TABLE OF CONTENTS

INTRODUCTION TO S.F.G.

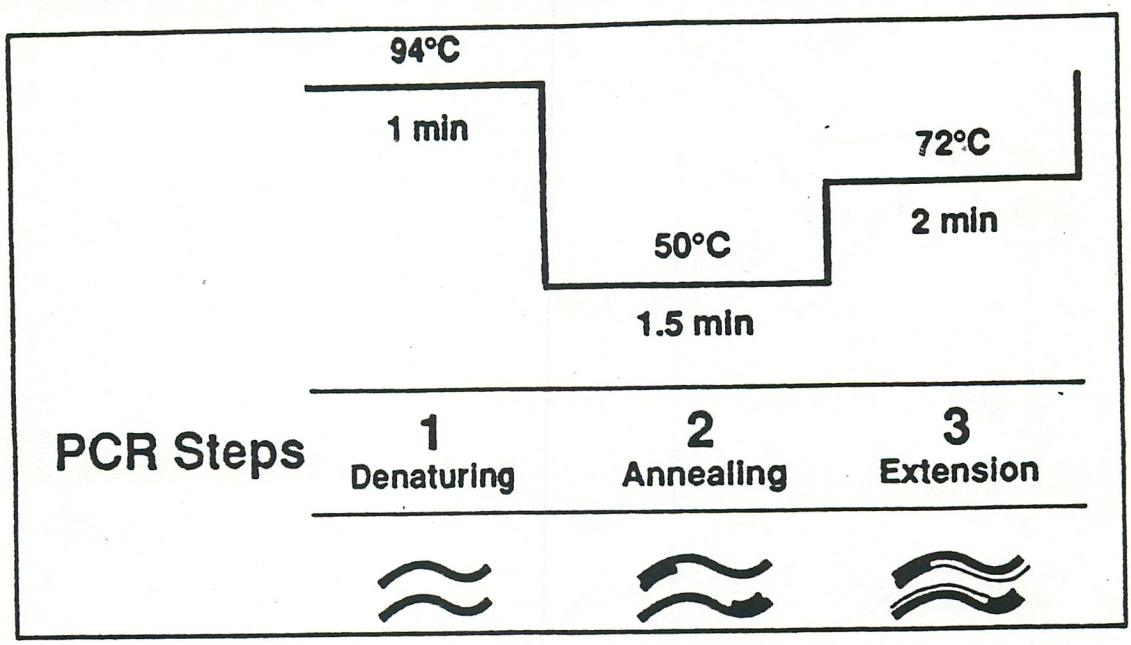
The Polymerase Chain Reaction, or PCR, is a method that allows enzymatic amplification of minute quantities of DNA and RNA for sequencing or other forms of genetic analysis. PCR has largely been developed by workers at Perkin Elmer Cetus and we wish to thank them collectively for all their help. We also would like to point out that Perkin Elmer Cetus publishes a newsletter called "Amplifications" which often has PCR protocols and suggestions that are extremely useful.

The *Simple Fool's Guide to PCR*, a collection of PCR protocols and oligonucleotide primers, is an attempt to promote sharing of PCR protocols and primer sequences from different gene regions, so that redundant (and costly) effort in the refinement of PCR techniques and the design and making of primers is not wasted. The compilation of this "guide" is *not* an altruistic act. We are asking that other labs share their experiences with us. If you or your lab uses any of the techniques or primers that we have gathered together in this guide we ask that you let us know what works with what under what conditions. Subsequent versions of SFG (if there are any) will contain such information as is given unto us along with your name and affiliation in the "**Contributors**" section. Although PCR is extremely fast and easy, there are (in our minds) a number of mysterious manifestations and inconsistencies that rear their ugly heads when working with new species. We hope that while we might have encountered and solved some mysteries that others are running across, other workers may have solved ones with which we need help. Please keep in mind that these protocols in the first version are ones that we have had consistent success using on various taxa in our lab, but that does not mean that they will work perfectly with the DNA with which you are working or that other methods are not better. It is also not an attempt to make a complete reference for PCR techniques and primers, rather it is an attempt to collect *easy* PCR techniques and primers that just about anyone can use with success. We have gathered some of these methods from colleagues across the country. Some have been scribbled on beer-soaked coasters in dark Berkeley bars. Some have resulted from midnight airport rendezvous. We have found in general that people have been delightfully open and generous with their technique development and primers. One of our goals is to promote continued openness with this guide.

THE CYCLE

A. The Basic Cycle

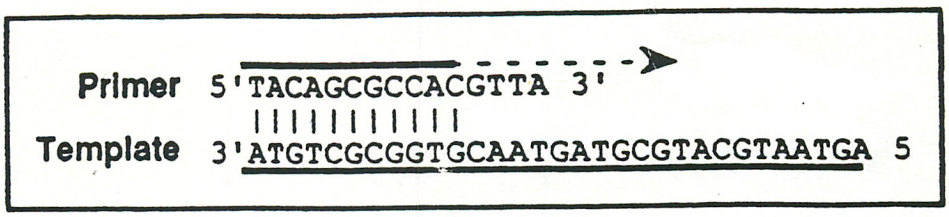
The PCR cycle is relatively simple and is composed of 3 major steps (diagramed below).



Step 1: The PCR reaction requires a single-stranded template. The first step denatures, or melts, the double-stranded template DNA so that all the DNA is single-stranded. This allows the oligonucleotide primers to anneal to the single-stranded template DNA at specific locations (i.e. the primers' complements). 94°C for 1 minute seems to work well. *Remember*, if the melting temperature is too low or time too short the double-stranded DNA may not denature between cycles — thereby reducing the efficiency of the reaction. If, on the other hand, the temperature is too high, the Taq polymerase enzyme will die.

Step 2: The second step of the cycle involves the actual annealing of the primers to the template DNA. Once the template DNA has been denatured, the temperature must be lowered to a level that allows the primers to anneal. The trick is to lower the temperature to a level that allows the primer to anneal to the complementary sequence — if the temperature is too low the primer will sit down randomly (non-specifically) and if too high the primer will not sit down at all. Standard temperatures seems to be about 50°C for 1-1.5 minutes. If you are having problems with getting any product at this annealing temperature, lower the temperature to 45-48°C (though sometimes as low as 37°C works).

Step 3: The Taq polymerase works best at temperatures between 72-74°C and so we raise the temperature from the relatively low annealing temperature to a temperature at which the Taq polymerase can function efficiently. The polymerase has to add nucleotides to the 3' end of the primer sequence annealed to the template DNA (see diagram below). The primers are necessary for the initiation of the reaction and the template DNA acts as a reference strand for the polymerase which adds the complementary nucleotide bases starting at the position just after the 3' end of the primer sequence (ADENOSINE pairs with THYMINE and GUANINE with CYTOSINE). The primers are incorporated into all subsequently amplified DNA templates insuring perfect priming sites in subsequent PCR cycles.



B. Variations in the Cycle

If a low annealing temperature is required in step 2 of the PCR cycle, often a lower extension temperature is required. We may use the following types of temperature cycles when dealing with various DNAs, primers and annealing temperatures:

94-50-72	for good or perfectly matched primers
94-48-68	for poorly matched primers (say 4-5 mis-matches)
94-45-65	for fishing expedition where the primer is of very questionable quality

Some feel it is particularly useful to extend the annealing time in addition to (or as opposed to) extensively lowering the annealing and (especially the) extension temperatures in step 2 and 3 of the PCR cycle (respectively). This gives a primer more of a chance of finding its complement and allows the Taq polymerase (even though not at optimal temperature) to extend the primer sequence a little, thus "locking" it to its complement on the template, before the temperature is raised to the optimal extension temperature of 72°C, which could potentially cause a poorly matched primer to melt off its complement on the target template DNA in the early PCR cycles.

Another helpful variation in the PCR cycle is to "ramp" the annealing to extension steps during the cycle. This is a minor variant on the basic PCR cycle that slowly raises the temperature from the low annealing temperature to the relatively high extension temperature over a 2-3 minute period (or so), thus allowing the Taq polymerase time to, again, "lock" the primer to the template as describe in the preceding paragraph.

DNA EXTRACTIONS FOR PCR

Since PCR uses sequence specific primers, it is possible to amplify any portion of the mitochondrial or nuclear DNA using total DNA extracts from tissue samples (though most prefer to work with pure mtDNA when possible in our lab). Below are a couple of very simple methods that we have successfully used to extract total DNA for PCR amplification of specific genes from mtDNA and nuclear DNA. We welcome comments on other methods that might work better or be easier for various species.

A. General Extraction Protocol for Total DNA

This general procedure has worked extremely well with everything that we have tried (e.g. shrimp, urchins, corals, sharks, spiders, gastropods, insects). Very little tissue is needed and it is fast and easily adapted to different quantities of tissue. We like to grind the tissue in a very dilute solution.

Lysis Buffer :

100 mM	EDTA
10 mM	Tris (pH 7.5)
1 % mM	SDS
1µg/ml	Proteinase K

1. Homogenize about 0.1 - 0.5 grams of tissue in 750 µl of *Lysis Buffer*. We do this step in an 1.5 microfuge tube and a micro-centrifuge sample pestle (which can be glass or made by pouring plastic casting resin into a micro-centrifuge tube and letting is harden with a glass disposable pipette inserted into it).
2. Incubate at 65°C for 1-3 hours (at least — overnight is OK).
3. Phenol (H_2O saturated phenol, of course) extract the sample twice or until clear
4. Phenol/Chloroform extract the sample once.
5. Chloroform extract the sample once.
6. Precipitate at room temperature with 2 volumes of 95% EtOH (let sit for 2-5 minutes). You can (but we have found it unnecessary) add a $\frac{1}{2}$ volume of 7.5 M Ammonium Acetate to help precipitate the DNA.
7. Spin in microfuge to pellet DNA and wash with 70% EtOH and spin again and let samples dry.
8. Resuspend dry pelleted DNA samples in 200-300 µl of H_2O (or $\frac{1}{10}$ TE)
9. Use 1 µl of this in a PCR double-stranded amplification to see if it works. If not, try other (more dilute more likely) concentrations of this template DNA in your PCR runs.

A. Variations on the Extraction Protocol

In our lab, people, when trying to PCR mtDNA without cesium purification of mtDNA, will often low-speed centrifuge ground tissue samples to pellet nuclei before lysing the resulting supernatant with 1 % SDS. This increases the relative proportion of mtDNA to nuclear DNA in the genomic extractions.

Russ Higuchi's suggests, in the May 1989 issue of the "Amplifications" newsletter , using a non-ionic detergent — such as Nonidet P40 from Sigma. While Russ Higuchi's protocol is directed at blood cell extractions, substituting a non-ionic detergent in place of the SDS in the above protocol for other tissue extractions should be possible (we have not tried it yet) and would eliminate the need to phenol extract and precipitate your extractions.

PCR PROTOCOLS

The following PCR protocols are ones that work well for most people in our lab with a variety of different DNAs. The Taq polymerase buffer is one that was being used by some workers at A.C. Wilson's lab in Berkeley when two of our workers went there to learn PCR and sequencing techniques. It works well, but there are a number of different buffers being used in other labs and in the literature that may work as well or better. For example, some recommend that the Taq buffer have twice the molar concentration of $MgCl_2$ as dNTPs used in the PCR reactions (the one listed below has almost 4 times the $MgCl_2$ concentration). Perkin Elmer Cetus recommends titrating the amount of $MgCl_2$ used in the PCR reactions for every different DNA template being used. If you are having problems, explore other buffers (particularly ones that change $MgCl_2$ concentrations).

A. Solutions

Note: 10X Stocks of the buffer solutions are usually made.

Taq Polymerase Buffer :

67 mM	Tris (pH 8.8)
3 mM	$MgCl_2$
16.6 mM	$(NH_4)_2SO_4$

TE Buffer:

10 mM	Tris (pH 7.6)
1 mM	EDTA (pH 8.0)

TBE Buffer:

89 mM	Tris (pH 7.8)
89 mM	Boric Acid
2 mM	EDTA

TAE Buffer:

40 mM	Tris (pH 7.8)
5.71 %	Glacial Acetic Acid
1 mM	EDTA

Dense-Dye:

200 µl	250 mM EDTA
300 µl	Glycerol
100 µl	2.5 % Bromophenol Blue
100 µl	2.5 % Xylene Cyanol
300 µl	H_2O

B. Double-Stranded DNA Amplifications

1. Prepare the following reaction mix for each template sample:
   - 10 µl 10x Taq buffer
   - 10 µl 8 mM dNTP's (i.e. 2 mM each of dATP, dGTP, dCTP, dTTP)
   - 5 µl each of two primers (10µM stock solutions)
   - 1 µl template DNA (in dH_2O or $\frac{1}{10}$ TE, 1-2 ng of mtDNA or 1-2 µg genomic DNA seems to work OK—try other amounts if need be)
   - 0.125 µl Taq polymerase (this is alot less then recommended but it often works perfectly—so save some money!)
   - —add dH_2O to make 100 µl per reaction (usually this means about 70µl or so)
2. Add 2 drops of mineral oil (common pure drug store variety) to prevent evaporation of sample (if condensation forms on the top of the PCR tubes during cycles our reactions do not seem to work), and spin in microfuge for ≈20 seconds
3. Set PCR machine for 40 to 50 cycles. Standard conditions are: 94°C for 1 min, 50°C for 1-2 min, 72°C for 2-3 min. These should be altered when different stringencies are desired (i.e. the annealing temperature, step 2, can be raised or lowered a couple of degrees for higher and lower stringency, respectively) or when the amplified piece is larger than 2 kb (i.e. the extension time, step 3, should be made a little bit longer with very large target pieces, > 2 kb — though this may not be that critical).

C. Single-Stranded DNA Amplifications

Single-stranded DNA can be produced from pure mtDNA, or from a double-stranded amplification made as described above.

1. For pure mtDNA samples with good primers, an asymmetric amplification can easily be used. Make PCR cocktail as above with the following differences. One of the primers (the "limiting primer") is $\frac{1}{100}$ the concentration used in double-strand amps. Use 5 µl of a 0.1 µM primer solution (instead of the normal 10 µM solution). Add 1-5 ng (probably 1 µl is fine in most cases) of pure mtDNA as template.

2. For single-strand amplifications from double-strand amplifications, there are three methods:

   Method A: Use 1-5 µl of the double-stranded amp in a new reaction with ***only*** one primer (use 5 µl of 10 µM solution of primer, as usual). The amount of template to add is determined by the amount of double-stranded DNA in the first amplification. If too much is added, the single-strand amplifications will smear. This method is particularly good for problem DNA samples.

   Note: When doing ss amps from ds amps there are now perfect primer sites on the DNA template and so PCR conditions should be stringent (50-52°C), *regardless* of the original conditions needed to obtain the ds amps.

   Method B: Take a $\frac{1}{100}$ dilution of the double-stranded PCR product and use that in a asymmetric single-stranded amplification as described in #1. If this fails, diluting the starting DNA even more often remedies the problem either because inhibitors are diluted (unlikely since the double-stranded amplifications worked) or because of a more favorable primer to template ratio.

   Method C: Run the double-stranded amplification on a 3-4 % Nusieve low-melting agarose gel. The band can be visualized after staining with EtBr. Cut out the appropriate band with a sterile blade. (This method is ideal if the double-stranded amp has multiple products.) Some recommend using TAE instead of TBE in these agarose gels but we have had consistent success with TBE. Soak the gel slice in 1 ml of sterile water for 1-3 hr to de-stain, and melt the gel slice in about 100 µl of water (or butanol extract the 100 µl solution containing the melted agarose band which removes the EtBr also). Use 1 µl of this solution in the single-strand amplifications. OR, take a tiny slice of out of the middle of the gel band that contains the DNA and use that directly in a PCR solution as template (the low-melting agarose melts in the first cycle and releases the template DNA).

D. Double-Stranded mRNA Amplifications

The following method can be used to generate a cDNA product from mRNA that can reliably be used in PCR amplifications. This method has been used by Deborah Hansell and Lilly Tashima with human and guinea pig relaxin mRNA (respectively) and by Ed Metz with sea urchin sperm bindin protein mRNA.

<u>cDNA synthesis</u>:

1. 1 µl of a 1 µg/µl mRNA solution (total RNA can be used) is heated to 92°C in a microfuge tube for 3 minutes.
2. Quench on ice.
3. Add the following to give a total reaction volume of 20 µl:

20 units	RNasin (from Promega)
100 pmoles	Random hexamer
2 µl	10 X PCR Buffer
4 mM	dNTP (1 mM each)
32 units	AMV reverse transcriptase

4. Incubate for 10 minutes at room temperature.

5. Incubate at 42°C for 30-60 minutes.
6. Heat to 95°C for 5 minutes.
7. Cool on ice.
8. Make up ~800 µl with H_2O and store in aliquots at -20°C.
9. Try 2 µl of solution #8 directly for a double-stranded amplification.

For the production of full length cDNAs and PCR products you can try the RACE method (PNAS 85:8998-9002). For us this method has *only* produced good results with tissue containing a high concentration of target mRNA. The hexamer method described above seems to provide more consistent results with various tissues.

E. To Visualize PCR Products

1. Run PCR'ed samples (1-2 µl *Dense-Dye* + 10 µl sample) on 2 % agarose gels in 1 x TBE buffer. The 2 % agarose gel works well for PCR products that are 300 bp to 2 kb in length. Use a more concentrated gel (3-4 %) for smaller fragments of DNA. Stain the gel for 15 minutes in the running buffer containing a couple of drops of 2 mg/ml of ethidium bromide (EtBr) and visualize the gel on a shortwave UV radiation transilluminator. Make sure to always run a standard to insure that the band of DNA amplified is the predicted size!

PCR PROBLEMS

A. No PCR product

Try diluting the starting template DNA.
Try lowering the annealing temperature (step 2) in the PCR cycle.
Check the primer concentrations!
Try doing more cycles on the PCR machine (increase from 40 cycles to 50 cycles)

Determining Primer Concentrations:
Read A_{260} and A_{280} (the ratio should be >1.5 or so if the primer is reasonably pure).

$$\frac{(A_{260}) X \left(\frac{1}{DF}\right) X \left(3 X 10^{4} \mu g/liter\right)}{(N X 330 amu)} = \text{Primer concetration in } \mu g/liter \text{ or } \mu M$$

DF = Dilution Factor; the amount that the primer was diluted for the OD reading.
N = The Number of nucleotides that are in the primer.
$3 X 10^{4}$ µg/liter might more accurately be $3.3 X 10^{4}$ µg/liter in the formula.
(see Wrischnik et. al. 1987)

B. Smearing of double-stranded PCR product or Multiple Bands

The most common cause seems to be too much template. Try less template.
If you get a lot of smearing, or multiple bands, it *may* indicate that the primer is annealing to other parts of the template DNA and you might try an annealing temperature that is ≈2-5°C higher.
Try fewer cycles. This is often recommended but it is probably not really the best solution. While there is less "junk" amplified this way (remember mis-priming results in exact primer sites being attached to non-target DNA and the non-target's subsequent amplification in cycles), subsequent amplification from this PCR reaction will amplify even minute quantities of non-target DNA to visible levels (unless gel slices are used). It seems best to optimize conditions to reduce mis-priming (eg. temperature and salt concentration in buffer — try a buffer with different $MgCl_2$ concentrations).

C. Smearing of single-stranded PCR product

If the smearing is large (maybe even looks like some DNA is stuck in the well) we find that this is indicative of over-amplification (see "E"). Try diluting the template DNA or raising the annealing temperature (step 2 of the PCR cycle) a couple of degrees Celsius. If the smearing is small, it might (???) indicate mis-priming. Again, try raising the annealing temperature. Small smearing is a rare phenomenon and, other then mis-priming, we are not really sure of the cause. Keep in mind that you can get get sequence from amplifications that look smeary on gels... at least sometimes!

D. Double-stranded amplifications work, but single-strandeds don't

If you are amplifying from original DNA preparations, it might indicate that the primers are not that great and it takes a few cycles for the DNA to start amplifying. This means that the limiting primer gets in low concentration late in the cycle and therefore little single-stranded DNA is produce. Try a few more cycles or try using one of the single-stranded amplification methods using double-stranded amplification reactions as template. You might even consider one of the double-stranded sequencing methods in this guide. You could also use primers which have restriction sites used in cloning built into them, and then clone the double-stranded product into M13 (ugh! Last resort!!). If the double-stranded DNA

amplifications worked fine when you tried them, don't fret too much if you do not see a single-stranded product, try sequencing anyway. We have sometimes been able to sequence single-stranded amplifications that you can not see.

E. Bright bands in well of gel

Almost surely over-amplification of the PCR product. We have often gotten this result if we re-amplify from a PCR product (either double or single-stranded reactions) and do not dilute it enough (usually 1 µl of a $\frac{1}{100}$ dilution from a PCR reaction is enough for a standard asymmetric single-stranded amplification). Try diluting the PCR product that you are using as a template more. Also, remember to do the new PCR reaction at 50-52°C (52°C works well for our 20mers and above) — the priming sites on a PCR product are now perfect. You also want to reduce mis-priming, which can be accomplished by raising the annealing temperature.

SEQUENCING PROTOCOLS

Sequencing is probably the goal of most people using PCR, though you can use it to amplify mtDNA and nucDNA and digest with restriction endonucleases as a diagnostic tool for quick and dirty identification of genotypes in individuals or species or for hybridization studies. Sequencing PCR products is fairly easy, but requires that you remove the PCR buffer, excess primer(s) and nucleotides left over from the PCR reaction mix and concentrate the DNA. You can accomplish this a number of ways (e.g. EtOH or spermine precipitation) but we use Centricon-30 microconcentrators — as it seems most people sequencing PCR products do. They are fast, easy, reliable *and* expensive! We also use the Sequenase® System from USBiochemicals. This is an simple and rapid sequencing kit, though we have modified it to make it even easier and fit our needs better (see below). Once the PCR product is centriconed (or what ever), one PCR primer is used to initiate a sequencing reaction on denatured template DNA (either "denatured" by single-stranded PCR amplification or as in one of the double-stranded sequencing protocols mentioned below). With double-stranded sequencing, either PCR primer can be used in the sequencing reaction. If you use single-stranded DNA from a single-stranded PCR amplification you must use the primer that was limiting or absent in the amplification! The reason for this is obvious, the primer that was more abundant in the PCR reactions will produce the most DNA. This DNA will remain single-stranded since there is not an equal amount of the other strand of DNA to complement with it. The "limiting" primer complements with this excess single-stranded DNA and, therefore, must be used in the sequencing reaction.

A. Sequencing Gel

40 % Acrylamide stock solution:
- 38 % Acrylamide
- 2 % bis-Acrylamide

Store in cool dark location

8 % Acrylamide gel:
- 8.0 M Urea
- 8.0 % Acrylamide (using 40% stock)
- 1.0 X TBE buffer (pH 8.3)

Store in cool dark location

Just prior to pouring the gel, add to the solution of acrylamide:
- 0.04 g Ammonium persulfate per 70 ml of acrylamide solution (let it dissolve by swirling gently—the acrylamide will not polymerize rapidly until *Temed* is added)
- 25.0 µl Temed per 70 ml of acrylamide solution and swirl gently to mix. Do not over swirl — if the acrylamide solution has too much O_2 mixed in it will be slow to polymerize.

Pour gel immediately!

B. Centricon PCR product for sequencing template

Note: *Centricon-30* microconcentrators are not sterile — so store centriconed DNA frozen at -20°C.

1. Place 2 ml of sterile dH_2O in top half of Centricon-30 tube; add the PCR product, avoiding mineral oil which will cling to the plastic pipet tip (before dispensing the product wipe the pipet tip with a *Kimwipe*).
2. Label conical cap of the Centricon apparatus, place this cap on the top of half of centricon apparatus, and spin (ours works at 1500 g for 15-20 min in desk-top centrifuge fixed ≈ 45° angle).
3. Repeat this 1-2 more times (at least), dumping water from bottom half when needed.
4. Invert tube and spin 20 sec. to bring concentrated sample into tip of conical tube.
5. This should yield about 20-50 µl of sample (ours yields about 20-25 µl). Store this at -20°C.

Optional: To recycle Centricon-30 microfuge tubes, stir in 50 % EtOH for a few hours. We have been doing it with no adverse effects *and* great savings! We usually recycle tubes only once.

C. Double-stranded sequencing template

Method 1: (This method is from Perkin ELmer Cetus)

1. Denature centriconed double-stranded PCR product by bringing it to 0.2 M NaOH.
2. Neutralize with 0.3 volumes of 7.5 M NH_4OAc.
3. Precipitate in 3 volumes 95 % EtOH.
4. Wash with 70 % EtOH.
5. Dry & re-suspend in 7 µl H_2O.
6. Make Buffer Reaction described below in step 1 of section "E".
7. Heat to 95°C for 2 min. then quench quickly on ice.
8. Go on to chain termination reaction.

Method 2:

1. Heat 7 µl of centriconed double-stranded PCR product, 1 µl of primer for sequencing reaction, and 2 µl of 5x Sequenase® buffer in an Eppendorf tube to 94°C for two minutes.
2. Immediately quench in dry-ice/ethanol for several minutes.
3. Put into a dry-bath block which has been chilled to 0°C and allow the block to come to room temperature.
4. Proceed to run the labeling reaction (Step 2 below in "E")

D. Sequencing Reactions

1. Use 7 µl of the Centricon-30 purified ssDNA template for sequencing (or from one of the double-stranded sequencing method). To this, add 1 µl of 10-100µM primer and 2 µl of *5X Sequenase® buffer.* Incubate at 65°C for 2-5 min and then let cool to room temperature slowly (for 15 min or so).
2. Add 2.5 µl of ddGTP (labeled *Mix G*), ddATP (labeled *Mix A*), ddTTP (labeled *Mix T*), and ddCTP (labeled *Mix C*) to separate tubes (or racks). These are the *termination mixes.* For each template there should be four separate tubes labeled G, A, T and C.
3. Make up the following reaction mixture for each template (always make 1 extra solution):

1.0 µl	DTT (Dithiothreitol, 0.1 M)
2.0 µl	dH_2O (that's right, no *Labeling mix* is used! We have found that there seems to be enough nucleotides left over from the centriconing and also in the ddNTP mixes to sequence just fine. Besides, you get right up next to the primer with this protocol! Use a $\frac{1}{100}$ dilution of the *Labeling mix* to sequence a little farther from the primer.)
1.75 µl	1 X TE (do not use the Sequenase® dilution mix—we have gotten better results with TE in side by side comparisons).
0.5 µl	$^{A}_{C}TP\text{-}S^{35}$ (S^{35} is recommended, but you can use P^{32} — if you are suicidal)
0.25 µl	Sequenase®

Note: Our modification of the Sequenase® protocol (i.e. adding H_2O in place of the *Labelling mix*) means that either radioactively labeled ATP or CTP can be used in the "Labeling" reaction.

4. Add 5.5 µl of the reaction mix from #3 (above) to template mix from #1.
5. Pipette 3.5 µl of the reaction mix from #4 to each of the four tubes from #2 and incubate for 2-5 min at room temperature (it is recommended that you do not let this reaction go longer then 5 minutes). If you get alot of non-specific termination in the sequencing reactions, try doing this step at 37°C.
6. Add 4 µl of *Stop Solution* to each ddNTP mix in #5.
7. Store reactions in the freezer until ready to load onto acrylamide gel (if you happened to use P^{32} you can not store this reaction for more than a day, reactions using S^{35}, however, can be stored for many days, if not weeks).

E. Running the Sequencing Gel

1. Pre-warm the sequencing gel to running temperature (usually take 20-30 minutes). The sequencing gel has to be run so that its temperature is warm (about 50-55°C) to keep the DNA in the gel from forming secondary structures. The high urea concentration in the gel also assists in this. Beware: if the gel get too hot you run the risk of cracking your glass sequencing plates.
2. Heat samples to 95°C for 2 minutes to make the sequencing samples single-stranded.
3. Rinse the sequencing gel's wells to remove urea that has diffused out of the gel and will keep the sequencing samples from entering the well. Load 3-4 µl of samples in gel well in the order of G, A, T and C. This way of loading the gel is not arbitrary. Depending on the direction (strand of DNA) that is sequenced with the primer used, you may be reading the complement of the message strand of DNA (the one that is usually published). By using this order when loading the sequencing gel, you merely flip the film over to read the complement ("G"s become "C"s and "A"s become "T"s and visa versa, i.e. their complement!).
4. Run the gel so that the leading dye just runs off the gel. This will hopefully result in you getting the bases very close to the primer. Run the gel longer if you want to get sequence further away from the primers or you can do 2-3 sequential loadings spaced a few hours apart (obviously run the gel a lot longer using sequential loadings — with the last loading running until the leading dye just falls off the gel).
5. If you used S^{35} in your sequencing reaction, after electrophoresing, the sequencing gel must be soaked in 5 % acetic acid and 5 % methanol solution for about 15-30 minutes. This removes the urea from the gel which blocks the beta particles emitted from the S^{35} and prevents the radiation in the gel from exposing the film. (omit this step if you used P^{32}.)
6. Dry the gel thoroughly (if the gel is still wet it will stick to the film and the liquid itself blocks the radiation from S^{35}) and expose to film for 1-2 days (usually 2 days).

PRIMER DESIGN TIPS

We have a fair amount of experience in making primers — both good and bad. Some of our tips are ones that were compiled by Chris Simon with input that she obtained from T. Kocher, C. Orgego, K. Thomas, S. Paabo, D. Irwin, and M. Stoneking while in A. Wilson's lab at U.C. Berkeley in the summer of 1988. These tips are for designing primers that have the best chance of working on a number of groups, not just the species designed from.

1. Primers can be as small as 18 nucleotides but usually run from 20 - 24 nucleotides. Primers larger then 25mers are not thought to be more specific than a 25mer.

2. If you are designing blind (without any sequence information for the species or group that you are interested in) align as much sequence information for the region that you are interested in from as many species available (use the complete mtDNA sequence references provided with this guide) and pick very conserved regions. RNA coding regions offer a lot of conserved regions even if you are interested in protein coding regions. Look in tRNA coding regions that often surround protein coding regions for conserved sequences (it would be wise to try and end the primer on the three nucleotide bases that code for the tRNA attachment since these will be extremely conserved). Otherwise, look for highly conserved amino acid sequences for primers within protein coding regions.

3. It is believed that the 3' end of the primer must match exactly for the polymerase to start adding nucleotides so do not end a primer on a third codon position in protein coding regions. Logically it seems best to end on a second codon position since these are least variable.

4. Try to pick primers that have codons that are non-degenerate (i.e. they belong to a one codon family). If your primer has four-fold degenerate sites that probably vary in the groups that you want to examine, you may want to use a degenerate primer as a last resort or non-Watson-Crick base pairing at those degenerate sites.There can be at least 3 mis-matches in at least one of the primers for the primers to work well during amplification (with a 22mer)

5. The primers should have an even G,A,T and C composition. Avoid repeats like the plague and also make sure your primers do not complement each other! It may also be important not to end on the 3' end of the primer with lots of G's or C's since even a few of them may form strong enough bonds to lead to mis-priming.

6. Some people design primers with a restriction site attached to the 5' end for later use in cloning.

7. A general rule of thumb for the estimated annealing temperature of any given primer is 4°C for every G or C and 2°C for every A or T, and the total number minus 10°C.

PCR PRIMERS

The mtDNA oligonucleotide primers that follow are all written in the 5' to 3' direction. A figure at the end of this section (page 21) diagrams the *approximate* primer locations and the direction they amplify in vertebrate, sea urchin and *Drosophila* mtDNA. Codon spacing is used when appropriate. Primers are compared to the human, mouse, cow, Xenopus (*Xenopus laevis*), urchin (*Strongylocentrotus purpuratus*) and Drosophila (*Drosophila yakuba*) mtDNA sequences with their locations as described in the references contained in the "References to Complete mtDNA Sequences" section (page #).

12s RNA Primers

12sai (25mer)	5'- AAACTAGGATTAGATACCCTATTAT -3'	Position
Human	•••••G••••••••••••C•C•••	1067
Mouse	•••••G••••••••••••C•C•••	485
Cow	•••••G••••••••••••C•C•••	843
Xenopus	•••••G••••••••••••C•C•••	2495
Urchin	••••C••••••••••••••G••••	491
Drosophila	•••••••••••••••••••••••••	14588

12sbi (20mer)	5'- AAGAGCGACGGGCGATGTGT -3'	Position
Human	G••G•T••••••••G•••••	1478
Mouse	G••G•T••••••••G•••••	901
Cow	G••G•T••••••••G•••••	1262
Xenopus	G••G•T••••••••G•••••	2897
Urchin	G••••T••••••••••••••	855
Drosophila	•••••••••••••••••••••	14214

Comments: 12sai and 12sbi were made for cicadas (Insects) by Chris Simon based on Kocher *et al.* (1989). Some people use the human versions of these primers (12sa & 12sb — the so called "universal" primers)

12sc (20mer)	5'- AAGGTGGATTTGGTAGTAAA -3'	Position
Human	••••••••••••A•C•••••	1416
Mouse	•••A•••••••A••••••••	839
Cow	•••A•••••••A•C•••••	1200
Xenopus	••••C•••••••A•C•••••	2834
Urchin	Does not exist!	?
Drosophila	•••••••••••••••••••••	14275

12se (20mer)	5'- ATTCAAAGAATTTGGCGGTA -3'	Position
Human	•C••••••G•CC••••••G	1154
Mouse	•C••••••G•C••••••••	581
Cow	•C••••••G•C•••••••G	938
Xenopus	•CC•••••G•C•••••••G	2573
Urchin	•••••••••G•••••••••T	547
Drosophila	•C•T•••A•••••••••••	14521

Comments: 12se primer was made from/for cicadas by Chris Simon.

12st (24mer)	5'- GGTGGCATTTTATTTTATT--AGAGG -3'	Position
Human	••C••TGC••C••A•CCCTCT•••••	1175
Mouse	••C••T•C•••••A•••ATCT•••••	594
Cow	••C••TGC•••••A•CC•TCT•••••	951
Xenopus	••C••TGC•CC••ACCCACCT•••••	2586
Urchin	••C••TT••CC•AACCTCCCTG•••A	560
Drosophila	••C••T••••••G•C••C•--••••••	14503

Comments: This primer was made by Henrietta Croom from for tetragnathid spiders.

Cytochrome oxidase II Primers

CO2a (23mer)	5'- GGG GCT AAC CAT AGA TTC ATG CC -3'	Position
Human	••A ••A ••• ••C ••T ••• ••• ••	8189
Mouse	••A T•• ••• ••• ••C ••T ••• ••	7713
Cow	••• T•A ••• ••C ••T ••• ••A ••	7974
Xenopus	••A ••A ••• ••C ••C ••T ••A ••	9709
Urchin	••• ••• ••• ••• ••• ••• ••• ••	8312
Drosophila	••• ••• ••T ••• ••T ••T ••• ••	3682

Comments: This primer was based on the urchin sequence. It is in a region of high amino acid conservation, but is not very useful for anything but sea urchins.

ATPase 6 Primers

ATP6 (22mer)	5'- G TGC GCT TGG TGT TCC CTG TGG -3'	Position
Human	• G•G TG• A•• ••• G•• T•• •••	8936
Mouse	• ••A AA• ••• A•• ••• T•• •••	8333
Cow	• ••G AG• G•• ••• ••• T•• •••	8698
Xenopus	A ••G TG• ••• ••• ••• T•C A••	9709
Urchin	• ••• ••• ••• ••• ••• ••• •••	9039
Drosophila	A AAT TGC A•• ••• A•• T•• A••	4478

Comments: Pretty poorly conserved at the 5' end, this primer works well only in Strongylocentrotid sea urchins, where it does a great job.

16s RNA Primers

16sa (20mer)	5'- ATGTTTTTGGTAAACAGGCG -3'	Position
Human	••••••••••••••••••••	3079
Mouse	••••••••••••••••••••	1948
Cow	••••••••••••••••••••	2306
Xenopus	••••••••••••••••••••	3976
Urchin	••••••••••••••••••••	5093
Drosophila	••••••••••A•••••••••	13398

16sar (20mer)	5'- CGCCTGTTTATCAAAAACAT -3'	Position
Human	••••••••••••••••••••	3079
Mouse	••••••••••••••••••••	1948
Cow	••••••••••••••••••••	2306
Xenopus	••••••••••••••••••••	3976
Urchin	••••••••••••••••••••	5093
Drosophila	••••••••••T•••••••••	13398

16sb (22mer)	5'- ACGTGATCTGAGTTCAGACCGG -3'	Position
Human	•••••••••••••••••••••••	3058
Mouse	•••••••••••••••••••••••	2600
Cow	•••••••••••••••••••••••	2852
Xenopus	•••••••••••••••••••••••	4573
Urchin	•••••••••••••••••••••••	5662
Drosophila	••A••••••••••••A•••••	12888

16sbr (22mer)	5'- CCGGTCTGAACTCAGATCACGT -3'	Position
Human	•••••••••••••••••••••••	3058
Mouse	•••••••••••••••••••••••	2600
Cow	•••••••••••••••••••••••	2852
Xenopus	•••••••••••••••••••••••	4573
Urchin	•••••••••••••••••••••••	5662
Drosophila	•••••T••••••••••••T••	12888

Comments: These 16s primers work for urchins, vertebrates, insects, corals, gastropods and just about anything else. The 16sar and 16sbr (the complements of 16sa and 16sb) primers face one another and can be used to amplify a 500-650 base fragment of the 16s RNA coding region from just about anything. Note that in *Xenopus*, there is a tandem repeat on the 3' side of the 16sa primer. The insertions in the repeat should make it difficult for the primer to anneal there, however.

tRNA Primers

t-arg (24mer)	5'- CGAAATCAGAGGTTCTCCTTAAAC -3'	Position
Cow	••••••••TTTA••T•AT••T••A	10203
Urchin	•••••••••••••••••••••••	7380
Drosophila	•••••CT•ACT•CAA•TAA•CGCT	6102

Comments: Clearly not a good primer for anything but urchins.

t-Phe (20mer)	5'- TCTTCTAGGCATTTTCAGTG -3'	Position
Human	C•G••••AA••••••••••••	625
Mouse	C•A••••A••••••••••••	49
Cow	••A•••••••••••••••••	411
Xenopus	••A•••CA••••••••••••	2182
Urchin	C•••TG•A••••••••••••	52

t-Pro (20mer)	5'- CTACCTCCAACTCCCAAAGC -3'	Position
Human	•C•••ATT•G•A••••••••	15980
Mouse	•AC•AC•AGG•A••••••••	15701
Cow	TC•••AT••••C••••••••	15753
Xenopus	•C••TATTG••C••••••••	17510
Urchin	TACAT•G••••••••••••	

Comments: t-Phe and t-Pro go through the entire vertebrate D-loop (control region) based on Kocher *et al.* (1989). Successful amplifications have been obtained for Fish.

Cytochrome oxidase I Primers

CO1a (21mer)	5'- AGT ATA AGC GTC TGG GTA GTC -3'	Position
Human	G•• G•• T•• A•• G•• ••• •••	7227
Mouse	G•• G•• ••• A•• ••• ••• •••	6651
Cow	T•• G•• T•• A•• ••• ••• •••	7010
Xenopus	T•• ••• ••• ••• ••• ••• •••	8720
Urchin	T•• ••• G•• ••• ••• A•• •••	7108
Drosophila	••• G•• ••• A•• A•• ••• A••	2791

CO1c (22mer)	5'- TC GTC TGA TCC GTC TTT GTC AC -3'	Position
Human	•• ••• ••• ••• ••• C•A A•• ••	6454
Mouse	•T ••• ••• ••• ••A C•• A•T ••	5878
Cow	•• ••A ••• ••• ••A A•A A•T ••	6237
Xenopus	•• ••T ••• ••A ••A ••A A•• ••	7947
Urchin	•• ••• ••• ••• ••• ••• ••• ••	6335
Drosophila	•T ••A ••• ••A ••A G•• A•T ••	2018

CO1d (27mer)	5'- GAA CAT GAT GAA GAA GTG CAC CTT CCC -3'	Position
Human	••• T•• ••• AGT ••• A•• G•T T•• GG•	7258
Mouse	••• ATA ••• •GA ••• ••• GGA T•• TG•	6582
Cow	A•• T•• A•• TGC ••• ••• G•T T•• GG•	6940
Xenopus	A•• T•• T•C TCC A•• A•• G•T T•• T•G	8650
Urchin	••• ••• T•• ••• ••• ••• A•• ••• T••	7039
Drosophila	T•• ACA T•A T•• T•• A•T G•• T••-TTA	2723

Comments: The CO1d primer was designed for Echinometrid sea urchins, and works well only within this group.

CO1e (23mer)	5'- CCA GAG ATT AGA GGG AAT CAG TG -3'	Position
Human	••T ••• •A• ••G ••A ••• ••• ••	7110
Mouse	G•T ••A •A• •AT ••• ••• ••• ••	6533
Cow	••T ••• •A• ••T ••• ••• ••A ••	6892
Xenopus	••• •TA •A• •AC ••• ••• ••• ••	8602
Urchin	••• ••• •AG ••G ••A ••C ••• ••	6992
Drosophila	••• •TA •A• •AT ••• T•• ••• ••	2672

CO1f (20mer)	5'- CCT GCA GGA GGA GGA GAY CC -3'	Position
Human	••C ••C ••• ••• ••• ••C ••	6569
Mouse	••C ••T ••• ••• ••G ••C ••	5990
Cow	••G ••• ••• ••• ••• ••C ••	6431
Xenopus	••• ••C ••• ••• ••T ••C ••	8061
Urchin	••• ••• ••• ••G ••• ••T ••	6451
Drosophila	••A ••T ••• ••• ••• ••T ••	2131

where Y = C or T

Comments: CO1e and CO1f make superb dsDNA amplification. ssDNA amplifications often smear for both primers. Sequencing is difficult with the CO1f primer, perhaps because of its small size and degeneracy. Successful amplifications have been obtained for sharks, lamprey, fish, sea urchins (CO1f only), corals. Note that CO1f is a degenerate primer.

NADH dehydrogenase Primers

ND4c (18mer)	5'- TAC TCC CTA TAC ATA TTT -3'	Position
Human	••• ••• ••C ••• ••• •••	11975
Mouse	••• ••A A•• ••• ••• A••	11187
Cow	••T ••T ••• ••• ••G C•A	11748
Xenopus	••• ••T ••C ••• ••• ••C	13488
Urchin	••• ••• ••• ATG ••• •••	11888

ND5a (21mer)	5'- GAA TTC TAT GAT CGA TCA TGT -3'	Position
Human	••• ••• ••• ••• G•• C•• •••	12650
Mouse	••G ••G ••• A•• T•• ••• •••	12055
Cow	••• ••• ••• A•• A•• ••• •••	12420
Xenopus	A•• ••• A•• ••• A•• ••• •••	14170
Urchin	••• C•• C•• ••• ••• ••• •••	12486

Comments: NADH primers are not really good primers. Amplifications are inconsistent and should be performed at annealing temperatures of 45°C or lower. However, we have obtained sequence for approximately 250 bp from the ND4 primer. Successful amplifications have been obtained for fish.

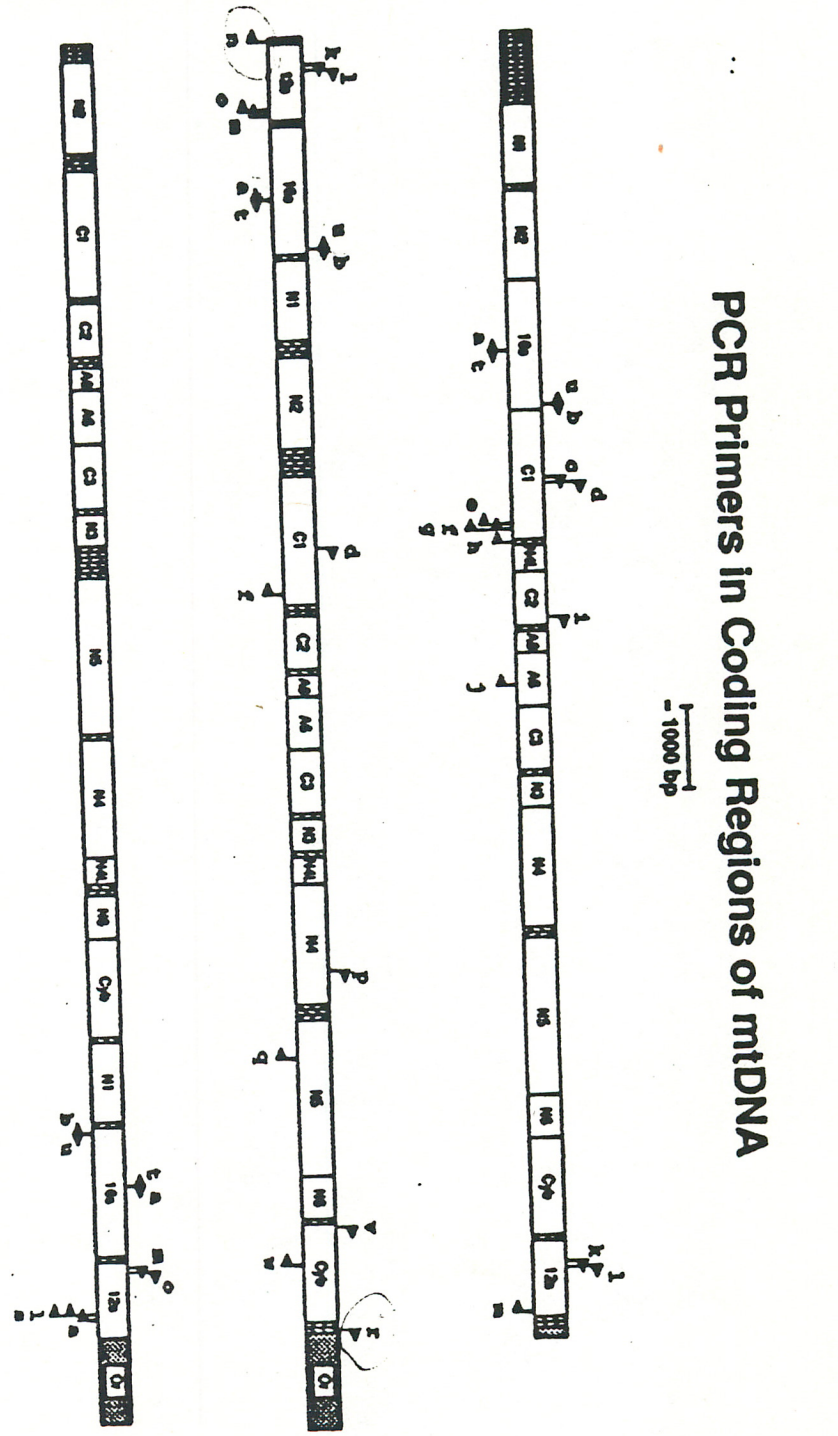
Cytochrome b Primers

Cyb1 (26mer)	5'- CCA TCC AAC ATC TCA GCA TGA TGA AA -3'	Position
Human	••• ••• ••• ••• ••C ••• ••• ••• ••	14817
Mouse	••• ••• ••• ••T ••• T•• ••• ••• ••	14208
Cow	••• ••A ••• ••T ••• T•• ••• ••• ••	15753
Xenopus	••• ••A ••• ••T ••• T•• •T• ••• ••	16321
Urchin	••C ••• ••• C•T ••C ATT ••G ••• ••	14581

Cyb2 (24mer)	5'- CCC TCA GAA TGA TAT TTG TCC TCA -3'	Position
Human	••• ••• ••• ••• ••• ••• ••• •••	15175
Mouse	A•• ••• ••• ••• ••• ••• ••• •••	14565
Cow	T•• ••• ••• ••• ••• ••• ••• •••	15753
Xenopus	••• ••• A•• A•• ••• ••• ••• •••	16677
Urchin	AG• ••• A•• G•• C•• ••• C•• C••	14937

Comments: These primers seem to work fairly well with most vertebrates. They are based on Kocher *et al.* (1989)

A linearized map of gene order in mtDNA of three taxa showing the approximate positions at which 23 oligonucleotide primers bind, and the direction of subsequent polymerase extension. Gene regions are named as in Jacobs *et al.* (1988), and tRNA genes are shown with hatching. Relative size of the gene regions are approximations!



CONTRIBUTORS

Cann, Rebecca
Department of Genetics
University of Hawaii

Croom, Henrietta B.
Department of Biology
University of the South

Hansell, Deborah
Department of Anatomy and Reproductive Biology
University of Hawaii

Kessing, Bailey D.
Department of Zoology
University of Hawaii

Martin, Andrew P.
Department of Zoology
University of Hawaii

McIntosh, Carl
General Science/Department of Zoology
University of Hawaii

McMillan, W. Owen
Department of Zoology
University of Hawaii

Metz, Edward
Department of Zoology
University of Hawaii

Palumbi, Steve R.
Department of Zoology
University of Hawaii

Simon, Christine
General Science/Department of Zoology
University of Hawaii

Tashima, Lily
Department of Anatomy and Reproductive Biology
University of Hawaii

Wilson, A.C. et al.
Department of Biochemistry
U.C. Berkeley
Berkeley, CA 94720

REFERENCES TO COMPLETE MTDNA SEQUENCES

Human..... **Nature 290:457-465.**

Mouse..... **Cell 26:167-180.**

Cow ..... **J. Mol. Biol. 156:683-717.**

Xenopus..... **J. Biol. Chem. 260:9759-9774.**

Urchin..... **J. Mol. Biol. 202:185-217.**

Drosophila ..... **J. Mol. Evol. 22:252-271.**

SELECTED REFERENCES TO PCR

Higuchi, R., von Beroldingen, C.H., Sensabaugh, G.F., and Erlich, H.A. 1988. DNA typing from single hairs. Nature 332: 543-546.

Erlich, H.H., Gelfand, D.H., and Saiki, R.K. 1988. Specific DNA amplification. Nature 331: 461-462.

Saiki, R.K., Gelfand, D.H., Stoffe, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich, H.A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239: 487-491.

Stoflet, E.S., Koeberl, D.D., Sarkar, G., and Sommer, S.S. 1988. Genomic amplification with transcript sequencing. Science 2139:491-494.

Saiki, R.K., Scharf, S.J., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A., and Arnheim, N. 1985. Enzymatic amplification of ß-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230: 1350-1354.

Wrischnik et. al. 1987. Nucl. Acids Res. 15:529-542

Perkin-Elmer Cetus also puts out a complete PCR reference list that we have found very helpful.

ABBREVIATIONS

amp - amplification
ds - Double-stranded DNA
ss - Single-stranded DNA
PCR - Polymerase Chain Reaction
3' - The three prime end of a strand of DNA or RNA
5' - The five prime end of a strand of DNA or RNA
dH_2O - distilled water (sterile)