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Creating a DNA Probe,Thermal Cycling with Degenerate Primers

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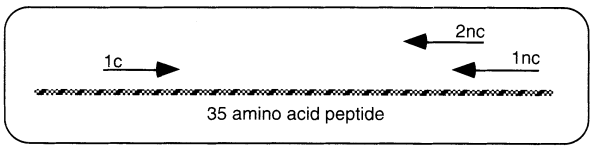
We are cloning the DNA from a structural protein in Xenopus leavis to further characterize it. A DNA probe was needed for Southerns, northerns and probing libraries for our gene of interest. The following is our procedure using the Air Thermo-Cycler to clone and amplify a fragment of DNA using degenerate primers. We found increased primer concentration as well as longer annealing times were beneficial in obtaining DNA products from degenerate primers.

Primer Design

The protein of interest was digested with endo-Asp-N to obtain protein fragments for amino acid sequencing. Of these, a 35 amino acid peptide was chosen to design degenerate primers for amplification of the peptide DNA. Coding (1c) and non-coding (1nc) primers were made from terminally located amino acids with minimal codon degeneracy. A third non-coding (2nc) primer was made internal to 1nc primer (see figure).



Each primer was 26 nucleotides long. All combinations of nucleotides at codon wobble positions were synthesized with the following exceptions: inosines were used for 4-fold degeneracy at the wobble position when appropriate

(according to Molecular Cloning, a Laboratory Manual. Sambrook et al., page 11.18); to accommodate a serine in 1c and a leucine in 1nc, primers had to be made in duplicate; for serine, the codons TCI and AGT/C were used; for leucine (1nc, 2nc), IAG and T/CAA were used. Each primer was synthesized with a GGC clamp and an EcoR1 site at the 5 ? terminus. The degeneracy of the 1c, 1nc, and 2nc primers were respectively 48 fold, 8 fold and 48 fold. The expected size of the product from the 1c and 1nc primers was 100 bp, and from the 1c and 2nc primers, 94 bp.

Reaction Mix

primers	7 μM working concentration; 1 μl each of 1c and 1nc
template	7.6 ng/μl Xenopus leavis oocyte cDNA; 1μl
[Mg2+]	30 mM; 1 μl
dNTP mixture	2 mM each dNTP; 1 μl
10 X buffer	500 mM tris pH 8.3, 2.5 mg/ ml BSA, 1 μl
enzyme	1 μl Taq polymerase diluted 1:12.5 in enzyme dilution buffer (10 mM tris pH 8.3, 2.5 mg/ml BSA)
water	to 10 μl total volume

Thermal Cycling Conditions

These conditions produced the expected 100 bp fragment in small amounts as visualized on a 4% Nusieve low melting temperature agarose gel. The band was cut from the gel (approximately 2X2X4 mm chunk) and used as a template in subsequent thermocycling reactions.

Each 10 ul reaction was done in heat sealed glass capillaries.

Initial hold -- 2 minutes 94°C

2 cycles	D: 94°C 0 sec,	A: 40°C 7 sec,	E: 74°C 5 sec
5 cycles	D: 94°C 0 sec,	A: 42°C 7 sec,	E: 74°C 5 sec
23 cycles	D: 94°C 0 sec,	A: 45°C 7 sec,	E: 74°C 5 sec

Confirmation of the 100 bp product

To confirm the accuracy of our 100 bp product, we attempted to amplify a smaller fragment using the 100 bp cycling product as a template with the internal non-coding primer (2nc) and the original coding primer (1c).

The 100 bp product isolated in agarose was heated at 100 °C until melted, and 500 µl TE was added. Two µl of this mixture were used as the template in a 10 µl reaction. One µl each of the 1c and 2nc primers was used, and the other parameters were as described above.

The thermal cycling reaction was run with an initial 2 minute denaturation at 94 ° C followed by 30 cycles: 0 sec at 94 °C (denaturation), 12 sec at 50 °C (annealing), 5 sec at 74 °C (elongation).

This reaction produced a 94 bp band as seen on a 4% Nusieve agarose gel indicating that the 100 bp fragment was the correct DNA sequence. A duplicate reaction to the one directly above was done with the 100 bp fragment as template and the original outside primers (1c and 1nc) to amplify the 100 bp fragment. One 10 µl reaction gave approximately 30 ng of product.

The 100 bp product was cloned directly into the pCRII® vector from the Invitrogen TA Cloning Kit. Subsequent DNA sequencing of this vector confirmed that this product coded for the original amino acid sequence and will be used as a probe for subsequent experiments.

Superior Quantitation of Rare mRNA's Using Rapid Cycling

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After a long period of skepticism, quantitative PCR is finally gaining acceptance in the molecular biology community. No one doubted that PCR could be quantitative in theory, but there was a general consensus that the efficiency of DNA amplification would be too sensitive to interference for practical quantitation. Small effects on the reaction's efficiency, it was argued, would destroy the quantitative value of PCR. Quantitation of mRNA added the additional complication of the reverse transcription step.

Despite these initial concerns, it has now been thoroughly demonstrated that the quantitative power of reverse transcriptase PCR (RT-PCR) is as good or better than the traditional methods of mRNA quantitation such as northern blot (1), dot blot (2), and in situ hybridization (3). Two recent papers from the John Weis lab report a sensitive RT-PCR assay using rapid air thermocycling (4,5). The Weis lab was trying to measure mRNA for the complement receptor Cr2, a rare mRNA in mouse spleens. They were unable to quantitate the message when they used slow heat block cyclers because of very low yields of product DNA and highly variable amounts of product. They switched to an Air Thermo-Cycler and solved both of these problems. The amount of DNA produced was at least 100 fold greater in the air cycler than in the heat block instrument and the variability problem disappeared (Figure 1).

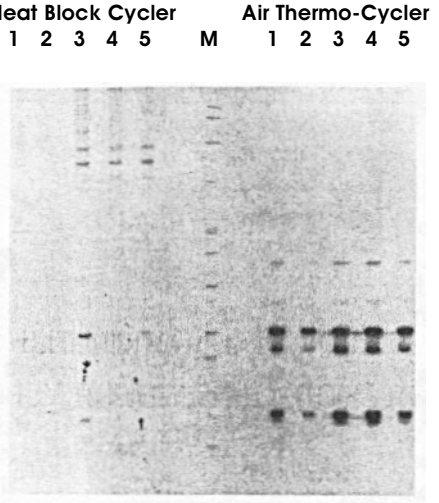

Figure 1. Comparison of RT-PCR products using heat block instrument and the Air Thermo-Cycler (ATC). Five different spleen cDNA samples were set up for PCR amplification and equally split between the standard heat block instrument (first 5 lanes) and the ATC (last 5 lanes) for the same number of cycles. Quantitation of these results (cutting the bands out of the gel and counting incorporated 32P-dCTP) indicated that there was at least 100 fold more product from the ATC than the heat block machine.

This short review will include some general considerations in quantitative PCR followed by the detailed Weis protocol.

The Linearity Problem

The amount of DNA produced in a PCR reaction is predicted by the well known equation:

$$y = x(E)^n$$

where **y** is the concentration of DNA produced by the amplification
x is the initial concentration of DNA
E is the efficiency of the reaction. For example, in a reaction where the amount of DNA is doubled every cycle, the efficiency is 2.
n is the number of amplification cycles

This equation can be linearized to:

$$\log(\mathbf{y}) = \mathbf{n}\log(\mathbf{E}) + \log(\mathbf{x})$$

The **y** intercept of this line gives the log of the starting concentration of DNA while the slope of the line gives the log of the efficiency of the reaction.

When the DNA concentration of an amplification is determined after varying numbers of cycles, the results fit quite nicely to the equation above during the early cycles. Efficiency is reduced during the later cycles of an amplification reaction (Figure 2). This is probably due to primers competing less effectively with template reannealing and a lower molar ratio of enzyme to product. The number of cycles after which these effects become important depends on the initial concentration of DNA.

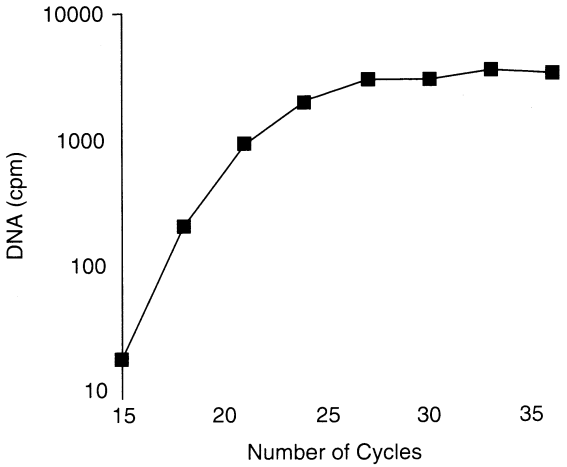

Figure 2. Amplification of mouse splenic cDNA with primers complimentary to the complement receptor Cr2. Eight identical samples were prepared with 250 ng of cDNA and removed sequentially every third cycle. After electrophoresis and autoradiography, bands were excised and quantitated by liquid scintillation counting (from Weis 1991).

When doing a quantitation experiment with the Air Thermo-Cycler, a typical experiment would include making up a large master mix, filling multiple capillaries from that master mix, and starting all the tubes at the same time. As the reaction goes on, tubes are pulled out at increasing numbers of cycles. The amount of DNA in each tube can be quantitated in various ways. The points that fall in the log-linear portion of the curve can be used to determine the amount of starting material and the efficiency of the reaction. For Figure 2, the efficiency of the reaction during the log-linear phase was about 1.7, which is typical for a real reaction.

The Quantitation and Detection Problem

The most common technique for detection and quantitation of DNA is radiolabeling with 32P. Amplified products can be labeled by incorporation of radiolabeled nucleotides or by end labeling one of the primers. End labeling tends to be more sensitive because a higher fraction of the product carries a label (6) , but labeling by incorporation is easier if you don't need the sensitivity. After the amplification, reactions are size separated by gel electrophoresis. The gels can then be directly quantitated by autoradiography using film or a PhosphorImager type system . The limited linear range of film (usually 3 orders of magnitude or less) makes this approach difficult. Phosphor Imager type systems are convenient and have extended linear ranges (5 to 9 orders of magnitude) but are very expensive. The Weis protocol uses labeling by incorporation of 32P-dCTP, location of the product by autoradiography, and quantitation by excision of the band and liquid scintillation counting.

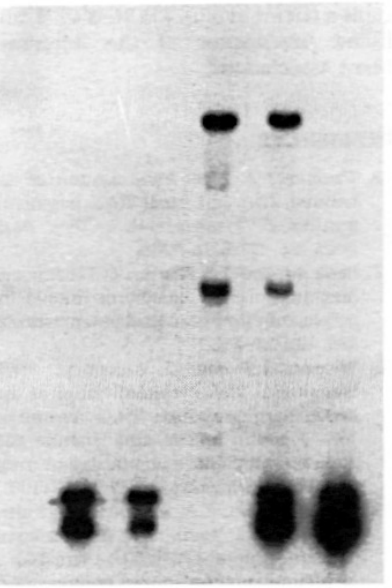

Figure 3. Effect of amplifying two different gene products in one reaction. PCR analysis of 100 ng of splenic cDNA with Cr2 and ß-actin oligos. Lanes 1-3 were done for 18 cycles. Lane 1, Cr2 oligos alone; lane 2, ß-actin oligos alone; lane 3, Cr2 + ß-actin oligos. Lanes 4-6 were done for 24 cycles. Lane 4, Cr2 oligos alone; lane 5, Cr2 + ß-actin oligos; lane 6, ß-actin oligos alone (from Tan 1992).

Some users of the Air Thermo-Cycler are hesitant to load glass capillaries with a radioactive reaction mixture

because of a fear of breakage. While Weis reports that this has not been a problem, plastic capillary tubes are now available (see "New from Idaho Technology" in this issue).

The Reverse Transcriptase Problem

In most protocols the reverse transcription is primed with the same primer that is later used for the amplification. The Weis group uses random hexamers to prime the cDNA synthesis and they report several advantages to this approach. First, it ensures that all RNA's are represented equally in the cDNA pool. Second, as reverse transcription is done at low temperatures, using 20-mers to 30-mers can lead to synthesis of cDNA's from non-specifically hybridized primers. These products might be specifically amplified during the quantitation.

The Relative versus Absolute Quantitation Problem

When measuring product by radiolabel, it is difficult to convert CPM's to absolute measures of DNA quantity. One solution to this problem is to set up an external standard curve by running known amounts of DNA each in their own reaction tube. Unfortunately this straightforward method has run into trouble due to large variation in the efficiency of different reactions. Further complications arise with RT-PCR because of the desire to control for the efficiency of the reverse transcriptase step. These problems have led to the use of internal standards of various types (7).

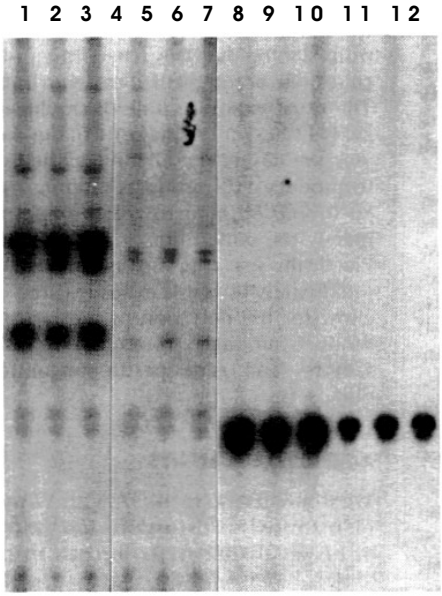

Figure 4. Reproducibility of PCR amplification for quantitation of products: multiple tissue samples. PCR analysis for 24 cycles with Cr2 oligos (lanes 1-6) and ß-actin oligos (lanes 7-12) with 100 ng of cDNA generated from three different spleens (lanes 1-3 and 7-9) and livers (lanes 4-6 and 10-12) (from Tan 1992).

The simplest internal standard is to simultaneously quantitate the sequence of interest with some more or less invariant "housekeeping" mRNA. If the level of the housekeeping gene's message is constant

between samples, then the amount of the unknown transcript can be reported in relative terms. Popular genes for standardization are ß-actin and HLA genes. All of these internal standard methods are based on the presumptions that: 1) the reverse transcription is not biased between the standard and test transcripts, 2) the amplification of the standard and the unknown occur with the same efficiency, and 3) the amplifications do not interfere with each other significantly.

Weis uses ß-actin mRNA as an internal standard (Figure 3). The autoradiograph shown in figure 3 shows that both products can be simultaneously amplified with minimal interference.

The Variability Problem

Sample-to-sample variability has long been a problem with RT-PCR. The efficiency of reverse transcription has been reported to vary from 5% to 90% (8), while the amplification itself may vary up to 200-300% between duplicate reactions. The Weis group reports good reproducibility not only between duplicate aliquots of the same cDNA but also between tissue samples (Figure 4).

The Protocol

1.Total RNA was prepared by the method of Chirgwin et al. (9)

2.RNA (5 μgs) was reverse transcribed in 1X RT buffer(GIBCO-BRL), 0.125 mM each dNTP, 0.5 μg random hexamers (New England Biolabs) and 400 units of Moloney virus reverse transcriptase (GIBCO-BRL) in a 50 μl reaction. The reaction was incubated at 37°C for 60 minutes. DNase free RNase was added and incubated for 5 minutes at 37°C. The reaction volume was adjusted to 270 μl with 0.4 M NaCl and was phenol extracted and precipitated with ethanol. cDNA concentration was determined by UV absorbance.

3.The optimal cDNA concentration and number of cycles was determined by a titration from 1 to 500 ng of cDNA and from 18 to 39 cycles. Optimal parameters were 200 ng of cDNA for 20 cycles. Each 10 μl reaction contained 200 ng of cDNA, 70 pmoles of each primer, 50 mM tris pH 8.3, 3 mM MgCl2, 20 mM KCl, 0.5 mg/ml BSA, 0.2 mM each dNTP, 2.5 uCi [32P]dCTP(3000 Ci/mmol; New England Nuclear), 0.72 units AmpliTaq DNA Polymerase (Cetus). To improve reproducibility, a master mix was prepared without primers and then aliquoted to separate tubes containing the different primer pairs. These mixtures were then aliquoted to the cDNA samples. Each 10 μl reaction was loaded into a glass microcapillary tube (Idaho Technology) and the ends were flame sealed. Capillaries were cycled in the 1605 Air Thermo-Cycler (Idaho Technology). Cycling parameters

were denaturation, 94°C for 1 sec; annealing, 59°C for 1 sec; elongation, 72°C for 4 seconds (products ranged in size from 80 to 200 base pairs). Total cycle time was 24 seconds.

4.Following amplification the ends of the capillary tubes were scored and the samples removed using a microaspirator and then 5 μl were electrophoresed in a 6% acrylamide gel. Radioactive bands were detected by autoradiography and then the bands were cut from the gel for quantitation by liquid scintillation counting. A 32P-end labeled MspI digest of pBR322 was used as a size standard.

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Rapid Cycle Amplification of VNTR Loci for Engraftment in Bone Marrow Transplantation.

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Bone marrow transplantation is now standard therapy for a range of diseases including many hematologic malignancies, some solid tumors, and some acquired or inherited hematologic and immunologic diseases. Many of these disorders result from a malfunctioning bone marrow, and the only cure is to inactivate the diseased bone marrow and replace it with healthy marrow. After the original marrow is destroyed, healthy marrow from a donor is infused into the recipient. Bone marrow transplantation may be: 1) autologous (where healthy stem cells have been previously harvested from the same individual), 2) syngeneic (where the donor is an identical twin), and 3) allogeneic (where the donor is different genetically from the recipient). In allogeneic transplantation, it is possible to determine the success of transplantation by monitoring the genotype of cells appearing in the peripheral blood. If the recipient type converts to the donor type, successful engraftment has occurred.

Variable number of tandem repeat (VNTR) loci are regions in the human genome where a short nucleotide sequence is repeated in tandem for a variable number of times. If flanking primers are placed outside of the repeats, the number of tandem sequences in any particular allele determine the length of the amplified product. Some VNTR loci are highly polymorphic with over 10 different alleles and are very useful for establishing individuality by genotype. For highly polymorphic loci, homozygosity is uncommon and two bands are expected at each locus because of the diploid nature of human cells. VNTR loci are commonly used in forensics to establish identity and can also be used to establish donor vs. recipient type in peripheral blood leukocytes after bone marrow transplantation. Since peripheral blood leukocytes originate in the bone marrow, the type of circulating leukocytes establishes the type of hematopoietic cells populating the

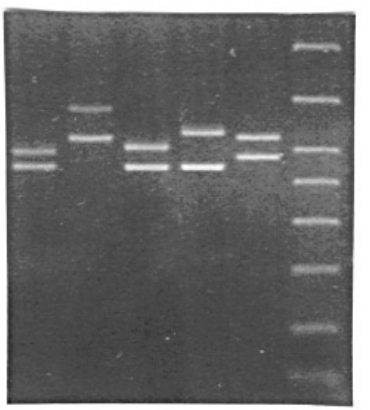

Figure 1. DNA samples from five unrelated individuals amplified with primers for the D1S80 locus2.

bone marrow. An example of DNA amplification of a VNTR locus in 5 unrelated individuals is shown in Figure 1.

Siblings are often used as donor/recipient pairs in bone marrow transplantation because they may match at HLA loci and have fewer problems with graft/host acceptance. HLA and VNTR loci are not linked and follow classical Mendelian inheritance. If siblings are matched at HLA loci for transplantation, they have a one in four chance of receiving the same parental VNTR alleles at any particular locus. If they do receive the same VNTR alleles at one locus, that particular locus is not useful for distinguishing donor vs. recipient type. However, most of the time, siblings will differ by either one allele (50% of the time) or two alleles (25% of the time). DNA from peripheral blood leukocytes needs to be isolated from donor and recipient before bone marrow transplantation, so that informative VNTR loci can be identified. Since there are many VNTR loci, finding differences between recipient and donor is not difficult, even for siblings. In the case of syngeneic or autologous transplantation, genotyping studies are not informative. The VNTR loci used here are HGM locus D17S301 and D1S802. All PCR reactions were run with standard rapid cycling techniques3-5 in an Idaho Technology 1605 air cycler with buffers and reagents supplied by Idaho Technology (1761 Optimizer Kit). The Mg2+ concentration was 2.0 mM. Cycling parameters were denaturation at 94° C for 0 sec, annealing at 55° C for 0 sec, and elongation at 73° C for 20 sec for 30 cycles. The total cycle time was 23.7 min. The samples were loaded directly on a 1.5% Agarose gel and electrophoresed at 5 V/cm.

VNTR Amplification in Bone Marrow Transplantation

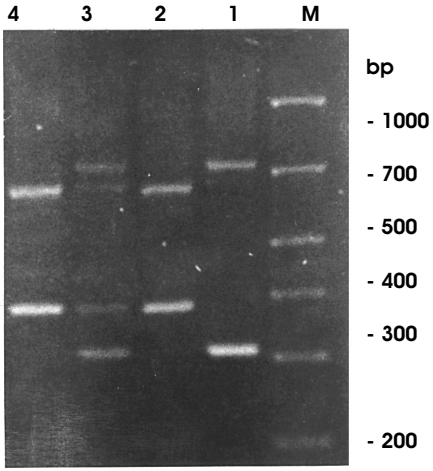

Figure 2. Bone marrow transplant engraftment by 1 month.
The VNTR locus HGM D17S301 was used.
Lane 1: Recipient before transplantation.
Lane 2: Donor.
Lane 3: Recipient 2 weeks after transplantation.
Lane 4: Recipient 4 weeks after transplantation.
In lane 3 the patient shows bands from both the recipient and the donor. In lane 4 the patient shows only donor bands suggesting successful engraftment.

Figure 2 illustrates a typical example of engraftment. This is a sibling transplant where all four alleles are different. At 14 days after transplantation, both donor

and recipient bands were observed. Residual recipient lymphocytes may circulate for 2-3 weeks after transplantation. However, recipient bands should disappear by 4 weeks if engraftment has occurred.

Figure 3 illustrates a typical example of disease recurrence after bone marrow transplantation. This is a sibling transplant where one allele is shared between donor and recipient types. At 36 days post bone marrow transplantation, both donor-specific and recipient-specific alleles are apparent. This indicates that the donor marrow has not entirely supplanted the recipient marrow at 36 days. At 100 days post bone marrow transplantation, only the recipient bands are present, indicating failure of engraftment and recurrence of disease.

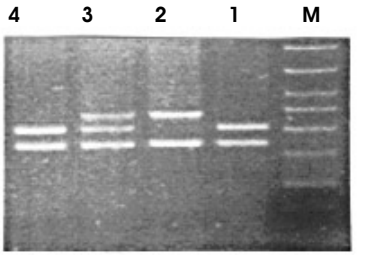

Figure 3. Disease recurrence after bone marrow transplantation. The VNTR locus HGM D1S802 was used.
1: Recipient before transplantation.
2: Donor.
3: 36 days after bone marrow transplantation.
4: 100 days after bone marrow transplantation.
In lane 3, alleles from both the donor and recipient are present at approximately equal amounts. After 100 days (lane 4), the unique donor band has disappeared and only the original recipient alleles are present.

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New From Idaho Technology

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Idaho Technology Inc.

Polycarbonate tubes

There has been a great deal of interest displayed by users of the 1605 Air Thermo-Cycler (ATC) in the possibility of using plastic capillary tubes to augment the glass capillary tubes currently standard in our instrument. The results of our tests on various plastic tubes have been very encouraging. Our selection for final testing is poly carbonate tubing which has thermal response characteristics almost identical to our 10 μl glass tubes. The polycarbonate does not interfere with the reaction and it should be of great help in those situations where the fragility of glass capillary tubes is an excessive hazard. However, those who are interested in using plastic capillary tubes should be aware that plastic tubes are not without their drawbacks.

Glass tubes can be easily loaded either singly or eight at a time by capillary action. However, hydrophobic plastic tubes require a loading mechanism such as a micro-aspirator or a similar device. We are working on ways of loading and sealing eight tubes at a time; but at present it can only be done one tube at a time.

The second drawback is sealing the ends of the plastic tubes. It is tricky but possible to flame seal plastic tubes by intentionally igniting the ends. For many people, this tends to be somewhat disconcerting; therefore, we have developed an electric tip sealer.

The last potential problem with plastic tubes is price. At a cost of approximately $80 per 1000, plastic tubes will be about twice as expensive as similar glass tubes. Even at that price, plastic tubes would still be less expensive than other second generation sample containers. We will know more about pricing after final testing on the tubes is complete.

We hope to be completely finished with the final tests on the plastic tubes and have the tubes and the sealers available in June of '94, barring major catastrophe. (10 μl tubes, part number 1714; tube sealer, part number 1740)

Modular Tops

In other hardware news, some of our earlier customers may be interested in a change made in the design of the 1605 cycler. The plastic top now has removable modules for loading and unloading tubes. The entire module is removable from the rest of the top to allow easier loading and unloading. Each module holds 16 tubes. To help ensure a good fit of all sizes of tube, modules are available in two sizes, 10 μl and 50 μl. An upgrade kit to a modular top is available from Idaho Technology, part number 1869.

Module Racks

A rack for holding the capillary tube modules is also now available. Each rack will hold three filled capillary tube modules. These module racks should help eliminate damage to capillary tubes when filled modules are set down prior to reinsertion into the instrument top. The part number is 1735.

Improved Buffer System

We have made several improvements to the buffers optimized for rapid cycling. Traditionally we have used Ficoll and tartrazine to increase the density of our buffer and make it visible for direct loading of product onto gels. We now recommend substituting sucrose for Ficoll, and cresol red for tartrazine.

For optimizations we have traditionally recommended using a three-by-three matrix of 3 mM, 2 mM, and 1 mM Mg2+ run at three annealing temperatures; 40°C, 50°C, and 60°C. However, our experience is that most reactions optimize at the higher end of the Mg2+ concentration, therefore we now recommend using 2 mM, 3 mM and 4 mM Mg2+ in the high, medium and low buffers.

We will include the new buffers free with all reagent orders for the next few months and if the reaction is positive, we will switch to the new system for individual buffer orders and the Optimizer Kit. As usual we are also publishing the reagent constituents in case you choose to make your own buffers. On the following pages are procedures for running individual reactions, making mastermixes, and making the reaction constituents themselves.

Reaction Mixes and Buffer Recipes

from Carl Wittwer's laboratory

Reaction Constituents for One 10 µl Reaction

Component	[10X]	[Reaction]	Separate	Combined
DNA (human genomic)	50 ng/µL or A 260 = 1.0	50 ng/10µl	1 µl	1 µl
Primers				
Separate				
Primer 1	5 µM	0.5 µM	1 µl	
Primer 2	5 µM	0.5 µM	1 µl	
or Combined				
Primer 1 + 2	5 µM each	0.5 µM each		1 µl
Nucleotides	2 mM each dNTP	200 µM each dNTP	1 µl	1 µl
Buffer	500 mM Tris, pH 8.3 2.5 mg/ml BSA 20% (w/v) Sucrose 1mM Cresol Red	50 mM Tris, pH 8.3 250 µg/ml BSA 2% (w/v) Sucrose 0.1 mM Cresol Red	1 µl	1 µl
Low Mg2+	20 mM MgCl2	2 mM MgCl2		
Medium Mg2+	30 mM MgCl2	3 mM MgCl2		
High Mg2+	40 mM MgCl2	4 mM MgCl2		
Enzyme	0.4 U/µL	0.4U/10µl	1 µl	1 µl
dH20/other			4 µl	5 µl

Amplification Procedure

1. Prepare master mix without DNA and without primers weekly:

For <50 runs at a 10 µl reaction volume:

Dilute Enzyme to 0.4 U/µl

11.5 parts Enzyme diluent
(10 mM Tris pH 8.3, 2.5 mg/ml BSA)
1 part Enzyme (5 U/µl)

For separate 5 uM primers:
4 parts dH20
1 part buffer
1 part 2 mM dNTPs
1 part 0.4U/µl Enzyme

For combined 5 uM primers:
5 parts dH20
1 part buffer
1 part 2 mM dNTPs
1 part 0.4U/µl Enzyme

Mix and store at 4°C for < 1 week.

For >50 runs at a 10 µl reaction volume:

For separate 5 uM primers:
308 (61.5 parts) dH20
63 µl (12.5 parts) buffer
63 µl (12.5 parts) 2 mM dNTPs
5 µl (1 part) 5U/µl Enzyme

For combined 5 uM primers:
370 (74 parts) dH20
63 µl (12.5 parts) buffer
63 µl (12.5 parts) 2 mM dNTPs
5 µl (1 part) 5U/µl Enzyme

Mix and store at 4°C for < 1 week.

2. For each run with a specific primer pair, make a primer-specific mix:

For separate 5 µM primers
1 part 5 µM primer 1
1 part 5 µM primer 2
7 parts master mix

For separate 5 µM primers
1 part 5 µM primer 1
1 part 5 µM primer 2
7 parts master mix

3. Add 1 µl of each sample DNA (for genomic DNA, 50 ug/ml or A260=1.0) to individual wells in a microtiter plate. Pipette 9 µl of the specific-primer mix into each well and mix by pipetting up and down. Load capillary tubes into the modular tops and aspirate 8 samples at a time by capillary action. Flame seal the loading end of the tubes, then seal other end. Place into the Air Thermo-Cycler and run at desired protocol. When reaction is complete, score each end of the glass tubes while still in the modular top, break glass and transfer directly into the gel wells.

Working Solutions

1. Primers and DNA are prepared in 1X TE':

50X TE' solution, pH 8.3 (500 mM Tris, 5mM EDTA)

10 m l 2 M Tris, pH 8.3
400 µl 0.5 M EDTA
dH2O to 40 ml

1X TE' solution, pH 8.3 (10 mM Tris, 0.1 mM EDTA)

200 µl 50X TE'	or	10 ml 50X TE'
dH2O to 10 ml		dH2O to 500 ml

2. Make 50 µM primer stocks with 1X TE'.

Make10X primers (5 µM) either separately or combined:

For 10X separate primers

40 µl (1 part) 50 µM Primer
360 µl (9 parts) 1X TE'

For 10X combined primers

40 µl (1 part) 50 µM Primer 1
40 µl (1 part) 50 µM Primer 2
320 µl (8 parts) 1X TE'

3. 10X Nucleotides (2 mM each dATP, dCTP, dGTP, dTTP)

250 µl 100 mM dATP (Sigma D4788)
250 µl 100 mM dCTP (Sigma D4913)
250 µl 100 mM dGTP (Sigma D5038)
250 µl 100 mM dTTP (Sigma T9656)
to 12.5 ml with dH2O

4. 10X Buffer

2.5 ml Tris, pH 8.3 (2 M stock)
0.5 ml BSA (50 mg/ml stock)
5.0 ml 40% Sucrose
1.0 ml 10 mM Cresol Red

Low Mg2+	200 µl (1M MgCl2) + 800 µl H2O
Medium Mg2+	300 µl (1M MgCl2) + 700 µl H2O
High Mg2+	400 µl (1M MgCl2) + 600 µl H2O

5. Enzyme diluent (10 mM Tris, pH 8.3, 2.5 mg/ml BSA)

50 µl 2 M Tris, pH 8.3
500 µl 50 mg/ml BSA
9.5 ml dH2O

Stock Solutions

All solutions are made from deionized, distilled water. No stir bars or pH meters are to be used in the preparation of stock or working solutions. Check pH by withdrawing 10 µl of solution and placing it on pH paper.

2 M Tris, pH 8.3

14.80 g Tris base (Sigma T1503)
12.28 g Tris HCl (Sigma T 3253)
to 100 ml with H2O

or

27.08 g TRISMA Preset, pH 8.3 (Sigma T5128)
to 100 ml with H2O

1 M MgCl2

20.3 g MgCl2 (Sigma M9272)
to 100 ml dH2O

or

Sigma M1028 (ready made)

50 mg/ml BSA

0.50 g BSA (Sigma A2153)
to 10 ml dH2O (use 15 ml tube)

10 mM Cresol Red

404 mg cresol red (Sigma C9877)
to 100 ml dH2O

40% (w/v) Sucrose

40.0 g sucrose (Sigma S5016)
to 100 ml dH2O

0.5 M EDTA, pH 8.3

18.6 g disodium EDTA (Sigma ED2SS)
10 ml 5 N NaOH (Baxter H369-1*NY)
to 100 ml dH2O

Rapid Cycle DNA Amplification – The 10 Most Common Mistakes

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Mistake #1.

Not having bovine serum albumin in the reaction. You will not get amplification in capillary tubes without BSA. Most buffers supplied by manufacturers of the enzyme do not include BSA. BSA is necessary to prevent surface adsorption/inactivation of the DNA polymerase on the large surface area of the capillary tubes. Yield increases with BSA concentrations up to 500 μg/ml in the reaction. Using gelatin gives a poor yield in capillary tubes. You can make up your own buffers. We recommend including 2.5 mg/ml BSA in the 10X buffer and 2.5 mg/ml in a 10X polymerase dilution. The grade of BSA is not critical. We use Sigma #A2153.

Mistake #2.

Using acetylated bovine serum albumin. It is expensive and does not work. Presumably, the same sites that are acetylated are those sites necessary to coat the glass walls and prevent polymerase inactivation.

Mistake #3.

Using Triton X-100. Some manufacturers of heat stable polymerases state that 0.1% Triton X-100 is needed for enzyme activation. Triton X-100 does activate some enzymes when BSA is not present and amplification occurs in microfuge tubes. However, Triton X-100 is not necessary when BSA is present. Furthermore, if Triton X-100 is added, yield substantially decreases in capillary amplifications that include BSA.

Mistake #4.

Adding polymerase to a microtiter plate before BSA. For convenience, many people mix reactions in a microtiter plate so they can be loaded simultaneously by capillary action into tubes already placed in modular tops. However, if the polymerase is added to a microtiter well before BSA, the polymerase can be adsorbed onto the plastic surface and not loaded into the capillaries. To prevent adsorption of polymerase during handling, we recommend diluting the polymerase to a 10X concentration with a diluent that includes BSA at 2.5 mg/ml. In addition, always block the well surface with BSA by adding the BSA-containing buffer before the polymerase. Microtiter plates that do not absorb protein can also be used and are available from Idaho Technology (microtiter plate, part

number 2590; lid, part number 2591) , but BSA is still necessary for the capillary tubes, whether glass or plastic.

Mistake #5.

Pulling tubes out near the denaturation temperature. If double stranded product is cooled rapidly (by pulling a tube out of an air cycler that is near denaturation temperatures), not all the product will reanneal and multiple apparent products may appear on gels.

Mistake #6.

Using excessive denaturation times. There is no reason for denaturation times longer than "0" sec at 94° C. The Tm of products in amplification buffer is around 85-90° C and complete denaturation of product at 94° C occurs faster than can be measured (< 1 sec. See Wittwer and Garling, 1991, BioTechniques, 10: 76-83, or Wittwer et al., 1994, in: The Polymerase Chain Reaction , Mullis, Ferre, and Gibbs, eds., pp. 174-181). The only possible exception is on the first cycle when high quality, complex genomic DNA is used as template. An initial denaturation of 5-15 sec at 94° C on the first cycle may allow more complete initial denaturation. However, extended times at high temperatures degrade DNA, and are particularly harmful in long product amplifications (CE Gustafson et al., 1993, Gene 123:241-244, and W.M. Barnes, 1994, PNAS, 91: 2216-2220).

Mistake #7.

Using nonstandard capillary tubes. The tubular metal-sheathed thermocouple that monitors temperature in the air cycler is precisely matched in thermal response to aqueous samples in the 10 μl capillary tubes sold by Idaho Technology. When nonstandard capillary tubes are used, the temperature of the sample will not correspond to the temperature indicated on the instrument readout. If you optimize a reaction in 10 μl tubes, and later run the reaction in larger tubes, you should not expect similar results. Larger tubes will not reach target temperatures without setting a hold time. If you insist on using larger or nonstandard tubes, you can monitor the sample temperature inside the tube with an IT-23 micro-thermocouple probe available from Sensortek (Clifton, NJ) and empirically adjust target temperatures and hold times. Be aware that some types of glass interfere with the reaction, presumably because ions on or near the surface of the glass are absorbed into the reaction buffer.

Mistake #8.

Forgetting to add a critical component. Accidental omission of polymerase, dNTP1s, or buffer components can be avoided by "master mixes" that include everything necessary for amplification except primers and template. Such a master mix, if sterile, lasts for 3-6 weeks at room temperature, >15 weeks at 4° C, and > 26 weeks at -20° C. Master mixes also minimize pipetting errors, particularly with small volumes.

Mistake #9.

Inappropriate Mg2+ concentration. Rapid cycling generally requires higher magnesium concentrations than slow cycling. For example, whereas 1.5 mM magnesium chloride is standard in slow cycling, 2-4 mM is more typical for rapid cycling. With 2-4 mM magnesium chloride, excellent yield and specificity can be obtained with annealing times of "0" sec. Magnesium chloride is hygroscopic and it may be difficult to prepare accurate solutions from the solid salt. We use a 1 M solution of magnesium chloride available from Sigma (#M1028).

Mistake #10.

Poor temperature/time optimization. Rapid cycle temperature/time parameters are very different from slower cyclers. It is a mistake to directly transfer a protocol like, "1 min at 94° C, 2 min at 55° C, and 3 min at 72° C," to a rapid cycler. Denaturation should be set at 94° C for 0" sec. The annealing time should almost always also be set at "0" sec. The extension temperature should be 70-74° C. The extension time should be "0" sec for products up to 100 bp, 5-15 sec for products up to 500 bp, and about 30 sec for a 1000 bp product. Most amplifications with 20-mer primers will work well using 3 mM MgCl2 at an annealing temperature of 50° C. Rapid cycling makes it feasible to rapidly test all combinations of 3 different annealing temperatures (40° C, 50° C, and 60° C) and 3 different Mg concentrations (2 mM, 3 mM, and 4 mM).