THE POLYMERASE CHAIN REACTION: DNA EXTRACTION AND AMPLIFICATION

Chris Simon[1,2], Adrian Franke[2], and Andrew Martin[2]

Ecology and Evolutionary Biology
University of Connecticut
Storrs, CT 06269

Zoology Program
University of Hawaii
Honolulu, HI 96822

The polymerase chain reaction (PCR) is a method of cloning DNA without the use of microorganisms (Saiki et al. 1985, Mullis et al. 1986). In five short years since the development of PCR, this technology has been modified for many uses (Innes et al. 1990) and has essentially revolutionized molecular biology (Guyer and Koshland 1989). PCR allows the rapid selection, isolation and amplification of DNA regions of interest from small amounts of tissue and can be used to help prepare DNA for sequencing. Because it works well for small amounts of tissue and for small pieces of DNA, PCR allows the examination of nucleotide sequences from ancient preserved specimens which have been dried, frozen, hidden in anaerobic sediments, or soaked in alcohol or formalin (Paabo 1990). The greatly increased speed of extraction, amplification, and sequencing has made nucleotide sequence data available on the large scale necessary for population biological and systematic studies. If sequencing studies turn up consistent differences in nucleotide sequence among populations or taxa, PCR can be combined with RFLP analysis to rapidly screen for these known differences. Dot blot analysis with allele specific probes or allele specific PCR primers can be used for a similar purpose (Innes et al. 1990).

PCR makes use of 18-25 bp pieces of DNA called primers which flank the region to be copied and create starting points for strand synthesis. These primers must be homologous or nearly homologous to segments of the sample mtDNA bordering the region to be cloned. If the nucleotide sequence of the sample mtDNA is not known, potential primers must be chosen based on sequences of close relatives. For taxa for which no close relatives have been sequenced, highly conserved regions of DNA can serve as "universal" primers for DNA amplification (Kocher et al. 1989, Simon et al. 1990). For

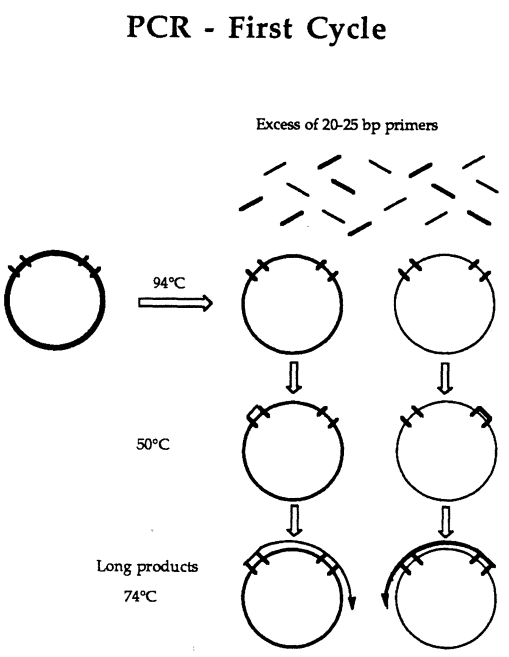
NATO ASI Series, Vol. H 57
Molecular Techniques in Taxonomy
Edited by G.M. Hewitt et al.
© Snrin er-Verlag Rerlin Heidelberg 1991

amplification of protein coding regions for unstudied species, degenerate primers can be used which are designed to include a mixture of possible primers all matching a known conserved amino acid sequence. The substitution of nucleotides by the non-specific base analog inosine in degenerate positions increases the universality of a primer (Linz et al. 1990). Tips for constructing PCR primers are provided in Appendix 1. References useful for designing primers are provided in Appendix 2. Useful PCR primers with varying degrees of conservation are provided in Appendix 3. A discussion of the choice of particular DNA regions appropriate for various taxonomic levels of investigation is given in part 1 of this book (Simon, this volume).

The PCR reaction takes place in three basic steps: template denaturation, primer annealing, and new strand extension (Figure 1). The reaction mixture contains template DNA, two primers each complementary to opposite strands, buffer, the four nucleotides in equal proportions, and a thermo-stable polymerase enzyme (Taq polymerase). In the first step, the temperature is raised rapidly to 92-96° C and held there for 15 seconds to 1 minute. This causes the double stranded template DNA to dissociate. An initial denaturation of 3-4 minutes prior to the first cycle improves the efficiency of the reaction (Adding the Taq polymerase after this initial denaturation step will prolong the life of the enzyme). For the second step, the temperature is dropped rapidly to approximately 50° C. This allows annealing of the oligonucleotide primers. The excess of primers assures that they will compete successfully with the double strands of the original template molecules for annealing. We use a range of annealing temperatures from 45 to 72° C; 50° C is a good starting point. The lower the temperature, the less specific the annealing, therefore we recommend using as high a temperature as possible to avoid mispriming. In the third, extension, step of the PCR cycle, the temperature is raised to 72-74° C for maximum efficiency of the Taq polymerase. At this temperature, Taq has been found to add between 35 and 100 nucleotides per second such that an extension time of one minute is sufficient to amplify up to 2 Kb (Innis and Gelfand 1990), although empirically we have found 2 minutes to work well. The cycle ends when the temperature is again raised to 92° C and the strands dissociate. The number of cycles necessary varies between 25 and 40 depending on the number of target molecules in the initial reaction.

Figure 1. Diagrammatic representation of the PCR Cycle. A) In the first cycle of PCR, dissociation at 94 degrees C is followed by annealing of the primers to the complementary strands, and finally by extension at 74 degrees C. Long products are produced from the dissociated circular mtDNA strands because extension is terminated only by a new cycle of denaturation or the Taq enzyme falling off. B) In the second cycle of a PCR reaction a second set of long products is generated for each molecule of starting template DNA. This is not shown because it is identical to the diagram in A. In addition, one set of short products is produced from the long products. Extension of the short product stops when the end of the long product is reached. C) In all subsequent cycles of PCR, one set of long products is produced from each molecule of starting template DNA (not shown) and short products are produced from long products and from existing short products. Thus, short products increase exponentially while long products accumulate linearly.

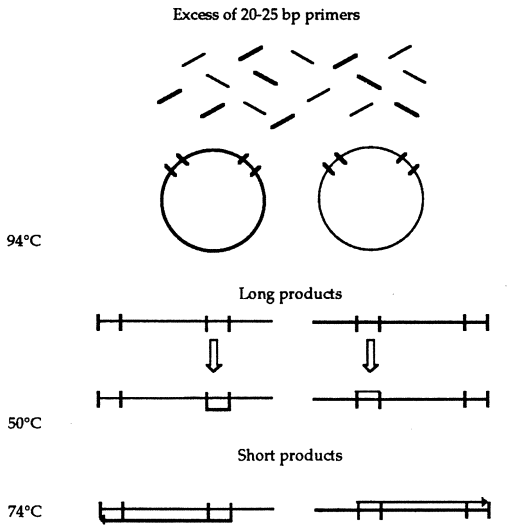
A.



B.

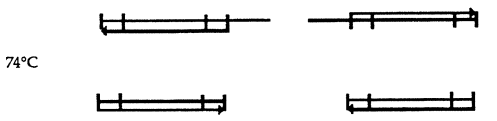
PCR - Second Cycle

Short Products Produced From Long Products



C.

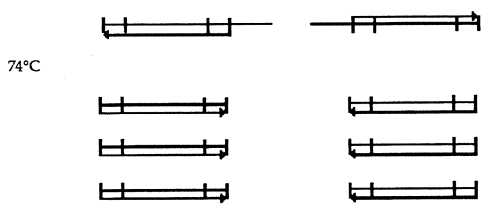
Short Products of Third Cycle



Short products: multiply exponentially

Long products: one set produced each cycle per molecule of starting template

Short Products of Fourth Cycle



In addition to the standard PCR reaction which produces a double stranded product, PCR can be modified to produce single stranded DNA (Gyllensten and Erlich 1988). In this "asymmetric amplification", one primer, the "limiting primer" is added in 50 to 100 times less concentration. Amplification proceeds exponentially until the limiting primer is exhausted. At this point, the reaction becomes linear and only one strand is produced. Two amplification mixtures are prepared each containing a limiting amount of one of the two primers. The limiting primer can be used for sequencing or a third, internal, primer can be used.

The protocols presented below were developed for extracting and amplifying mitochondrial DNA (mtDNA) from insect tissue but they vary little from the standard protocols used in PCR. The book, "PCR Protocols: A Guide to Methods and Applications" (Innes et al. 1990) provides detailed descriptions of basic PCR methodology as well as a wide variety of modifications. The protocols provided here emphasize simplicity, efficiency, and applications to mitochondrial DNA, especially that of invertebrate taxa.

EXTRACTION PROTOCOLS

Most extraction protocols share the same basic steps: homogenization, destruction of extraneous or harmful proteins (such as nucleases), lysis of membranes containing DNA, and the separation of DNA from cell debris, digested proteins and other extraneous materials. Because PCR primers are specific, simple genomic DNA preps (containing mitochondrial and nuclear DNA) work well for mtDNA amplification. Thus, time consuming ultracentrifugation of DNA in a cesium gradient can be avoided. Below we present two protocols: A standard genomic protocol and a simplified genomic protocol for use with small amounts of tissue. In the standard protocol, we have noted where amounts of certain components can vary. Obviously, these amounts are not critical. Success of each recipe may vary depending on the sample organisms. In the second protocol, the ability of PCR to work from small amounts of tissue allows the omission of centrifugation, unpleasant phenol/chloroform extractions, and ETOH precipitation. We have just begun using this technique and have obtained excellent amplifications for periodical cicadas for the 12A & B primer set (Appendix 3).

Extraction of Genomic DNA (Periodical cicada used as an example) for Mitochondrial DNA Amplification.

1. Homogenize entire insect (trim wings, legs, excess chitin). Flight muscles and egg masses are excellent sources of mitochondrial DNA. Amount of homogenization buffer will depend on the amount of tissue used.

HOMOGENIZATION BUFFER:	10 mM NaCl	.584 g	
	50 mM Tris HCl	6.05 g	(pH 7.5 in 1M soln.)
	100 mM EDTA	37.22 g	
	250 mM Sucrose	85.57 g	
	Sterile Distilled Water	to 1 L	

2. Centrifuge 10 minutes at 3.5K rpm. Transfer supernatant to new tube discard pellet (mostly nuclei and cell debris). This and the following step increase the ratio of mitochondria to nuclei and are optional.
3. Centrifuge 20 minutes at 13K rpm. At this speed the mitochondria will pellet. Save pellet and dissolve in 700 microliters sterile distilled water.
4. Transfer to 1.5 ml eppendorf and add: 70 µl 10.0% SDS (to make 1%), and Proteinase K (to make 100 µg/ml; extraction protocols are highly variable in amount of Proteinase K from 1 µg/ml to 500 µg/ml).
5. Incubate at 60° C overnight. (Other protocols: 37° C for 10 - 20 hours; or 55 - 65° C for 2-3 hours).
6. Extract once with 500 µl Phenol (equilibrated with Tris HCl pH 8 and containing 0.1 % 8-Hydroxy quinolin), then extract 3 times with 500 µl Chloroform/Isoamyl Alcohol (24:1).
7. Pipette aqueous phase into new 1.5 ml eppendorf and ppt. DNA with 2 volumes 95% ETOH. Spin at 10K rpm for 2 minutes.
8. Wash pellet with 70% ETOH and dry
9. Resuspend in 300 µl sterile distilled water.
10. Use 5 µl in PCR reaction. Note: mtDNA content varies among species and among individuals (see "Sample Concentration," below).

Simplified Extraction Protocol for Small Amounts of Tissue Modified from Kawasaki (1990).

1. Remove three eggs from one periodical cicada (or small amount of other tissue) and place in 35 µl lysis buffer:

LYSIS BUFFER: 10 mM Tris HCl pH 7.5-8.0
1 mM EDTA
1 % NonIdet (preferentially lyses mitochondria)
100 µg/ml Proteinase K

2. Add an equal volume of sterile distilled water.
3. Heat to 95° C for 3 min.
4. Use 1 µl in PCR reaction.

AMPLIFICATION PROTOCOL

10 X PCR Buffer: 670 µl 1M Tris HCl pH 8.8
20 µl 1M $MgCl_2$
83 µl 2M $(NH_4)_2SO_4$
227 µl sterile distilled water
1000 µl total

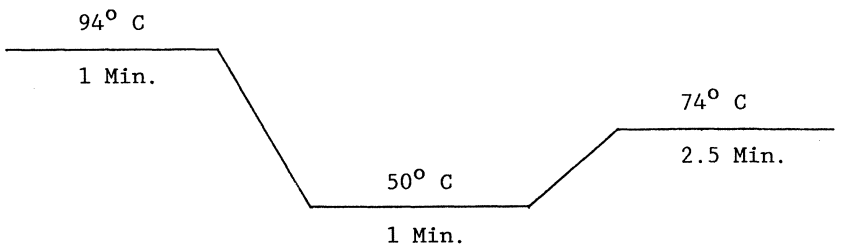
In a .5 µl eppendorf tube, make a 100 µl rxn using:

Volume		Final Concentration
1-5 µl extracted DNA		unknown (see below)
10 µl 10X PCR Buffer:		67.0 mM Tris HCl
		2.0 mM $MgCl_2$
		16.6 mM $(NH_4)_2SO_4$
5 µl primer 1	10µM stock	0.5 µM
5 µl primer 2	10µM stock	0.5 µM
10 µl dNTP's	10mM stock	1.0 mM total or .25 mM each
.5 µl Taq		1 unit

Optional:

	Bovine Serum Albumin	2 - 100	µg/ml (nuclease free)
or	Autoclaved Gelatin	100	µg/ml
	β-Mercaptoethanol	10	mM

Overlay with 2 drops mineral oil and amplify 30-35 cycles (Denature: 94° C; Anneal: 50° C; Extend: 72° C) as illustrated.



Following the reaction, run 10 µl of product with 4 µl dense dye on a minigel, with a known size standard (Phi-X HAE III). Stain with EtBr. A water control (all rxn components except template DNA) should always be run because contamination is a serious problem in PCR (see Kwok 1990 for procedures to minimize contamination). Minigels can be run with TBE or TA buffer.

		Amount	10X Concentration
10X TBE Buffer:	Tris base	215.60 g	890 mM
	Boric Acid	110.05 g	890 mM
	EDTA	14.80 g	20 mM
	Sterile dH_2O to 2.0 L		

Template Concentration. When extracting total DNA it is difficult to know the concentration of mitochondrial versus genomic DNA. Probing of each sample with a mitochondrial probe on a southern blot would provide an estimate of relative mtDNA concentration but is too time consuming for routine procedures. We have found considerable variation in the amount of total DNA from individual to individual. Total DNA concentrations can be equalized by eye by running 15 µl of each extraction on a minigel, staining with EtBr, comparing staining intensities among individual samples and diluting more concentrated samples. Although PCR is supposed to work from one or two molecules of starting DNA, often when comparing samples of the same species extracted in parallel, some samples simply will not amplify. A sample may be either too concentrated or too dilute to work. We recommend titration of sample concentration in both directions.

PCR Buffer. The PCR buffer may contain the salt $(NH_4)_2SO_4$, or KCl or no salt other than $MgCl_2$ (e.g. Pääbo 1990). Magnesium concentration effects primer annealing and enzyme activity. It may have to be re-adjusted for each primer pair for optimum amplification. Cetus recommends $MgCl_2$ titration over the range 1.5 - 4.0 mM and that the final magnesium concentration be at lest 0.5 to 1.0 mM higher than the total dNTP concentration (Oste 1989). We have found that it is useful to titrate each primer separately for the single stranded reactions.

Primer Concentration. If primer concentrations are too high, non-specific priming can occur (resulting in multiple products). High primer concentration can also increase the incidence of primer-dimers (see primer construction tips).

dNTP Concentration. The four dNTPs should be in equal concentrations. Innis and Gelfand (1990) recommend comparing nucleotide concentrations between 20 and 200 µM each dNTP. Low concentrations minimize mispriming.

Taq Concentration. Taq concentrations can be varied between .5 units per reaction to 2.5 units per reaction with good success. Concentrations necessary will vary with template and primers. Because Taq is expensive, it pays to try a titration series. High concentrations of Taq may result in non-specific products.

Asymmetrical amplifications. As described above, asymmetrical (single stranded) amplifications can be performed using the same protocol as for symmetrical (double stranded) reactions and varying the concentration of one of the primers to 1/100 or 1/50 that of the other. The number of cycles is increased from 35 to 40 to increase the amount of single stranded product produced during the linear phase of the reaction. Template DNA for the asymmetrical amplification can come directly from the original genomic extraction, from a double stranded PCR reaction, or from a band of double stranded product cut or melted (diluted 100X in water) from a 2-4% NuSieve agarose gel slice. If the template DNA for the asymmetrical amplification is contaminated with primers from the double stranded reaction or if the starting amount of amplified double stranded DNA is high enough, it may not be necessary to add limiting primer in order to obtain sufficient single stranded DNA for sequencing. We have tried all of these techniques and found that the relative success of each varies across species and primer pairs. It is not uncommon that in single stranded reactions, one primer will amplify better than its opposing partner. If this is the case, there are several options: sequence double stranded rather than single stranded template; kinase one primer and then digest one strand of a double stranded PCR reaction to produce single stranded DNA (see Kreitman, this volume); or add a third primer, external to the problem primer, for single stranded amplification. Amplify double stranded with the three primers. The problem strand will thus increase more than exponentially. Use 1 µl of this solution for single stranded reactions with the each of the two original primers, respectively (use no limiting primer). Sequence the single strands with the appropriate original primers. We have found that the problem primer as well as its opposing partner will sequence well with this technique.

To Prepare DNA for Sequencing: To purify DNA for sequencing, the reaction mixture can be concentrated (to approx. 30-40 µl) using a Centricon 30 microconcentrator[1] (Amicon, Danvers, MA) or an Ultrafree Micrọcentrator 30,000 (Millipore, Bedford, MA) which remove salts, excess nucleotides and primers. Both methods retain more DNA than ethanol precipitation and the latter is less expensive, faster to use, and works well. In our laboratory sequencing is performed using approximately 5-7 µl of the purified, concentrated single stranded product in a dideoxy sequencing reaction using the Sequenase enzyme system (U.S. Biochemical Co, Cleveland, OH).

ACKNOWLEDGEMENTS

Ideas presented in this paper benefited from discussions with and/or contributions from the following people: R. Cann, H. Croom, R. DeSalle, R. Harrison, D. Irwin, B. Kessing, T. Kocher, C. McIntosh, E. Metz, O. McMillan, C. Orego, S. Paabo, S. Palumbi, A. Phillips, A. Sidow, M. Stoneking, K. Thomas, and A.C. Wilson. This work was supported by NSF BSR 88-22710 to CS.

REFERENCES

Guyer RL, Koshland Jr. DE (1990) The molecule of the year. Science 246:1543-1544

Gyllensten UB, Erlich HA (1988) Generation of single-stranded DNA by the polymerase chain reaction ad its application to direct sequencing of the HLA-DQA locus. Proc. Natl. Acad. Sci. USA 85:7652-7656

Innis MA, Gelfand DH, Sninsky JJ, White TJ (1990) PCR Protocols. A guide to methods and applications. Academic Press, NY

Innis MA, Gelfand DH (1990) Optimization of PCRs. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR Protocols. Academic Press, NY, p 3

Kawasaki ES (1990) Sample preparation from blood, cells and other fluids. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR Protocols. Academic Press, NY, p 146

Kocher TD, Thomas WK, Meyer A, Edwards SV, Pääbo S, Villablanca FX, Wilson AC (1989) Dynamics of mitochondrial DNA sequence evolution in animals: Amplification and sequencing with conserved primers. Proc. Natnl. Acad. Sci. USA 86:6196-6200

---

[1] Amicon now recommends their new 100,00 MW filter which cleans up PCR products faster and more efficiently and retains pieces up to 125 bp with 90% efficiency. Millipore still recommends their 30,000 MW filter for PCR clean up stating that their filter is "not as tight."

Kwok S (1990) Procedures to minimize PCR-product carry-over. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR Protocols. Academic Press, NY, p 142

Linz U, Rubsamen-Waigmann H, Roesch R, and Seliger H. (1990) Inosine-substituted primers prevent false-negative PCR results due to 3' mismatches. Amplifications 4:14-15

Mullis K, Faloona F, Scharf S, Saiki RK, Horn G, Erlich H (1986) Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. Cold Spring Harbor Symposium on Quantitative Biology. 51: 263-273

Oste C (1989) Optimization of magnesium concentration in the PCR reaction. Amplifications 1:10-11

Pääbo S (1990) Amplifying ancient DNA. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR Protocols. Academic Press, NY, p 159

Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N (1985) Enzymatic amplification of B-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230: 1350-1354

Simon C, Pääbo S, Kocher T, Wilson AC (1990) Evolution of mitochondrial ribosomal RNA in insects as shown by the polymersase chain reaction. In: Clegg M, O'Brien S (eds) Molecular Evolution. UCLA Symposium. Alan R. Liss, Inc., NY, p 235

Appendix 1. Tips on primer construction. The first version of these primer tips was compiled by CS during a research visit to the laboratory of A.C. Wilson in July of 1988 and includes input from D. Irwin, T. Kocher, C. Orego, S. Pääbo, M. Stoneking, and K. Thomas. This updated version incorporates information from our subsequent experiences plus insights compiled in Innis et al. (1990).

1. Oligonucleotide primers should be approximately 18-25 bp long. Above 25 bp there is not much increase in primer annealing specificity.

2. To choose primers for unsequenced organisms, try regions which are known to be highly conserved. Remember to take into account possible gene rearrangements and differences in direction of transcription when choosing opposing primers. Use references in Appendix 2 for these purposes.

3. A convenient size DNA segment for PCR amplifications and sequencing is 400-500 bp. Amplifications of up to 10 Kb have been performed successfully and produce enough product to be visible on a EtBr stained gel. Pieces of 20 kb have been amplified and visualized by probing (Dan Clutter, pers. comm.). Once a reference sequence is obtained for the species under investigation, A useful strategy is to choose universal primers 1000 bp apart for the initial amplification and then to sequence with internal primers which are more specific to your particular organism.

4. The 3' end of the primer is most critical. The first three bases should be complementary. It has been shown that a match at the 3' end of a primer is essential for the success of PCR because Taq polymerase lacks a 3'-5' exonuclease activity (Linz et al. 1990). For this reason, it is best not to end a primer on the third position of a codon. A "T" at the 3' end of a primer has been shown to pair with any base (Kwok et al. 1990).

5. The 5' end of a primer is less critical. Some people add a restriction site to the 5' end (for later cloning or expression vector analysis). This site, of course, does not anneal in the first round of polymerization. Thereafter it is copied. In fact, any mismatches in the primer will be copied such that in later runs primers will match the newly created attachment sites perfectly. As a result, annealing temperature can be increased after about 5 cycles of PCR to increase priming specificity.

6. The base composition of a primer should be roughly equivalent for all four bases. G's and C's form stronger bonds that A's and T's. The annealing temperature should be as high as possible to prevent non-specific priming. For standard conditions annealing temperature should be 10 degrees C less than the melting temperature.

$$T_{melting} \text{ (for 20bp or less)} = (4 \times (G+C)) + (2 \times (A+T)),$$

where G+C = the total number of G's and C's in the primer, etc. Computer programs are available for calculating bonding energy of oligonucleotide primers (see Kreitman, this volume).

Appendix 1 (continued)

7. Runs of nucleotides should be avoided because they can pair with runs of nucleotides outside of the target sequence or complementary runs within the same primer. Avoid palindromic base sequences which could cause the primer to fold and pair with itself. Secondary structure in the template can also interfere with priming. Substitution of 7-deaza-2'-deoxyGTP for dGTP can help solve this problem (Innis and Gelfand 1990).

8. Primer pairs must not have complementary 3' ends. Primers with complementary ends can pair with each other and amplify to form what is known as primer artifacts or primer dimers (in multiples of the size of the original primers). During amplification, primer dimers compete for nucleotides, primers and enzymes and if they form early, can take over a reaction. Watson (1989) reports that, "primer artifacts may form even in the absence of obvious complementarity." He suggests that the Taq enzyme may hold the primers together long enough for extension to take place. Higher annealing temperatures can reduce primer dimers.

9. Primers can be made less specific to match variable positions in the target DNA by substituting inosine (which will pair with any base) or by making degenerate primers--a mixture of primers derived from an amino acid sequence containing all possible combinations of bases at degenerate codon positions (Compton 1990). Degenerate primers should not be degenerate on the 3' end (use the single-codon amimo acids methionine and tryptophan for this purpose).

10. If at first you don't succeed, repeat the same amplification. If the reaction fails, lower the annealing temperature. If still no product appears, dilute the template DNA. If this fails, try a different primer combination. Two oligonucleotides that look equally good sometimes are not.

Additional References:

Compton T (1990) Degenerate primers for DNA amplification. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR Protocols: A Guide to Methods and Applications. Academic Press, Inc. NY

Kwok S, Kellog DE, McKinney N, Spasic D, Goda L, Levenson C, Spinsky JJ (1990) Effects of primer template mismatches on the polymerase chain reaction: Human Immunodeficiency Virus Type I model studies. Nucleic Acids Research 18:999-1005

Watson R (1989) The formation of primer artifacts in polymerase chain reactions. Amplifications 2:5-6

Appendix 2. References useful for constructing PCR primers for mitochondrial DNA. Comparative gene order, direction of transcription, and level of conservation of nucleotides across species must be taken into account when designing primers.

Complete Mitochondrial DNA Sequences

HUMAN — Anderson S, Bankier AT, Barrell BG, de Bruijn MHL, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BP, Sanger F, Schreier PH, Smith AJH, Staden R and Young IG (1981) Sequence and organization of the human mitochondrial genome. Nature 290:457-465

COW — Anderson S, De Bruijn MHL, Coulson AR, Eperon IC, Sanger F and Young IG (1982) Complete sequence of bovine mitochondrial DNA. Conserved features of the mammalian mitochondrial genome. J Mol Biol 156:683-717

RAT — Gadaleta G, Pepe G, DeCandia G, Quagliariello C, Sbisa E, and Saccone C (1989) The complete nucleotide sequence of the Rattus norvegicus mitochondrial genome: cryptic signals revealed by comparative analysis between vertebrates. J Mol Evol 28:497-516

MOUSE — Bibb MJ, Van Etten RA, Wright CT, Walberg MW, and Clayton DA (1981) Sequence and gene organization of mouse mitochondrial DNA. Cell 26:167-180

CHICKEN — Desjardins P, and Morais R (1990) Sequence and gene organization of the chicken mitochondrial genome. J Mol Biol 212:599-634

FROG — Roe BA, Ma DP, Wilson RK, and Wong JFH (1985) The complete nucleotide sequence of the Xenopus laevis mitochondrial genome J Biol Chem 260(17):9759-9774

URCHIN — Jacobs HT, Elliott DJ, Math VB, and Farquharson A (1988) Nucleotide sequence and gene organization of sea urchin mitochondrial DNA. J Mol Biol 202:185-217

URCHIN — Cantatore P, Roberti M, Rainaldi G, Gadaleta MN, and Saccone C (1989) The complete nucleotide sequence, gene organization, and genetic code of the mitochondrial genome of Paracentrotus lividus. J Biol Chem 264:10965-10975

DROSOPHILA — Clary, DO and Wolstenholme DR (1985) The mitochondrial DNA molecule of Drosophila yakuba: Nucleotide sequence, gene organization, and genetic code. J Mol Evol 22:252-271

NEMATODE — Wolstenholme, et al. in prep. Gene order for Ascaris suum given in: Wolstenholme DR, MacFarlane JL, Okimoto R, Clary DO, Wahleithner JA (1987) Bizarre tRNAs inferred from DNA sequences of mitochondrial genomes of nematode worms. Proc. Natl. Acad. Sci. USA 84:1324-1328

Appendix 2 (cont.)

Compilations

srDNA — Neefs J-M, Van de Peer Y, Hendriks L, and DeWachter R (1990) Compilation of small ribosomal subunit RNA sequences. Nucl Acids Res 18 supplement:2237-2318

lrDNA — Gutell RR, Schnare MN, and Gray MW (1990) A compilation of large subunit (23S-like) ribosomal RNA sequences presented in a secondary structure format. Nucl Acids Res 18 supplement:2319-2330

tRNA — Sprinzl M, Moll J, Meissner F, and Hartman T. (1987) Compilation of tRNA sequences and sequences of tRNA genes. Nucl Acids Res 15 supplement:r53-r188

Partial mtDNA Sequences for Invertebrate Taxa

CICADA: Simon C, Pääbo S, Kocher T, and Wilson AC (1990) Evolution of the mitochondrial ribosomal RNA in insects as shown by the polymerase chain reaction. pp 235-244 In M Clegg & S O'Brien (eds.), Molecular Evolution. UCLA Symposia on Molecular and Cellular Biology, New Series, Vol. 122. Alan R. Liss, Inc., NY

LOCUST: Haucke H-R and Gellissen G (1988) Different mitochondrial gene orders among insects: exchanged tRNA gene positions in the COII/COIII region between an orthopteran and a dipteran species. Curr Genet 14:471-476

McCracken A, Uhlenbusch I, Gellissen G (1987) Structure of the cloned Locusta migratoria mitochondrial genome: restriction mapping and sequence of its ND-1 gene. Curr Genet 11:625-630

Uhlenbusch I, McCracken A, and Gellissen G (1987) The gene for the large (16S) ribosomal RNA from the Locusta migratoria mitochondrial genome. Curr Genet 11:631-638

CRICKET: Rand DM and Harrison RG (1989) Molecular population genetics of mitochondrial DNA size variation in crickets. Genetics 121:551-569

BEE: Crozier RH, Crozier YC, and Mackinlay AG (1989) The CO-I and CO-II region of honeybee mitochondrial DNA: Evidence for variation in insect mitochondrial evolutionary rates. Mol Biol Evol 6:399-411

Vlasak I, Burgschwaiger S, Kreil G (1987) Nucleotide sequence of the large ribosomal RNA of honeybee

Appendix 2 (cont.)

MOSQUITO: HsuChen C-C, Dubin DT (1984) A cluster of four transfer RNA genes in mosquito mitochondrial DNA. Biochem Int 8:385-391

HsuChen C-C, Kotin RM, Dubin DT (1984) Sequences of the coding and flanking regions of the large ribosomal subunit RNA gene of mosquito mitochondria. Nucl Acids Res 12:7771-7785

Dublin DT, HsuChen C-C, Tillotson (1986) Mosquito mitochondrial transfer RNAs for valine, glycine, and glutamate: RNA and gene sequences and vicinal genome organization. Curr Genet 10:701-707

DROSOPHILA: Clary DO, Wolstenholme DR (1987) Drosophila mitochondrial DNA: conserved sequence in the A+T rich region and supporting evidence for a secondary structure model of the small ribosomal RNA. J Mol Evol 25:116-125

DeSalle R, Freedman T, Prager EM, Wilson AC (1987) Tempo and mode of sequence evolution in mitochondrial DNA of Hawaiian Drosophila. Proc Natl Acad Sci USA 83:6902-6906

Garesse R (1988) Drosophila melanogaster mitochondrial DNA: Gene organization and evolutionary considerations. Genetics 118:649-663 (extends known sequence to 90% of coding regions)

Satta Y, Ishiwa I, Chigusa SI (1987) Analysis of nucleotide substitutions of mitochondrial DNAs in Drosophila melanogaster and its sibling species. Mol Biol Evol 4:638-650

URCHINS: Thomas WK, Maa J, and Wilson AC (1989) Shifting constraints on tRNA genes during mitochondrial DNA evolution in animals New Biologist 1:93-100

STARFISH: Jacobs HT, Asakawa S, Araki T, Miura K-I, Smith MJ, Watanabe K (1989) Conserved tRNA gene cluster in starfish mitochondrial DNA. Current Genetics 15:193-206

Smith MJ, Banfield DK, Doteval K, Gorski S, Kowbel DJ (1989) Gene arrangement in sea star mitochondrial DNA demonstrates a major inversion event during echinoderm evolution. Gene 76:181-185

FLATWORM: Garey JR and Wolstenholme DR (1989) Platyhelminth mitochondrial DNA: Evidence for early evolutionary origin of a $tRNA^{ser}$ AGN that contains a dihydrouridine arm replacement loop, and of serine-specifying AGA and AGG

Appendix 3. Mitochondrial PCR Primers. The following primers were designed for use with mtDNA. Many were designed to be universal. This compilation was updated from Kessing B, Croom H, Martin A, McIntosh C, McMillan WO, Palumbi S. 1989. The Simple Fools Guide to PCR. Version 1.0. Available from the authors.

Primers are named for the gene in which they are located followed by a letter designation indicating the order in which they were created. All primers are written in the 5' to 3' direction. Codon spacing is used when appropriate. Arrows following primer designation indicate direction of priming (opposing primers are on opposites strands). Direction of priming will be consistent across genes only within the taxa for which the primers were designed (e.g. Insects, Echinoderms, or Vertebrates). If opposing primers are located in different genes and are used for a taxon other that that for which they were designed, direction of priming should be checked by locating the primers in the most closely related sequenced species. Primers are compared to the human, mouse, cow, frog (Xenopus laevis), urchin (Strongylocentrotus purpuratus), fly (Drosophila yakuba) and miscellaneous other mtDNA sequences with their 5'-end positions on the light strand (with reference to humans) numbered as in the references contained in Appendix 2 (unless unnumbered in publication). Approximate sizes of pieces to be amplified can be obtained by subtracting location numbers of opposing primers. The figure at the end of this appendix illustrates gene order and direction of transcription in various taxa.

Primers ending in "r" are the reversed complements of existing primers which someone has tried and found to work (sometimes modified slightly to avoid any lack of conservation which may have been present at the 5' end of its reverse analog). All primers could theoretically be reversed.

12S RNA Gene Primers (Summary figure with locations follows 12S primers)

12Sai (25mer)►	5'- AAACTAGGATTAGATACCCTATTAT -3'	Position
Fly	.....	14588
Cicada	.....	Simon & Franke, unpubl.
Cricket (Gryllus)	.....	Rand & Harrison, unpubl.
Human	.....G.....C.C...	1067
Mouse, Rat	.....G.....C.C...	485, 486
Cow	.....G.....C.C...	843
Frog	.....G.....C.C...	2485
Urchin	....C.....G....	491

12Sair (20mer)◄	5'- AGGGTATCTAATCCTAGTTT -3'	Position
Cicada, Drosoph.	.....	as above

12Sbi (20mer)◄	5'- AAGAGCGACGGGCGATGTGT -3'	Position
Fly	.....	14214
Human	G..G.T.....G.....	1478
Mouse	G..G.T.....G.....	901
Cow	G..G.T.....G.....	1262
Frog	G..G.T.....G.....	2897
Urchin	G....T.....	855

Comments: 12Sai, 12Sair and 12Sbi were made for cicadas (insects) based on comparisons of Drosophila yakuba and Gryllus. The human versions of these primers are one set of the "universal" primers of Kocher et al. 1989. 12Sa has an 11 base pair core (in two pieces) which is conserved across eukaryotic, bacterial, plastid, and mitochondrial small ribosomal RNA genes in 103 out of 106 species examined. The 12Sb primer has a 14bp 3' end which is conserved across 96 of the same 106 species, with the exception of the A in the sixth position of D. yakuba (Simon et al. 1990) and S. purpuratus (Jacobs et al. 1988) The insect primer set 12Sai & 12Sbi will amplify vertebrate DNA and the vertebrate primer set 12Sa & b will amplify insect DNA, despite the mismatches near the 3' end in the 12Sa primers. Because of the 14 bp match of the 3' end of the 12Sb primer between nuclear and mitochondrial DNA, we had some concern that nuclear DNA might compete for this oligonucleotide (although we have obtained excellent sequences from this primer set, it does not work 100% of the time for cicadas). To avoid this potential problem, we designed a new primer, 12Sfi which was moved 8 bases upstream (3' end matches cicada):

				Position
12Sfi (20mer)◄	5'- CGGGCGATGTGTACATAATT -3'	Fly		14221
12Sbi (20mer)◄	5'- AAGAGCGACGGGCGATGTGT -3'	Fly		14214

Comments: 12Sfi works well for cicada, cricket, & planthopper.

12Sc (20mer)►	5'- AAGGTGGATTTGGTAGTAAA -3'	Position
Fly	.....	14275
Cicada	..AC.....AA.....	Simon et al. 1990
Human	.....A.C.....	1416
Mouse	....A.....A.....	839
Cow	....A.....A.C.....	1200
Frog	....C.....A.C.....	2834
Urchin	Does not exist!	----

Comments: Wilson lab primer modified to match Drosophila.

12Se (19mer)►	5'- ATTCAAAGAATTTGGCGGT -3'	Position
Cicada	.....	Simon et al. 1990
Fly	.C.T...A.....	14521
Human	.C.....G.CC.....	1154
Mouse	.C.....G.C.....	581
Cow	.C.....G.C.....	938
Frog	.CC.....G.C.....	2573
Urchin	.....G.....	547

Comments: The 12se primer is located at the beginning of Domain III of the 12S gene and works well for cicada.

12Sgi (18mer)►	5'- AAGTTTTATTTTGGCTTA -3'	Position
Fly	.....	14939
Human	.G....GG.CC.A..C.T	651
Cow	.G....GG.CC.A..C.T	434
Mouse	.G....GG.CC....C.T	72
Rat	.G....GG.CC....C.T	71
Frog	.G....GC.CC.A..C.T	2205
Urchin	GGTCC.AGTCCCAATC.T	82

Comments: 12Sgi primer was made for cicadas based on a plant rRNA sequencing primer of Elizabeth Zimmer's. It is located only a few bases away from the beginning of Domain I of the small ribosomal subunit RNA gene. It is not universal but a vertebrate primer could be made at this site which would work for a wide variety of vertebrates.

12Sg could be used in combination with 12Sb to amplify most of the 12s gene (about 750 bp), then the other 12S primers could be used as internal sequencing primers operating from this one amplification product. The diagram on the following page shows the location of all 12S primers on the 13-year cicada sequence (Simon & Franke, unpubl.)

12Sh (21mer)►	5'- GACAAAATTCGTGCCAGCAGT -3'	Position
Cicada	.....	Simon & Franke, unpubl.
Drosophila yakuba	...C.....G.....	14756
Drosophila virilis	...C.....G.....A.	
Human	.GTC..T.....CAC	877
Chimpanzee	.GTC..T.....T...CAC	Hixson & Brown, 1976
Gorilla	.GTC..T.....CAC	" " " "
Mouse, Rat	.GT...T.....CAC	296, 295
Cow	.GT...TC.....CAC	656
Frog	.GTC..TC.....-...CGC	2294

Comments: 12Sh primer was made for cicadas. Removing three bases from the 3' end would make it "universal."

12Sj (21mer)◄	5'- TACAAAACAGATTCCTCTG -3'	Position
Fly	.....G.....	14490
Cicada -Okanagana	..T.G.....	Simon, Paabo, McIntosh, unpubl.
-Dicerop.	..T.G.....	" " " "
-Magicicada	.....	Simon et al. 1990
Human	....G.....GC.....A	1195
Chimpanzee	....G.....GC.....A	Hixson & Brown, 1976
Gorilla	....G.....GC.....A	" " " "
Mouse, Rat	..T.G.....GC.....A	614, 614
Cow	..T.G.....GC.....A	971
Frog	....G.....GC.....A	2606

Comments: The 12Sh primer was made for cicadas. This primer could be made more versatile by omitting the 3' base.

12S REGION IN MAGICICADA TREDECIM SHOWING PRIMERS

```
      12SG➤
AAGTTTTATTTTGGCTTAAA~13 BASES AAAATTTTAATTATATTACATGCATAACTT 51


TAAATTGGTTGAAAATTATCTGAATGATAATTATCCAGAGCGCAGTATTAAGTATTATTA 111


                                                    12SH➤
ATTATTAATTTTAGAGGTAATAAATTTATTTATAGAGGACAAAATTCGTGCAGCAGTTGC 171


GTTATACGATTTTCTAAATTTAACTATGTTAGTTTCAGTTAAAAAGTGTGTTGATATTCA 231


ATTTTTTTAAATTTTGGTGGAATAAAATATAAATATGTGTTTAATTTTATGTCTGAGAAA 291


                    12S A➤
CTTTTATATAAAACTAGGATTAGATACCCTATTATTGAGAGTGTAAATAAAATAACTAGA 351
          TTTGATCCTAATCTATGGGA
               ◄12S Ar
                                    12S E➤
TTATTAATAGTTATGATCTTTAAATTCAAAGAATTTGGCGGTAATTTATCTAATCAGAGG 411
                                                          GTCTCC


AATCTGTTTTGTAATTGATAATCCACGATAGATTCTATTTTAAATAAATTTGTATACCTC 471
TTAGACAAAACAT
◄12S J

TGTCAAGAATGTTTTATCAGAATAATTTTCATTTGTTTTATTAATAAAATGTCAGGTCAA 531


GGTGCAGTTAATTTTAAAGAAAAAATGGATTACATTATTGTAAAAAATGAATTGTTTTCT 591


                     12S C➤
ATAATGAAAATCATGAAACTGGATTTGAAAGTAAATTTCATTAAATATGTGTTTTTGAAT 651


TTAGGTTCTAAATT                                                665
        ◄TTAATACATGTGTAGCGGGC  12S F
              ◄TGTGTAGCGGGCAGCGAGAA  12S B
```

16s RNA Primers

16sa (20mer)◄	5'- ATGTTTTTGTTAAACAGGCG -3'	Position
Fly	.....	13398
Locust	.....	690
Human	.....G.....	2491
Mouse	.....G.....	1927
Cow	.....G.....	2306
Frog	.....G.....	3976
Urchin	.....G.....	5093

16sar (20mer)►	5'- CGCCTGTTTAACAAAAACAT -3'	Position
Fly	.....	13398
Locust	.....	690
Human	.....C.....	2491
Mouse	.....C.....	1948
Cow	.....C.....	2306
Frog	.....C.....	3976
Urchin	.....C.....	5093

16sb (22mer)►	5'- ACGTGATCTGAGTTCAGACCGG -3'	Position
Human	.....	3058
Mouse	.....	2501
Cow	.....	2852
Frog	.....	4573
Urchin	.....	5662
Fly	..A.....A.....	12888
Locust	..A.....	163

16sbr (22mer)◄	5'- CCGGTCTGAACTCAGATCACGT -3'	Position
Human	.....	3058
Mouse	.....	2501
Cow	.....	2852
Frog	.....	4573
Urchin	.....	5662
Fly	.....T.....T..	12888
Locust	.....A..	163

Comments: The 16Sa primer was developed for cicadas from comparisons of crickets and Drosophila. The 16Sb primer is modified from the Wilson lab 16S1 primer. The 16Sar and 16Sbr primers face one another and can be used to amplify a 500-650 base fragment. 16Sa can be used with 12Sa in insects to amplify amplify approximately 1200 bp. These 16S primers work for urchins, vertebrates, insects, corals, gastropods and probably many other taxa as suggested by the extreme conservation illustrated above. In fact, there are many conserved sites in this gene which would make good universal primers and many labs have taken advantage of this (e.g. within 100 base pairs downstream of our 16Sa primer, the Wilson lab has their 16S2 primer and the Templeton lab has their 16SMid primer). Uhlenbush et al. (1987) illustrate these conserved regions in relation 16S rRNA structure.

Cytochrome oxidase I Primers

COla (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
Frog	T..	8720
Urchin	T.. ... G.. ... ... A.. ...	7108
Fly	... G.. ... A.. A.. ... A..	2791

COlc (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
Frog	.. ..T ... ..A ..A ..A A.. ..	7947
Urchin	.. ..	6335
Fly	.T ..A ... ..A ..A G.. A.T ..	2018

COld (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
Frog	A.. T.. T.C TCC A.. A.. G.T T.. T.G	8650
Urchin	... ... T.. ... ... A.. ... T..	7039
Fly	T.. ACA T.A T.. T.. A.T G.. T.. TTA	2723

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

COle (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
Frog	... .TA .A. .AC	8602
Urchin	... ... .AG ..G ..A ..C	6992
Fly	... .TA .A. .AT ... T..	2672

COlf (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
Frog	... ..C ... .T ..C ..	8061
Urchin	... ...G ... ..T ..	6451
Fly	..A .T ... ... .T. ..	2131

where Y = C or T

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

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
Frog	..A ..A ... ..C ..C ..T ..A ..	9709
Urchin	... ...	8312
Fly	... .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.

Cytochrome oxidase III Primers

Co3a (20mer) ➤	5'- TTATTTATTGCATCAGAAGT -3'	Position
Locust	.....	2038
Fly	.....TT.....	4995
Human	C.....A.C.....	9459

Co3b (20mer) ➤	5'- TCAACAAAGTGTCAGTATCA -3'	Position
Locust	.....	2503
Fly	.....A.....	5460
Human	..T.....A..C.....	9924

Comments: Co3a & b primers were made for cicadas using sequence information from Haucke and Gellissen (1988). These primers face each other. They should work well for most insects. Many more COIII primer sites can be found in this same reference as well as primers for COII and ATPase 6 & 8.

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
Frog	A ..G TG. ... ... T.C A..	9709
Urchin	. ...	9039
Fly	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.

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
Frog	... ..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
Frog	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, sequence has been obtained 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
Frog	... ..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
Frog	... ... 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).

tRNA Primers: As illustrated in the figure at the end of this appendix, tRNA genes are rearranged considerably among major groups of animals (Jacobs et al. 1988, Wolstenholme et al. 1987). In addition, they may exhibit minor variation in location within animal classes (e.g. Insecta: Crozier et al. 1989, Haucke and Gellissen 1988; Mammalia: Paabo, pers. comm.). Thus even when tRNA sequences are highly conserved between two organisms, the same primer pairs may not function together due to their relative locations. Note also that there are instances where some tRNA's have lost their

function and evolve at higher than normal rates (e.g. Thomas et al. 1989, Cantore et al. 1987), that mitochondrial tRNA are degenerate in nematodes (Wolstenholme et al. 1987) and that Chlamydomonas and Tetrahymena lack some tRNAs and trypanosomes lack all tRNAs (Attardi and Schatz, 1988).

t-Arg (24mer) ◄	5'- CGAAATCAGAGGTTCTCCTTAAAC -3'	Position
Cow	.....TTTA..T.AT..T..A	10203
Urchin	.....	7380
Fly	.....CT.ACT.CAA.TAA.CGCT	6102

Comments: Made to match urchins. Clearly not a good primer for anything else.

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

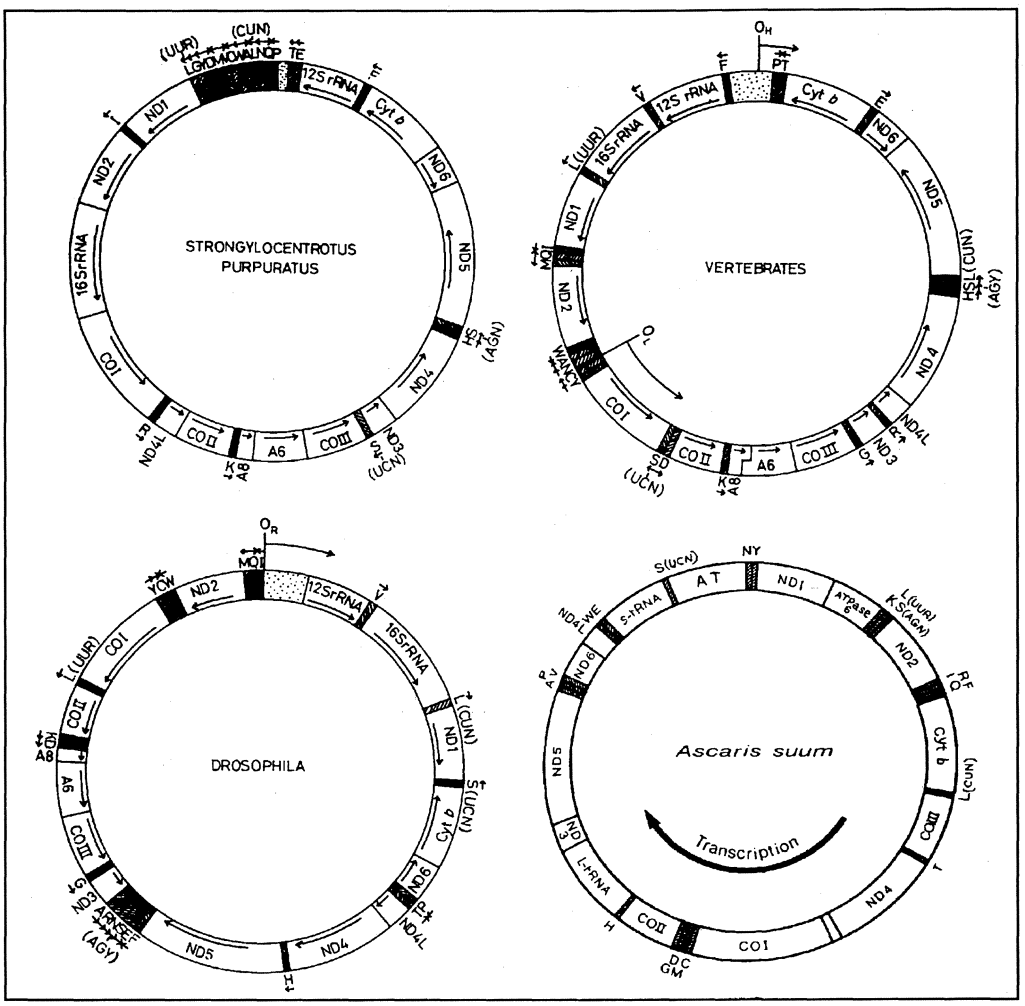
t-Pro (20mer) ►	5'- CTACCTCCAACTCCCAAAGC -3'	Position
Human	.C...ATT.G.A.....	15980
Mouse	.AC.AC.AGG.A.....	15701
Cow	TC...AT....C.....	15753
Frog	.C..TATTG..C.....	17510
Urchin	TACAT.G.....	

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.

t-Iso (19mer) ►	5'- ATTT-ACCCTATCAAGGTAA -3'	Position
Fly	....-.....	42
Human	....-..T.......A....	4303
Mouse	....-..T.......A....	3728
Cow	....-..T.......A....	4079
Frog	....C..T.......A..G.	

Comments: The above primer can be used in combination with one of the 12S primers to amplify the A+T-rich region in insects It has worked for Drosophila virilis. It did not work for Magicicada. This may be because in this cicada, the t-RNA is transposed or because the A+T-rich region can be up to 4Kb (Martin and Simon 1990). Considerable length variation has been found in this region in invertebrates (Rand and Harrison 1989; Snyder et al. 1987); in weevils the A+T-rich region is known to vary between 9 and 13Kb (Boyce et al. 1989). Note that tRNA iso is transposed in vertebrates and Xenopus has an insertion and a substitution one base from the 3' end.

Appendix Figure 1. Mitochondrial gene order in Strongylocentrotus (urchin), vertebrates [except in chicken where tRNA (Glu) and ND6 lie between the D-loop and tRNA (Pro) (Desjardins and Morals 1990)], Drosophila (fly), and Ascaris (nematode). tRNA genes (dark bars) are indicated by the one letter code of the amino acid they correspond to. Arrows indicate direction of transcription. O indicates origin of replication (for heavy [H], light [L], or both [R] strands). The origin of replication of Strongylocentrotus remains unmapped. The hypervariable, non-coding regions are indicated by stippling in all but Ascaris where it is labeled "A T" for A+T-rich region. Redrawn from Wolstenholme et al. 1987 and Jacobs et al. 1988.



Additional References for Appendix 3:

Attardi G, Schatz R, (1988) Biogenesis of mitochondria. Ann. Rev. Cell Biol. 4:289-333

Boyce TM, Zwick ME, Aquadro CF. 1989. Mitochondrial DNA in the bark weevils: size, structure and heteroplasmy. Genetics 123: 825-836

Cantore P, Gadaleta MN, Roberti M, Saccone C, Wilson AC (1987) Duplication and remoulding of tRNA genes during the evolutionary rearrangement of mitochondrial genomes. Nature 329:853-855

Hixson JE, Brown WM (1986) A comparison of the small ribosomal RNA genes from the mitochondrial DNA of the great apes and humans: sequence, structure, evolution, and phylogenetic implications. Mol. Biol. Evol. 3:1-18

Snyder M, Fraser AR, LaRoche J, Gartner-Kepkay KE, Zouros E. (1987) Atypical mitochondrial DNA from the deep-sea scallop Placopecten magellanicus. Proc. Natl. Acad. Sci. USA 84:7595-7599