range 3-5 Kb. A major problem when generating molecules of this size is the tendency of non-specific smaller products to deplete reactants. Such parasites arise through false priming events and become favored, as efficiency per cycle is inversely correlated with product size. The short dwell times used by the Air Thermo-Cycler during annealing discourage false priming. Nevertheless, single primer controls should always be run to verify that a given product is dependent on both primers.

I eliminate primer dimer formation and false priming prior to the first denaturation step by including TaqStart antibody (Clonetech, Catalog #5400-x; 4030 Fabian Way, Palo Alto, CA 94303-4607; 800 662-2566). This temporarily inactivates the polymerase, which reactivates as increasing temperature denatures the antibody. To use the reagent, combine 1 volume Taq polymerase (5 units/ml), 1 volume of TaqStart (7 uM) and 10.5 volumes of enzyme dilution buffer (2.5 mg/ml bovine serum albumin [BSA] in 10 mM Tris pH 8.3). This is then used as normal Taq polymerase stock at 0.4 units/ul.

Primer Design

Best results always correlate with well designed primers (20-30 nucleotides with approximately 50% G+C content; no 3' terminal complementarities; no internal palindromes; no runs of G or C near 3' end). Primers should be "balanced " in the sense that overall lengths and compositions are about the same. I often include 5' tails for special purposes and find little, if any detrimental effect if the 3' end is at least 20 bases long. Trailing sequences can be amazingly long.

Reamplification

I usually use reamplified template DNA for sequencing. I first prepare a starter by amplification of genomic DNA (either purified, crude or encapsulated in cells). Subsequent template preparations are reamplified from a 1:100 dilution of the starter. This is especially important when genomic DNA (prepared according to Ausubel, F.A. et al. (eds), 1990, Current Protocols in Molecular Biology, Greene Publishing and Wiley-Interscience, New York, pp. 2.4.1 - 2.4.2) is used in the primary reaction.

I have also had excellent success using cells scraped form a plate in place of genomic DNA. In this case, I either simply touch the cells directly to the reaction mix, or resuspend them in 50 uL TE (10 mM Tris, pH 8.3; 1 mM EDTA), heat 2' at 95°, spin down and use the supernatant, diluted zero to 100-fold. The remainder can be frozen for future use.

More Tricks for Large Products

Purified genomic DNA should never be preboiled. Too many nicks are introduced and long products are more difficult to amplify. Preceding a primary genomic amplification with a 30 sec hold at 94° and following PCR with a 5 min hold at 72° improves yield. When characterizing a new primer pair, I always optimize according to the simple "3 x 3" scheme of Rasmussen (Rapid Cyclist vol .1, no. 1, 1-5, 1992). This takes only a couple of hours and pays great dividends.

PCR Reaction for a Typical Analytical Amplification

Use 10 - 15 uL of the following in a single capillary. For a preparative run, scale by 5 and load into 6 capillaries.

A Typical Amplification Program

For products longer than 1 kb, assume an elongation rate of 20 bases/sec for the elongation time. The annealing temperature can vary from 40° to 65°, and is determined empirically in a "3 x 3" optimization. For products longer than 3 kb, a denaturation time of 5 sec frequently improves the yield. If primers are poorly balanced or imperfectly match their sites, a ramp constant "S" of 6 will sometimes help.

For single product preparative runs, use Wizard PCR Prep (Promega; 2800 Woods Hollow Road, Madison, WI 53711-5399; 800 356-9526) for rapid cleanup. Elute with H2O and store at -20°. When more than one band is present, excise the correct one and purify with GeneClean (Bio 101; PO Box 2284, La Jolla, CA 92038-2284; 800 424-6101). Again, elute with H2O and store frozen.

8 uL water
2 uL 10X PCR Reaction Buffer
2 uL 4 dNTP's, each at 2 mM
2 uL primer 1 at 5 uM
2 uL primer 2 at 5 uM
2 uL DNA, diluted 1:100
2 ul Taq + TaqStart (see above)

Total = 20 uL

10X PCR Reaction Buffer: 500 mM Tris, pH 8.3, 2.5 mg/ml BSA, 5% Ficoll and 10 mM Cresol Red. MgCl2 added to 10, 20 or 30 mM.

1. Hold 30 sec at 94°

2. Cycle parameters
(as they occur on Air Thermo-Cycler screen):

D94	A50	E72	C30	S9
d0	a0	e1'0"		

3. Hold 5 min at 72°

Magnetic Strand Separation & Purification of Both DNA Strands

There are several methods for preparing single-stranded DNA from PCR products, such as asymmetric amplification, exonuclease digestion and magnetic separation. I prefer the last as it is fast and the magnetic beads lend themselves to a number of other techniques. It requires that one and only one of the two PCR primers be biotinylated. Generally, there is little additional cost for this service.

DynaBeads or "Beads" - DynaBeads M-280 Dynal

"Acetate Solution" - Potassium acetate, pH 4.8
(5 M acetate, 3 M K)
294 g KCH3CO2
115 ml HCH3CO2
H2O to 1 liter (no need to check pH)

HBWB - high-salt "Binding & Wash Buffer"
10 mM Tris, pH 7.5
1 mM Na2 EDTA
3 M NaCl

TE
10 mM Tris, pH 7.4
1 mM Na2EDTA

Glycogen Solution - 20 mg/ml
Boehringer Mannheim, Catalog # 901-393

The protocol yields two DNA strands, separated and purified in a manner suitable for direct sequencing. The "W" strand is biotinylated and the "C" is its complement. Preparation of the "W" strand is a modification of the Dynal protocol which conserves beads with no apparent sacrifice in yield. The method for preparing "C" strand is new . Typically, 5 uL of either strand preparation is used in a single sequencing reaction.

1. Wash the beads:
vortex bead stock
add 10 uL beads to 20 uL HBWB in tube "W" vortex
magnetically separate, discard supernatant
resuspend beads in 30 uL HBWB

2. Bind DNA
add 5 -20 uL PCR product to bead pellet mix by flicking
incubate 15 min RT with occasional resuspension
magnetically separate, discard supernatant
resuspend beads in 40 uL HBWB
magnetically separate, discard supernatant

3. Denature DNA:
load tube "C" with 30 uL 3 M acetate solution
resuspend beads in 15 uL 0.2 N NaOH
incubate 15 min x RT with occasional resuspension
magnetically separate, add supernatant to tube "C"

4. More strand separation - repeat 3x
resuspend beads in 50 uL 0.2 N NaOH
magnetically separate, add supernatant to tube "C"
resuspend beads in 40 uL HBWB
magnetically separate, discard supernatant
resuspend beads in 50 uL TE
magnetically separate, discard supernatant

5. "W" strand cleanup
resuspend beads in 25 uL H2O
store at -20 °

6. "C" strand cleanup
add 1 uL glycogen to tube "C" and mix well
add 500 uL 95% ethanol - mix well
30 min x ice
microfuge 10 min
wash once with 400 uL 70% ethanol
draw off ethanol in vacuum jar; avoid fully drying pellet
resuspend in 25 uL H2O
store at -20°

Sequencing Reactions

This is a synopsis of my current sequencing methods. I use "Sequenase Version 2 with Pyrophosphatase" (USB, Catalog # 70175) and the "Manganese Reagent" Sequenase Kit (USB, Catalog # 70130), which employs extensions and slight modification of commonly used dideoxy technology.

This protocol will yield sufficient material for fully redundant loading of 1.5 uL samples on two gels. "Mix" quantities are given for a single primer/template pair. Multiply amounts by the number of such sequences plus one. Wherever temperature blocks are called for, each cavity used is filled with water.

1. Annealing - combine in a small Eppendorf vial:
7 uL DNA at 0.1 - 1 ug/mL
1 uL primer at 5 uM
1 uL 10X MOPS: included in kit
1 uL 10X Mn Solution: included in kit
Total = 10 uL
2 min at 65° - temp. block
30 min at 42° - small oven or temp. block

2. Extension - add:
5.5 uL EMix
Total = 15.5 uL

0 -5 min at room temperature (Shorter times allow reads closer to template. 5 min is the norm.)

During this time, add 2.5 uL termination mixes to a preheated (37 ° C) mini tray. Distribute each mix to its own column, filling as many wells as there are reactions. Each row corresponds to one primer/template pair and can be color coded on the reverse side of the tray.

"Manganese Reagent" Kit
USB, "Mn2+ Reagent Kit for DNA Sequencing", catalog # 0130

"Enzymes"
USB, "Sequenase Version 2 with Pyrophosphatase", catalog # 70175

EMix
1.6 uL H2O
1.0 uL 0.1 M DTT: included in kit
0.4 uL Sequence Labeling Mix (incl. in kit), diluted 1:5 with H2O
0.5 uL labeled dATP (32P, 33P, or 35S)
2.0 uL enzymes
Total = 5.5 uL

"Mini Trays"
InterMountain Scientific, 1610 S. Main,Suite H, Bountiful, UT 84010, (801) 298-7884;
"Micro Well Mini Tray", cat. #438733.

TdT Mix
3.4 uL H2O
0.3 uL 4 dNTP's, each at 2 mM
(Pharmacia, "Ultrapure dNTP Set", cat. # 272035)
0.3 uL terminal deoxynucleotidyl transferase @20 u/uL (BRL, cat. # 8008SB)

Total = 4.0 uL

3. Termination - add to each well of appropriate row:
3.5 uL extension reaction
Total in each well = 6 uL
10 min at 42° - small oven or temp. block

4. TdT Extension - optional; used to resolve premature stops.
Add to each well:
1 uL TdT Mix
Total in each well = 7 uL

5. Electrophoresis: place tray in vacuum jar, evacuate 15 min.
add to each well:
4 uL stop solution: included in kit
Total = ~6 uL
3 min at 75° - place covered tray under slug of 75° temperature block load 1.5 ul sample per well

Rapid PCR Fingerprinting of Bacterial Genomes with REP Primers in Capillary Tubes Using the Air Thermo-Cycler.

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Introduction

DNA fingerprinting of genomes using PCR methods has been intensively used during the last years to characterize genomic diversity and to search for specific DNA markers (1-9). Different DNA banding patterns have been obtained using primers with length ranging from 8 to 25 nucleotides containing either arbitrary (2, 3, 5, 8, 9) or specific sequences (4, 6). In particular, the use of bacterial Repetitive Extragenic Palindromic sequences (REP) and Enterobacterial Repetitive Intergeneric Consensus (ERIC) have been proved to be practical and appropriate to fingerprint a number of different bacterial species (4, 6). Although REP and ERIC primers do not lead to amplification patterns as complex as those obtained with the random primed DAF (1), visualization of amplified DNA fragments can be easily achieved by agarose gel electrophoresis/ethidium bromide staining. Thus, classic REP and ERIC PCR amplifications may be efficiently used to characterize Gram-negative and Gram-positive bacterial genomes in a 10 hours experimental procedure. Here, we report a simple, rapid and reproducible protocol to perform REP DNA amplifications in 2 h using capillary tubes in air thermo-cyclers.

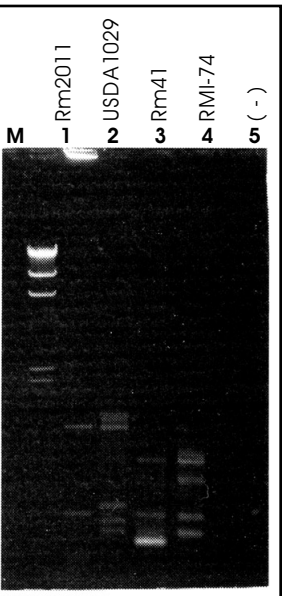

Figure 1. amplification patterns of four distinct R. meliloti strains using whole bacteria as the source of DNA in the Air Thermo-Cycler (ATC). Lane 1, Rm 2011; lane 2, Rm 1029; lane 3, Rm 41; lane 4, Rm 1-74 and lane 5, control without template. Molecular weight marker: lambda/HindIII.

Methods

The amplification mixture composition was as follows: 50 mM Tris, pH 8.3; 500 μg/ml BSA; 3mM MgCl2 (1x high magnesium buffer-Idaho Tech.); 200 μM dNTPs; 1U Taq DNA polymerase (Promega Corp.); 15 μM of each REP primers; and 1 μl of bacterial cells from a fresh isolated colony as the source of DNA template in a final volume of 25 μl. The cycling conditions were as follows: 95°C for 5 min; 30 cycles at 94°C for 10 sec., 40°C for 10 sec. and 65°C for 2 min.; 1 final step at 65°C for 4 min. All PCR amplifications were carried out using an Idaho 1605 Air Thermo-Cycler (ATC) in 25 μl capillary tubes. Ten μl of each sample were electrophoresed in 0.8-1.5% agarose gels added with 150 μg/l ethidium bromide.

Results

We set up the experimental conditions using 4 strains of the soil bacteria Rhizobium meliloti, Rm 2011 (Dr. J. Dénarie, Toulouse, France), Rm USDA 1029, Rm 41 (Dr. A. Kondorosi, Paris, France) and Rm 1-74 (Dr. A. Pühler, Bielefeld, Germany). The rapid transfer of heat in the capillary sample container allowed the shortness of amplification cycles from the required 600 sec. in metal block thermo-cyclers (MBTC), to 140 sec. in the ATC. Thus, the whole protocol could be carried out in less than 2h using either 1 μl of intact bacterial cells or purified DNA as template. The obtained DNA amplification patterns were all different among the strains (Fig 1) and allowed us to identify any of them in subsequent screenings. To validate this protocol designed for the ATC, we compared REP amplifications in MBTC with those obtained with the conditions here described. Figure 2 shows that DNA amplification products were comparable and tended to parallel each other when BSA was present in the reaction indi-

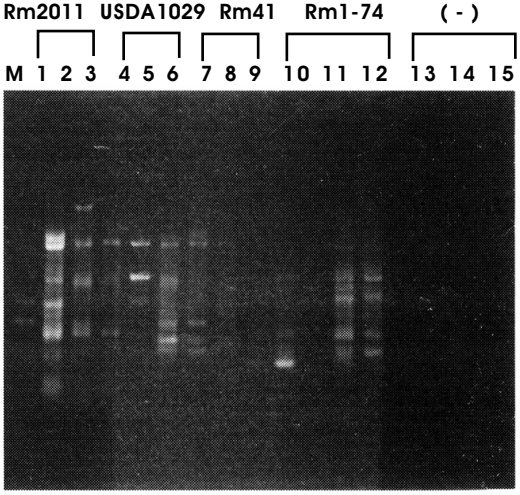

Figure 2. REP amplification patterns using metal block (MBTC) and capilary air thermo cyclers (ATC). Lane 1, Rm 2011 MBTC: lane 2 Rm 2011 MBTC/BSA; lane 3 RM 201 ATC/BSA; lane 4, Rm 1029 MBTC; lane 5 Rm 1029 MBTC/BSA; lane 6 Rm 1029 ATC/BSA; lane 7, Rm 41 MBTC; lane 8 Rm 41 MBTC/BSA; lane 9 Rm 41 ATC/BSA; lane 10, Rm 1-74 MBTC; lane 11 Rm 1-74 MBTC/BSA; lane 12 Rm 1-74 ATC/BSA; lanes 13, 14 and 15, controls without template for MBTC, MBTC/BSA and ATC/BSA, respectively. Molecular weight marker: pUC 9/HaeIII.

cating that BSA has additional effects other than the enzyme protection in the capillary system. Moreover, the presence of BSA allowed the amplification of DNA from the strain Rm 1-74. Total DNA preparation from these rhizobia was systematically contaminated by a yet unknown pigment which strongly inhibited conventional PCR amplifications.

The system here described for the characterization of bacterial genomes is fast, reproducible, strain specific, and suitable for amplification of samples containing natural PCR inhibitors not removed during the cell heating or template DNA preparations. The high number of individual isolates in strain collections represent a limiting factor during the selection of molecular characterization methods. The possibility to obtain reproducible DNA fingerprints in a short time represents a valuable alternative for programs of germoplasm characterization.

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Comparison of Different PCR Cycler Machines for Rapid and Sensitive Detection of Pathogens

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Abstract

In order to find a rapid PCR method to detect bacterial pathogens, we compared different PCR cycler machines. The total cycle time to complete the PCR amplifications were: 5 hours in the BioOven (BioTherm Co.) PCR cycler; 1.5 hours in the MiniCycler (MJ Research, Inc.), 2.5 hours in the Perkin Elmer Cycler 480; 3 hours in the PHC-2 Cycler (Techne Inc.), but only 30 minutes in the 1605 Air Thermo-Cycler (Idaho Technology). Using the 1605 Air Thermo-Cycler with our rapid and simple sample preparation method, the total detection and identification time was 1.5 - 2 hours including 30 minutes for the PCR cycles and 40 minutes for electrophoresis. Eight bacterial species have been tested with this protocol in the 1605 Air Thermo-Cycler, all of them gave good results.

Introduction

Rapid and specific methods for detection and identification of pathogens are essential for food safety and clinical diagnosis of human and animal diseases. Antibody-based test methods are the most often used technique. However, PCR based methods should be faster and more specific. A traditional PCR protocol takes about 5 hours in Perkin Elmer Cycler 480. Previously, we reported a protocol for the PHC-2 cycler machine (Techne Inc., Princeton, NJ), which shortened detection time to 3 hours (2,3,4,5). In this article, we report the results of comparison of different PCR cycler machines for the rapid and sensitive detection of pathogens.

Materials and Methods

The bacterial cells were collected from liquid cultures by centrifugation. The cells were washed twice with phosphate buffered saline (PBS), distilled water (dH2O), and resuspended in dH2O at 107 cells per μl. Just before the PCR assay, the samples were diluted to the desired cell concentration of 105 CFU in 50 to 100 μl of 1% Triton X-100. The cells were then heated at 100°C for 5 minutes, immediately cooled in ice water, and tested by PCR without isolation of the DNA. Two μl of above sample were added to 23 μl of a PCR mixture.

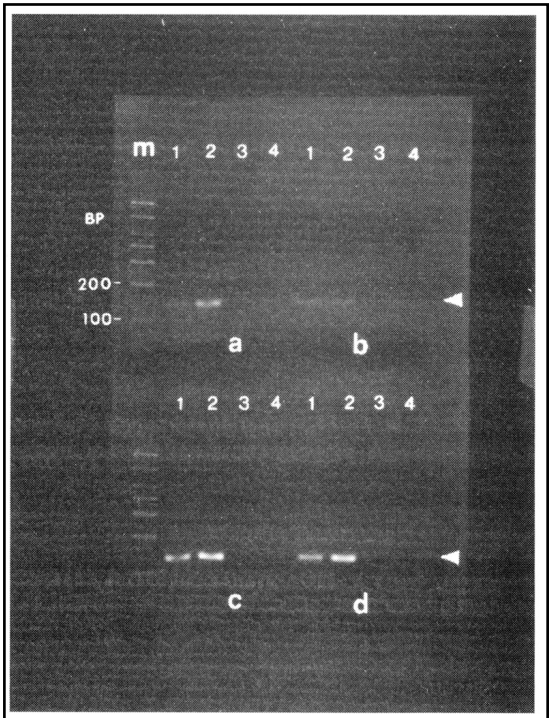

Figure 1. PCR results in different thermal cycler machines. PCR primers are specific for Mycoplasma gallisepticum (unpublished data). The PCR product is 138 base pair DNA fragment. 3% agarose gel was used for the electrophoresis. Lane m: molecular size marker. Lane 1: Mycoplasma gallisepticum strain K23. Lane 2: M. gallisepticum strain K730. Lane 3: Mycoplasma synoviae strain FMT. Lane 4: H2O for control. Panel a: The MiniCycler was used with a total cycle time of 1.5 hours. Panel b: The BioOven was used with a total cycle time of 5 hours. Panel c: The PHC-2 Cycler was used with a total cycle time of 2.8 hours. Panel d: The 1605 Air Thermo-Cycler was used with a total cycle time of 30 minutes.

For the BioOven, MiniCycler, Perkin Elmer Cycle 480, and PHC-2, the PCR mixture contained 50 mM Tris-HCl (pH 8.5), 50 mM NaCl, 1 mM MgCl2, and 2 mM dithiothreitol, 0.1% Triton X-100, 0.22 mM of each dATP, dTTP, dCTP, dGTP, 0.28 μM of each primer, and 0.9 U of Taq polymerase (Promega, Madison, WI). The program consisted of one cycle of 3 minutes at 94°C, then 40 cycles of 20 seconds at 94°C, 20 seconds at 55°C, 40 seconds at 72°C, and finally one cycle of 3 minutes at 72°C. For the 1605 Air Thermo-Cycler (Idaho Technology), the PCR mixture contained 50 mM Tris-HCl (pH 8.5), 20 mM KCl, 3 mM MgCl2, 0.05% bovine serum albumin (BSA, No. A-4378, SIGMA, Chemical Co., St. Louis, MO), 0.25 mM of each dATP, dTTP, dCTP, dGTP, 0.25 μM of each primer, and 0.9 U of Taq polymerase. The program consisted of one cycle of 15 seconds at 94°C, then 30 cycles of (5 seconds at 94°C, 5 seconds at 55°C, 15 seconds at 74°C), and finally one cycle of 2 minutes at 74°C, 2 seconds at 45°C. The fastest transition speed (S-9 on the 1605 Air Thermo-Cycler and 2.0 on the Rapidcycler) was chosen.

For the MiniCycler, Perkin Elmer Cycler 480, and PHC-2, the PCR reaction has to be covered with 50 µl of mineral oil, but for the BioOven and the Air Thermo-Cycler, no oil was needed.

The PCR products (6 - 10 µl each) were separated by gel electrophoresis in a 2 - 3% agarose gel containing ethidium bromide (1 µg/ml).

Results and Discussion

Figure 1 shows the PCR results using the four different PCR cycler machines. Only the two Mycoplasma gallisepticum strains gave 138 bp PCR products (lanes 1 and 2). M. synoviae and H2O (lanes 3 and 4) were negative. The most intense bands were in panel d (Air Thermo-Cycler) and panel c (PHC-2) compared with less intense bands in panel a (MiniCycler) and panel b (BioOven).

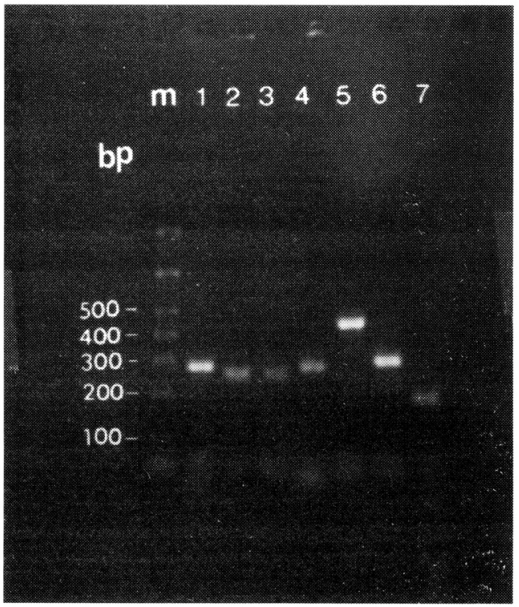

Figure 2. PCR results for different bacterial species in the 1605 Air Thermo-Cycler. 105 cells of each bacterial species was used for this test. 2% agarose gel was used for the electrophoresis. Lane m: molecular size marker. Lane 1: Clostridium perfringens. The product is 280 bp. Lane 2: C. leptum. The product is 257 bp. Lane 3: C. clostridiiforme. The product is 255 bp. Lane 4: Bacteroides distanonis. The product is 273 bp. Lane5: B. thetaiotaomicron. The product is 423 bp. Lane 6: B. vulgatus. The product is 287 bp. Lane 7: Bifidoacterium sp. The product is 190 bp.

Cycle times were 50 seconds in Air Thermo-Cycler, 2 minutes 5 seconds in MiniCycler, 4 minutes in PHC-2, and 7 minutes 10 seconds in BioOven. The total cycle times were 30 minutes in 1605 Air Thermo-Cycler, 1.5 hours in the MiniCycler, 2.5 hours in the Perkin Elmer Cycler 480 (data not shown), 2.8 hours in the PHC-2, and 5 hours in the BioOven.

High concentrations of BSA were essential for the PCR assay in the 1605 Air Thermo-Cycler, BSA is thought to prevent denaturation of the Taq polymerase on the large internal surface area of the glass capillary tubes (6).

We have already used this protocol and the 1605 Air Thermo-Cycler to detect many other bacteria, such as Clostridium perfringens, C. Clostridiiforme, C. leptum, Bacteroides distasonis, B. thetaiotaomicron, B. vulgatus, and Bifidobacterium. Figure 2 shows the results. Different primers were used for different bacterial species, but the same program and same 1605 Air Thermo-Cycler

were used. All of them gave good results.

We directly used the bacterial cells lysed in 1% Triton X-100 for DNA template of the PCR, so the final concentration of the Triton X-100 in the reaction tubes was about 0.1%. This concentration of Triton X-100 did not interfere with the PCR assay (data not shown).

In general, the optimal annealing temperature used in the 1605 Air Thermo Cycler was 5°C lower than the other machines and gave better sensitivity and better specificity (Figure 3).

In conclusion, the 1605 Air Thermo-Cycler is the fastest and most sensitive PCR machine for the detection and identification of microbial pathogens.

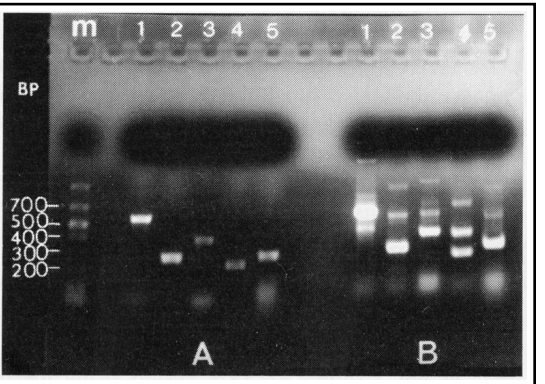

Figure 3. Comparison of the results of 5 PCR methods for 5 different bacterial species in the 1605 Air Thermo-Cycler (Idaho Technology) and the Cycler 480 (Perkin Elmer). Panel A: the 1605 Air Thermo-Cycler (25 μl tube, one cycle of 94°C for 15 sec, 30 cycles of 94°C for 3 sec, 50°C for 10 sec, 74°C for 15 sec and finally one cycle of 74°C for 2 min and 45°C for 2 sec). Panel B: the Cycler 480 (one cycle of 3 min at 95°C , then 35 cycles of 20 sec at 94°C, 20 sec at 55°C, 40 sec at 72 °C. and finally one cycle of 3 min at 72°C and 2 sec at 20°C). Line m, molecular marker. Lane 1: Escherichia coli, the primer set is CACACGCTGACGCTGACCA; with GACCTCGGTTTAGTTCACAGA, PCR product is 585 bp. Lane 2: Eubacterium limosum, the primer set is GGCTTGCTGGACAAATACTG; with CTAGGCTCGTCAGAAGGATG, the PCR product is 274 bp. Lane 3: Vibrio vulnificus, the primer set is CTCACTGGGGCAGTGGCT; with CCAGCCGTTAACCGAACCA, the PCR product is 383 bp. Lane 4: Listeria monocytogenes, the primer set is CGGAGGTTCCGCAAAAGATG; with CCTCCAGAGTGATCGATGTT, the PCR product is 234 bp. Lane 5: Staphylococcus aureus, the primer set is GCGATTGATGGTGATACGGTT; with CAAGCCTTGACGAACTAAAGC, the product is 276 bp.

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New From Idaho Technology

Kirk Ririe
Idaho Technology Inc.

Introducing the 1002 Rapidcycler

In March of 1995, we began shipping a new version of our capillary based temperature cycling system, the Rapidcycler. This system offers numerous advantages over the previous model.

Improved Temperature Control.

The Rapidcycler is able to run a broader range of temperature cycle protocols including two-temperature cycling. It is also less likely to overshoot elongation temperatures.

The temperature ramp rate between the annealing and elongation temperatures is now entered in degrees per second and is linear within and between runs.

Quiet Operation

The actuator used to control air flow through the Rapidcycler is a soft shift solenoid as opposed to the AC solenoid used in the 1605. This offers two advantages. Besides being much quieter, the new actuator allows intermediate door settings hence variable airflow. This is in contrast to the solenoid in the 1605 which had just two settings, open and closed. The new actuator allows the control software to more effectively dampen the temperature oscillations that tend to occur when driving rapid temperature changes.

Improved Programming

The Rapidcycler user interface is a significant improvement over the 1605. The readout size has been increased so that everything is printed in clear English instead of abbreviations. The three user modes, Cycle, Hold and Link, are now accessible by a single button from the keypad.

Maneuvering around programming screens has been simplified by the addition of cursor keys. There are now 99 Cycle programs, 99 Hold programs, and 99 Link programs available. Many of them come preprogrammed for the more commonly used reaction profiles.

New Optimizer Kit

The Optimizer kit has been modified to allow more flexibility and to reduce waste. There are now four base buffers available ranging in Mg++ concentration from 10 mM to 40 mM . Either of two gel loading additives (Ficoll/tartrazine or sucrose/cresol red) can be added along with dNTPs and other reaction constituents into a master mix which will keep for months in a refrigerator.

Blazer mini-torch

We now ship a Blazer butane torch with the Rapidcycler. The torch is fast igniting, light-weight, and burns very hot. It is a good general purpose lab torch and is ideal for sealing glass capillaries. We recommend that everyone using glass capillaries keep one handy. They are available either directly from us or from some sporting-goods outlets.

Research in Progress at IT

Idaho Technology Inc., together with the University of Utah has received generous funding from the National Institutes of Health STTR program and from the University of Utah. This joint research project is an effort to develop a system to continuously monitor the progress of an amplification reaction. The use of capillary tubes lends itself to fluorescent analysis of reaction product during the course of a reaction. By combining a fluorimeter and thermal cycler into a single mechanism it is possible to essentially "watch" a reaction occur. It is hoped that this research will lead to extremely rapid detection systems as well as becoming a general purpose window into reaction mechanics.

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