THE SIMPLE FOOL'S GUIDE TO PCR

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INTRODUCTION TO S.F.G.

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. 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 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 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. 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.

We start in the spirit of simple foolishness by describing DNA extraction, the basic PCR cycle, and some general guidelines we have found useful. Suggestions (like those in the "variations" section) are followed up in greater detail below. Good luck.

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DNA EXTRACTION FOR PCR

Because PCR uses sequence specific primers, it is possible (in theory) to amplify any portion of the mitochondrial or nuclear genome using total genomic extracts. These extracts may be obtained from fresh or wet/dry preserved tissues. Protocols for the extraction of total DNA from any of these tissue sample types are generalizable to most sample types after the performance of a few initial steps specific to each type. Presented below, in detail explicit enough for even your mother to follow, is a description of a general method for extracting total DNA from fresh tissue samples. Additions/deletions to this protocol for preserved samples are noted following "Mom's recipe for homemade DNA extracts". We welcome comments on other methods that may be superior to these.

A. General Extraction Protocol for Total DNA

This procedure has worked well for fresh tissue samples obtained from animals representing a variety of phyla from corals to urchins to sharks to even mammals.

1. Procure/dissect out roughly 0.1 - 0.5 grams of the the desired tissue type and place in a 1.5 ml micro-centrifuge tube. DON'T BE GREEDY (it is easier to use too much tissue than too little).

   NOTE: If you are working with a tissue that is especially DNA dense, such as sperm, do not start with "generous" quantities of gonads or cells. Obtain sparing samples and wash them in a water/sea water/Ringer's etc. solution before putting them in the *Lysis Buffer*.

2. Add an approximately equal volume of *Lysis Buffer* to the tissue swatch.

   Lysis Buffer:
   100mM EDTA
   10mM Tris (pH 7.5)
   1% SDS

3. Macerate/grind the tissue. We use a "micro-pestle" which is made by pouring plastic casting resin (used to repair surfboards, easily available in most labs in Hawaii) into a micro-centrifuge tube and letting it harden with a stiff rod inserted into it for a handle. Insert the pestle gently into the microfuge tube and rotate it. Make about three "passes" at the tissue (or until it appears milky).

   NOTE: Samples that are too DNA dense at this point have an extremely high viscosity and exhibit impressive viscoelasticities (= snotlike for non-biomechanics) after maceration. This physical property hinders the effectiveness of subsequent separation procedures so dilute such samples in *Lysis buffer* and macerate until the viscoelastic behavior of the solution is greatly reduced.

4. Microcentrifuge this solution for approx. 3 min at 14,000 rpm.
5. Decant the supernatant and save.

   NOTE: If you are working with more than one preparation its a good idea to keep the samples on ice between performing steps of the extraction procedure. This precaution may slow down any enzymatic digestion of the DNA that may occur before it is isolated.

6. Add an approximately equal volume of buffer-equilibrated phenol to the decanted supernatant and invert gently.
7. Microcentrifuge for 10 min at 14,000 rpm.
8. After centifuging, the DNA should be suspended within the upper aqueous layer. Immediately under this layer and above the bottom phenol layer there may be a whitish layer that contains proteins and carbohydrates. Pipet off only the topmost layer and save. If your sample consists of only two layers remove all of the upper layer.

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NOTE: Occasionally, when DNA density is high, the DNA containing layer is heavier than the phenol. Therefore the *bottom* layer after centrifugation will contain the DNA. For this reason always save the bottom layer after decanting the upper layer. (It will become obvious in subsequent steps if you've decanted the phenol layer and should be working with the bottom layer instead.)

9. Add an approximately equal volume of 1:1 phenol/chloroform to the decanted liquid and invert gently. If you are working with the DNA containing layer it will not mix with the phenol/chloroform. If it does mix, you've got the wrong layer, so try adding the phenol/chloroform to the bottom layer from step 8.
10. Microcentrifuge for 5 min at 14,000 rpm.
11. Pipet off and save the clear/whitish DNA containing layer off the top of the phenol/chloroform.
12. Add an approximately equal volume of chloroform to the decanted liquid and invert gently.
13. Microcentrifuge for 3 min at 14,000 rpm.
14. Pipet off the top layer into well labeled, pre-weighed microcentrifuge tubes. These will be the final sample containers.
15. Weigh each sample and determine the volume of each by assuming 1 gram of the liquid = 1 ml.
16. Add a volume of 7.5 M NH_4OAc equal to 1/2 sample volume.
17. Add to this solution either (1) enough 2-propanol to make the solution 50% 2-propanol, or (2) enough ethanol to make the solution 66% ethanol.
18. Invert gently and let the solutions sit for at least 10 min at room temperature.

    NOTE: The DNA will precipitate out of solution in this step as will unwanted salts if the waiting period is prolonged too much. We've had no problem however after leaving the samples out overnight unrefrigerated. We don't usually refrigerate samples as this appears unnecessary and also increases salt precipitation.

19. Look for precipitated DNA which will appear "fluffy", salts are white and often sink. The DNA is not always visible at this stage even in successful extractions.
20. Microcentrifuge for 10 minutes at 14,000 rpm.
21. CAREFULLY pour off the liquid. Don't lose the pellet!
22. Finger flick the tube to dislodge the pellet and then almost fill each sample tube with 70% ethanol.
23. Let the solution sit for 10 min.
24. Microcentrifuge for 10 min at 14,000 rpm.
25. CAREFULLY pour off the ethanol and use a Kimwipe to swab out any remaining liquid adhering to the side of the tube -- don't touch the pellet, it'll stick! (Eliminate the Kimwipe step if contamination is a worry.)
26. Vacuum dry the pellet.
27. Add 100µl of sterile distilled H_20 (or TE) to each sample and freeze until needed.

B. Variations on the Extraction Protocol

MtDNA enhancement-If you plan to PCR mtDNA without prior cesium purification you may want to increase the relative yield of mtDNA to nuclear DNA in your extractions. One method of doing this is low speed centrifugation of ground tissue samples in order to pellet the nuclei before adding the *Lysis Buffer*.

Tiny tissues- We have successfully employed the following super-quick-and-dirty extraction method to extract DNA from pelagic fish eggs. We suspect it may work for invertebrate eggs, as well as other small amounts of tissue:

1. In a microcentrifuge tube suspend 1 to 3 eggs (1-5mm in diam) in 30µl of PCR buffer (50mM KCL, 10mM Tris (pH 8.3), 1 µg/ml Protein kinase K and BSA).
2. Add enough NP40 to make the solution 1% NP40.
3. Heat the solution to 95° C for 5 min.

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4. Dilute the solution in enough sterile distilled H_20 to bring the final volume to 100 µl.
5. Run the PCR reaction with a buffer containing no detergents.

Alcoholic tissues- For specimens collected elsewhere and preserved in alcohol we alter steps 1 - 6 above and begin the extractions the following way:

1. Place 0.1-0.5 grams of the tissue sample in a microcentrifuge tube containing sterile distilled H_20.
2. Invert the tube a few times, and drain the water. Add ca. 300µl of 0.1M Tris, 0.15M NaCl(Ph 7.5) -- or-- 100mM EDTA, 20mM Tris.
3. Macerate/grind the tissue as described in the general protocol above.
4. Centrifuge this liquid at low speed for 1 min. to remove cell debris
5. Decant the top layer into a microfuge tube and add 300µl of 0.2M NaCl, 0.02M Tris, 1mM EDTA (pH 7.5) as well as 25µl of 20% SDS.
6. Mix this soluiton well, centrifuge for 5 min at 14,000rpm.
7. Decant the top layer into a microfuge tube and proceed with the general protocol starting with the phenol extraction.

Chelex- For very small (1-5µl)/dry/abused as well as more typical sample types Walsh et al. (1991 Biotechniques **10**:506-513) report on a new extraction method that does not require protein kinase treatment or phenol-chloroform methods. The method utilizes a chelating resin called Chelex® 100 that was developed for extracting DNA from forensic-type materials for use with the PCR. Complete protocols for a variety of sample types are documented in the article. This technique sounds especially attractive to researchers wishing to amplify DNA derived from bloodstains since the authors have found that Chelex prepared DNA samples are less likely to have PCR inhibitors than extracts prepared by protein kinase or phenol/chloroform methods.

Snot and other types of slimy contaminants- Corals, molluscs and algae often present a slimy problem when it comes to DNA extraction. We have been able to overcome this problem in corals by using a few simple tricks. Try one of the following:

A. Freshly collected corals with small polyps (fist-sized colonies)
   1. Rinse in distilled water and freeze at -70°C.
   2. Break up in small pieces and put in as small a volume as possible of 100 mMEDTA, 10mM Tris (pH 7.5) with one drop of "Foam Fighter" (Dimet hylpolysiloxane antifoam, Crescent Research Chemicals, 4331 East Western Star Blvd., Phoenix, AZ, 85044 -- it helps reduce mucus) on ice. Agitate this slurry periodically for 2 hours. This serves to dissociate the coral tissue cells from the skeleton.
   3. Centrifuge the slurry at 3000 rpm for 10 min.
   4. Pour off the supernatant. Suspend the pellet in Lysis Buffer. Proceed with step 4 of the general protocol. Step 9 may be repeated two more times if the supernatant remains pigmented. Also at this point add about 200 µg BSA (bovine albumin) to the supernatant and heat at 65°C for 10 minutes (this aids in absorbing protein contaminants.) While the sample is still in the heating block add chloroform and proceed.

B. Freshly collected corals with large polyps should be rinsed. At this point it is possible to dissect tissues out of the polyps with forceps and follow the general protocol for DNA extraction

Another method (developed by Dr. Stephen Miller) that has worked for snails is to heat the genomic DNA extraction at 60°C for an hour, add 8M $LiCl_2$ (which precipitates the DNA and dissolves the mucopolysaccharides) to bring the solution to 4M. Let stand for an hour then centrifuge at 8000 rpm to pellet DNA and keep the mucopolysaccharides in solution. The supernatant is removed and the DNA pellet resuspended in water.

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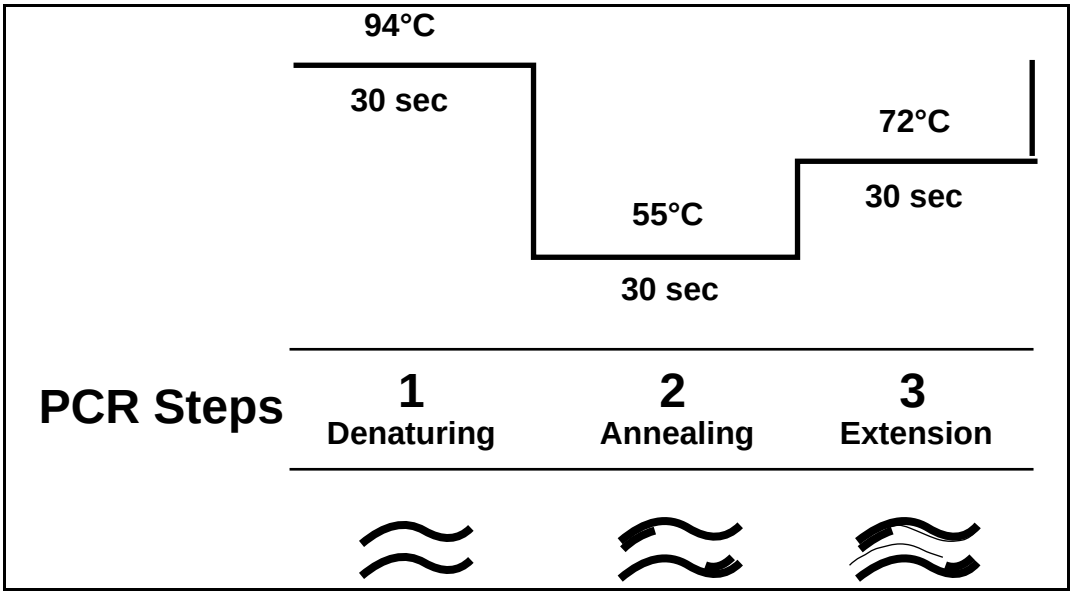
THE CYCLE

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-strand template. The first step denatures, or melts, the double-strand template DNA so that all the DNA is single-strand. This allows the oligonucleotide primers to anneal to the single-strand template DNA at specific locations (i.e. the primers' complements). 94°C for 30 seconds seems to work well, but shorter times have also been recommended. *Remember*, if the melting temperature is too low or time too short the double-strand DNA may not denature — thereby reducing the efficiency of the reaction. This is especially true for the first cycle in which the goal is to denature high molecular weight DNA. Some protocols suggest a long initial denaturation. On the other hand, Taq polymerase, unlike the legendary Phoenix, can't rise out of the ashes of your denaturation cycle *too* many times. Eventually, the enzyme becomes less active. So, there needs to be a balance between denaturation of the DNA and of the enzyme.

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 55°C for 30 - 60 seconds. 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).

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Step 3: The Taq polymerase works best at temperatures between about 72-75°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. 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 (**A**DENOSINE pairs with **T**HYMINE and **G**UANINE with **C**YTOSINE). The primers are incorporated into all subsequently amplified DNA templates insuring perfect priming sites in subsequent PCR cycles.

```
Primer   5'TACAGCGCCACGTTA 3'
          |||||||||||
Template 3'ATGTCGCGGTGCAATGATGCGTACGTAATGA 5
```

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 also required. We may use the following types of temperature cycles when dealing with various DNAs, primers and annealing temperatures:

94-55-72	for good or perfectly matched primers (higher temperatures can be used, see primer section to calculate maximum annealing temp).
94-48-68	for poorly matched primers (say 4-5 mis-matches out of 20)
94-45-65	for fishing expedition where the primer is of very questionable quality
94-40-65	last gasp before giving up. These conditions often give uncontrolled results- who knows what DNA will be amplified!.

Some feel it is particularly useful to extend the annealing time in addition to (or as opposed to) extensively lowering the annealing and extension temperatures in step 2 and 3. 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. The down side is that this also greatly reduces specificity of the reaction, and smeary products can be the result.

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 described in the preceding paragraph.

See the PCR cycle for other variant cycles that have proved helpful in forcing PCR to work.

PCR PROTOCOLS

A. PCR Hygene: Avoiding PCR problems

Because PCR products are so concentrated and easily volatilized (opening a microfuge tube or pipetting, for instance), cross contamination of samples is potentially a serious problem. Certain simple precautions can be taken to avoid contamination or at least minimize it if it occurs.

We have found that aliquoting solutions makes it possible to easily contain and relatively easily resolve any contamination problems that do arise. Each person working in the lab can have their own set of solutions:

-PCR reagents prepared in large amounts should be distributed in 1.5 ml microfuge tube and stored at -20°C.

-Water used for PCR reagents, DNA and primers should be double-distilled, sterilized, and then distributed in 1.5 ml microfuge tubes and stored at -20°C.

When primers are made, the stock solution is usually highly concentrated. From this highly concentrated stock solution, we make a 100µM stock solution which is used in making 10µM solutions for personal use. The different stock solutions are stored separately. This system allows each individual using a certain primer to have their own supply. Again, in this way massive, lab-wide contamination problems, that can be very costly in terms of time and money, can be avoided and any contamination problems that do arise can be efficiently contained.

Different sets of pipettors should be designated for different procedures. One set of pipettors should be designated for preparing PCR reactions. These pipettors should not come in contact with any amplified DNA at all. Another set of pipettors can be designated for post-PCR use. One pipettor should be designated to be used only in loading samples in agarose gels. Another set of pipettors should be designated for use with radiation only.

Often, in spite of infinite precautions, the PCR gremlins are successful and contamination problems occur. In order to have a headstart at figuring out what's going on, it is very useful to run a negative control and a positive control (some DNA that works every time with the primers being used) along with experimental samples. Bands that appear in a negative control indicate a problem to be dealt with. See below (DS amplifications) for some ideas on how to approach this.

B. 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 the one currently recommended by IBI, and is similar to Perkin-Elmer Cetus' buffer. Inclusion of the detergents has been very important to consistent success in our lab. A note about enzyme. Although we use IBI's buffer recipe, we are using only Perkin-Elmer enzyme, having in the past tried IBI, and Promega. In our hands, the P-E enzyme is more consistent and functions well in a greater variety of settings. Because we use so many different types of cycles, and types of templates, this versatility is important to us. Others may have different needs and experiences.

There are a number of different buffers being used in other labs and in the literature that may work as well or better than ours. 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. Some recent reports

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suggest adding other organic solvents like DMSO, PEG up tp 2-5% concentration to the reaction buffer. If you are having problems, explore other buffers.

Some Commonly Used Solutions

Note: these recipes are for 1X solutions, but they are usually made at the concentrations listed and diluted for use.

Taq Polymerase Buffer (usually made as 10X)

10 mM	Tris (pH 8.3)
1.5 mM	$MgCl_2$
50 mM	KCl
0.01%	Gelatin
0.01%	NP-40
0.01%	TritonX 100

TE Buffer (pH 7.6, usually made as100X)

10 mM	Tris
1 mM	EDTA (pH 8.0)

TBE Buffer (pH 8.3, made as 5X)

89 mM	Tris
89 mM	Boric Acid
2 mM	EDTA (pH 8.0)

TAE Buffer (pH 7.8, made as 50X)

40 mM	Tris
0.114 %	Glacial Acetic Acid
1 mM	EDTA (pH 8.0)

Dense-Dye (full strength)

50 mM	EDTA
30 %	Glycerol
0.25 %	Bromophenol Blue
0.25 %	Xylene Cyanol

dNTP mix (pH 7.0)

2.0 mM	dATP (USB 14244)
2.0 mM	dGTP(USB 14314)
2.0 mM	dCTP (USB 14279)
2.0 mM	dTTP (USB 22324)

Aliquot in 0.5 ml lots in sterile tubes

B. Double-Strand DNA Amplifications

1. Prepare the following reaction mix for each template sample:
   - 2.5 µl 10x Taq buffer
   - 2.5 µl 8 mM dNTP's (i.e. 2 mM each of dATP, dGTP, dCTP, dTTP)
   - 1.2 µl each of two primers (10µM stock solutions)

1 µl template DNA (in ddH_2O or $\frac{1}{10}$ TE, 1-2 ng of mtDNA or 1-2 µg genomic DNA seems to work OK—if in doubt use *less*)

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0.03 µl Taq polymerase (this is alot less then recommended **but** it works perfectly—so save some money!)

—add ddH_2O to make 25 µl per reaction (usually this means about 18µl or so)

2. Add 1 drop 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 ≈5 seconds.

3. **Standard-** Set PCR machine for 40 to 50 cycles. Standard conditions are: 94°C for 15-30 seconds, 50°C for 15-30 sec, 72°C for 15-60 sec. 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 1 kb (i.e. the extension time, step 3, should be made a little bit longer with very large target pieces).

4. **Double Whammy-** Add a third primer to the cocktail. This primer should anneal outside the other two, and be added in about 1/5th the normal amount. We have found that this greatly enhances the yield of product from the interior two primers, often by an order of magnitude. These double strand double whammies are often particularly useful for generating single-strand template with asymmetric amplification.

5. **To Visualize PCR Products**-Run amplified samples (1-2 µl *Dense-Dye* + 10 µl sample) on 2 % agarose gels in 0.5X 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!

6. **Problems and some solutions**

Ideally, when you look at the agarose gel with your PCR products you will see a nice, bright, tight band of the correct size from each reaction and the lane with your negative control should be clean. Unfortunately, this is not always the case - there are many other possibilities!!!

a. **No PCR product**

-Try it again.
-Try diluting the starting template DNA (try dilutions 1:10 or 1:100).
-Try lowering the annealing temperature (step 2) in the PCR cycle.
-Try 5 or 10 cycles at a very low annealing and extension temperature (42°C and 68°C for instance), then another 40 cycles at higher temperatures. This gives poorly matched primers a chance to sit down and generate some good double-strands while hopefully avoiding amplification of too much junk.
-Another technique similar to the above is to run PCR reactions with ramped cycles such that a minimum amount of time is spent at the annealing temperature (allowing primers to sit down at only "the best" sites). Extension occurs over a long period of time at lower temperatures so that extension is starting while primers are still sitting down, allowing poorly fitting primers to be locked onto the template. The profile for such a cycle would be 30 seconds at 94°C, 10 seconds at 55°C, a 2-5 minute ramp from 55 to 72°C and 10 seconds at 72°C.
- Check the primer concentrations.
-Try doing more cycles on the PCR machine (increase from 40 cycles to 50 cycles). You can even put the same tubes back in for another 10-20 cycles. This works with faint bands but does not when there is nothing there.
-It is possible that something in your DNA template is interfering with the PCR reaction. This can be determined by setting up a reaction with two templates, one that is known to work well with the primers being used and the other one that is the one that you are having problems with. The mucopolysaccharides present in the mucus of corals and snails for instance are

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known to co-precipitate with DNA. Once the mucopolysaccharides are eliminated, successful amplification of template is possible. See extraction protocols for solutions to the slime problem.

b**. Bright bands in well of gel**

-These are caused almost surely from over-amplification of the PCR product. We have often gotten this result if we re-amplify from a PCR product (either double or single-strand reactions) and do not dilute it enough (usually 1 µl of a 1:100 dilution from a PCR reaction is enough for a standard asymmetric single-strand amplification). Try diluting the PCR product that you are using as a template. Also, remember to do the new PCR reaction at >55°C (55°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.

c. **Smearing of double-strand PCR product or Multiple Bands**

-Try less template. The most common cause seems to be too much template.

-Try annealing temps 2-5° higher. 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.

-Try varying $MgCl_2$ concentrations. Do a dilution series to determine which concentration results in the best bands.

-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 gel purifying the double strands (only take the brightest part of the band) and then reamplifying (with stringent conditions) the purified double strand product as double strands. We have been able to get a good double-strand product from bands that were originally not very bright using this method.

-If bands appear to be the wrong size (in comparison to your standard) or if there are multiple bands from one reaction, try doing the same reaction with only one of the primers in order to determine if you are having problems with primer artifacts.

d. **Bands in the blank**

-Detective work: where have your solutions been lately? Investigate and systematically change your solutions, water is usually a good place to start (and its the cheapest....)

-If all else fails, digest the DNA in the blank with a few 4-base restriction enzymes. If you find an enzyme that cuts the DNA, you can use this to "clean up" your reactions by digestion of your buffer + primers + dNTPs solution with the restriction enzyme. After incubation for 1 to 2 hrs at 37°C (or whatever the recommended temperature), denature the enzyme by incubation at 95°C for 5 minutes. The TAQ polymerase can then be added (when hot if you like) and the buffer aliquoted into your tubes with your DNA. You can also clean house with DNAse [see Furrer et al. (1990) Nature 346: 324].

-Wash the pipettors well.

-Stop sneezing in the lab. Sneeze only in the fume hood or at your desk.

e. **If after trying some or all of these solutions you are still having problems, go to Kona for the weekend!!! (Reactions tend to start working when you get back.....)**

C. Single-Strand DNA Amplifications

In single strand or asymmetric PCR amplifications, single strand DNA template for sequencing is produced by limiting one of the two primers. During the initial cycles of a PCR run, double strand DNA is produced as in a normal amplification. However, during the final cycles the "limiting" primer runs out. The other primer continues to initiate amplification but only single strand products are produced with

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each cycle. The trick to this method is in adjusting the concentration of the "limiting" primer so that it runs out after enough double strand product has been produced and before your PCR run is complete.

Single-strand DNA can be produced from pure mtDNA, or from a previous double-strand PCR amplification. Typical reaction mixtures are in 100 µl instead of 25 µl.

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. Thus, use 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. We one of the four methods outlined below to generate single-strand product from initial double-strand amplifications. In all four primer, annealing temperatures are stringent (55-60 °C) regardless of the conditions originally needed to generate the double strand product.

Method A: Use 1-5 µl of the double-strand amplification 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-strand DNA in the first amplification.

Method B: Take a $\frac{1}{100}$ dilution of the double-strand PCR product and use that in an asymmetric single-strand amplification as described in **#1**.

Method C: We have found that using a third primer, annealing inside those used in the original double-strand amplification often gives superior single strand amplifications. This is probably because use of the third primer adds a new layer of specificity to the single-strand reaction that is not added by using one of the previous primers. This appears particularly important for ribosomal RNA gene primers, or others that tend to give smeary double strand products.

Method D: Gel purify the double-strand band. Run the double-strand amplification on a regular 2% agarose gel. Cut out the appropriate band with a sterile blade. (This method is ideal if the double-strand 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, replace with 50 µl sterile water and freeze the gel slice. Thaw it immediately, and repeat the freeze/thaw cycle two more times. 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 OR use low melt agarose in the original gel, cut out the band, and melt it at 80° C or so. The agarose can be removed by phenol extraction, followed by a chloroform extraction and EtOH precipitation.

3. **Single -strand problems**

Ideally, single strand products will appear on a mini-gel as a fuzzy band that runs either a little smaller or larger than the double strand piece it is made from. Unfortunately, we do not live in an ideal world and most of us have never experienced this type of result. In fact we have seen just about every pattern imaginable, from doublets to smears to absolute blanks, in our single-strand mini-gels. Generally, amplifications which produce a doublet seem to work the best. However, practically all patterns have yielded good sequence at one time or another. The pattern that works best appears to be template specific.

Because of this variability it is often difficult to predict which amplification patterns will sequence and which will not. We suggest that you initially try sequencing at least one of every pattern you see. In this manner you can let the sequencing reaction help you to build a better image of what patterns work and what patterns don't.

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If none of your initial sequencing attempts work, try toying with the concentration of the limiting primer. Lab wisdom suggests that many of the problems that occur when attempting to generate single strand DNA from a double strand product are a direct result of either too much or too little "limiting" primer. Too much and you never generate single strand template! Too little and you do not generate enough double-strand product from which to produce single-strand templates! There are a number of ways to adjust your reaction conditions so that you are in the "happy zone" where you peel off endless single-strand copies. Remember: Don't lose your sense of humor.

- Run a dilution series with your double-strand product to determine the template concentration that works the best.
- Re-do your double-strand amplification at higher stringency. It may be that your orginal double-strand amplification is tainted with non-specific products. Raise the annealing temperature of your double-strand reaction so that you are amphlifing only the DNA segment that you wish to sequence. This is especially helpful if your single-strand products are smeary.
- Adjust the concentration of your limiting primer in the initial double-strand amplification. It may be that you are simply starting with just too much primer. Along those lines, it is probably a good idea to double check the concentrations of your primers (see **PCR PRIMERS**).
- Try gel-purifying double-strand templates (see **Method D**). Gel-purification, in principle, should elimate all excess primers. As a result, you need to add both primers to your single-strand reactions. The concentration of your "limiting" primer should be about $\frac{1}{100}$ of your second primer. However, run a dilution series with the "limiting" primer to determine the concentration that works best for you.
- Try using an internal primer to generate single-strand template (see **Method C**).
- Try a different sequencing technique. Some of us have been so often frustrated and had such difficulties getting good sequencing templates from double-strand PCR reactions that we have been trying different sequencing techniques (see SEQUENCING PROTOCOLS). These techniques are becoming reasonably successful and consistent.

D. Double-Strand 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 is known as the RACE method (PNAS 85:8998-9002)

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-strand amplification.

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SEQUENCING PROTOCOLS

Use of conserved primers has made PCR amplification of the DNA of many species relatively straightforward. This has opened the door to population level analysis of DNA variation in diverse species. However, there are important questions in population biology that can only be answered by sequencing hundreds of individuals. In these cases, even mild problems can represent a logistic nightmare. It is our experience that most of these nightmares arise when trying to sequence your PCR amplified gene product. The single most important factor contributing to "nice" sequences is generating ample single strand DNA template. Obtaining the proper amount of sequencing template is not a trivial matter and we suggest that you experiment with the procedures outlined below to determine which methods work consistently for you.

We generate single-strand DNA templates for sequencing primarily in three ways. By far the simplest of these methods is to let the PCR machine do it for you. This is done by limiting one of the two PCR primers and sequencing directly from this reaction (see Single-Strand DNA Amplifications). Alternatively, you can sequence directly from a normal double strand PCR reaction by denaturing the double strand product or by "tagging" one strand of DNA with either biotin or phosphate. In the last method, the "tagged" strand can then be peeled or digested away leaving ample pure single strand DNA. template for sequencing

Sequencing Single-strand PCR products

When single-strand PCR products are consistently obtained, single-strand sequencing is the preferred method of sequencing. It is the easiest, quickest, and least expensive of all the sequencing methods we generally use. However, and here's the rub, obtaining the proper amount of single sequencing template consistently is tricky (see Single-Strand DNA Amplifications). We suggest that you begin with this method of sequencing and move on to more cumbersome methods if you are unable to produce readable sequences consistently.

I. Purification and concentration of template:

Prior to sequencing the PCR buffer, excess primer(s) and nucleotides left over from the PCR reaction mix must be removed, and the DNA concentrated. We accomplish this in two ways; propanol precipitation or Centricon-30 tubes. Both methods work equally well. We initially used Centricon tubes but moved to propanol precipitation because it was much less expensive.

A. Propanol precipitation

1. For a 100µl of PCR reaction add:
   50µl of 7.5M NH_4OAc
   150µl of 2-propanol
2. Mix well and incubate at room temp. for at least 10 min.
3. Microcentrifuge for 8-10 minutes.
4. Remove supernatant. Wash with 500µl of 70% EtOH.
5. Microcentrifuge for 2 minutes. Dry pellet.
6. Resuspend in 8-20µl of 0.1X TE buffer.

B. Centricon purification

1. Place 1.5 ml of sterile dH_2O and 0.5 ml of of 7.5M NH_4OAc in top half of a 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 centrifuge it at 1500 g for 15-20.
3. Repeat this 2 more times with 2 ml of dH_2O, dumping water from bottom half when needed.

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4. Invert tube and spin 20 sec. to bring concentrated sample into tip of conical tube. This should yield about 20-40 µl of sample.
5. Store sample at -20°C.

II. Primer Annealing:

Once the PCR product is purified and concentrated you are ready to anneal the primer to the template to initiate sequencing. When you are generating single-strand template via asymmetric amplification **you must use the primer that was limiting or absent in the amplification**! The primer that was more abundant in the PCR reactions will produce the most DNA. This DNA will remain single-strand because there is not an equal amount of the other strand of DNA to complement with it. The "limiting" primer complements with this excess single-strand DNA and, therefore, must be used in the sequencing reaction.

Use 7 µl of the purified ssDNA template. To this, add 1 µl of 10 - 100 µM primer and 2 µl of *5X Sequenase® buffer*. Incubate at 65°C for 2 - 5 min and then let cool to room temperature in the heating block (for 30 min or so). Many people shorten the annealing step by incubating the annealing reaction for 5 min at 65°C and simply moving to the sequencing steps.

III. Sequencing:

We use the Sequenase® System from USBiochemicals. We have modified it slightly to make it cheaper than following their protocol exactly.

1. 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.
2. Make up Labeling cocktail. For each sample add:
   a) 1.0 µl DTT (Dithiothreitol, 0.1 M)
   b) 2.25 µl dH_2O.
   c) 1.75 µl 1 X TE (We do not use the *Sequenase® dilution mix*. We have gotten better results with TE in side by side comparisons).
   d) 0.25 µl ATP-S^{35}
   e) 0.25 µl *Sequenase®*

   Note: Using this procedure, we do not normally use the *Labeling mix* provided by USBiochemicals. There seems to be enough nucleotides left over from the purification step to sequence fine. We generally get right up next to the primer with this mixture.

3. Add 5.5 µl of the labeling cocktail to the annealed templates.
4. Pipette 3.5 µl of this mix into each of the four tubes ddNTP tubes and incubate for 2-5 min at room temperature (Do not let this reaction go longer than 5 minutes).
5. Add 4 µl of *Stop Solution* to each ddNTP mix.
6. Store reactions in the freezer until ready to load onto acrylamide gel.

Sequencing from double strand PCR products

In the past, we have had trouble with this method of sequencing. Gene-cleaning the double-strand product coupled with the addition of a nonionic detergent during the primer-anealing step (Nucleic Acids Res. Vol. **18**: 1309 (1990)) seems to have cleared up most of the previous problems. Recently, we have been consistently generating sequences in excess of 400 base pairs using this method.

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Unfortunately, this method is really sensitive to "junk" left over from the PCR reaction. As a result, double-strand templated must be prepared very carefully. We generally cut the DNA product out of a 1% TAE gel and Geneclean (Bio101, Inc) this slice. While this initial "work" is tedious, the final pure, concentrated template is generally sufficient for 4 or more sequencing reactions. In addition, because both DNA strands are present it is possible to sequence in either direction. Thus, there is the potential to double the amount of data that can be collected with each PCR amplification. The details of this procedure were worked out by Grace Tang.

I. Purification and concentration of template:

A. Precipitation of DNA

1. Following PCR, remove oil by adding 100 µl of chloroform to a 100 µl PCR reaction, vortex, and transfer the aqueous layer to a clean vial.
2. Concentrate by precipitating DNA using linear polyacrylamide as a carrier (Nucleic Acids Res. Vol. **18**: 378 (1990)). We typically mix:

   80 µl of PCR product
   20 µl of 0.5 M KCl-0.125% polyacrylamide
   250 µl 95% EtOH (-20° C).

3. Incubate at -70°C for 30 min or -20°C over night.
4. Microcentrifuge for 10 minutes.
5. Wash with 70% EtOH (-20°C).
6. Resuspend in 10 µl of 1 x TE buffer.
7. Run in a 1% TAE gel.
8. Stain gel in EtBr (see To visualize PCR products). Locate and excise band.
9. Place in a 1.5 µl Eppendorf vial.

B. Geneclean (Bio 101)

1. Add 0.5 ml *NaI solution* to each vial.
2. Incubate at 50°C for 5 min.
3. Add 3-5 µl of *glassmilk* to absorb DNA. Mix well on ice for 5 minutes.
4. Wash *glassmilk* with 0.3 ml *NEW wash*. Spin this solution quickly (20-25 sec). The *glassmilk* will pellet easily. Pour off the supernatent. Add more *NEW wash* and resuspend the glass milk. Repeat this step 2 more times. The final spin should run for about 2 min.
5. Add 7-40 µl of TE. The amount of TE you add depends on how much DNA you started with. For bright double strands we normally add about 28 µl. This is enough for four sequencing reactions.
6. Let this solution soak at 50°C for 5 min. to 1 hour.
7. Spin for 2 min. and gather supernatant. This is your template.

II. Primer Annealing:

Once the PCR product is purified and concentrated you are ready to anneal the primer for sequencing. You can use either of the original PCR primers for sequencing.

1. Mix together:

   7 µl DNA template
   2 µl *Sequencing buffer*
   1-2 µl 5 % NP-40
   1 µl 10 or 100 µM primer

2. Boil mixture for 3 minutes.
3. Plunge into liquid nitrogen. 95% EtOH at -70°C also works well.
4. Warm to room temperature in a metal block initially chilled to -20°C.

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III. Sequencing:

1. 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 or positions labeled G, A, T and C.
2. Make up Labeling Cocktail. For each sample add:

   1.0 µl DTT (Dithiothreitol, 0.1 M)
   0.4 µl 5X *Labeling mix*.
   1.1 µl H_2O.
   1.0 µl 5% NP-40
   0.25 µl ATP-S^{35}
   0.5 µl *Mn buffer* (emphasizes the region close to the primer and tends to reduce shadowing problem)
   1.5 µl *Sequenase dilution buffer*
   0.25 µl *Sequenase*®

3. Add 5.5 µl of the Labeling Cocktail to each annealed template. DO NOT LET LABELING REACTION PROCEED LONGER THAN 5 MIN.
4. Pipette 3.5 µl of this mix to each of the 4 ddNTP tubes. Incubate for 2-5 min at room temperature. If you get a lot of non-specific termination in the sequencing reactions, try doing this step at 37°C-45°C.
5. Add 4 µl of *Stop Solution* to each ddNTP mix.
6. Store reactions in the freezer until ready to load onto acrylamide gel.

Solid Phase Stripping with Biotinylated DNA primers:

As an alternative to both these techniques, we have successfully used solid phase DNA stripping to make single strand DNA directly from double strand templates. The method combines the power and sensitivity of double strand PCR reactions with the clean sequencing results of asymmetric amplifications. The techniques have been described in the literature (Mitchell and Merrill, *Analyt. Biochem.* **178**: 239-242 (1989)), and the necessary reagents are commercially available.

The key to solid phase sequencing is the attachment of one of the strands of a DNA duplex to a solid support. In our case, we have attached biotin to one of our PCR primers. A normal double strand PCR reaction then produces a product that is "tagged" on one end. Our reaction products have the biotin attached at the 5' end of the coding strand. The biotin binds to strepavidin in a nearly irreversible reaction. Therefore biotin "tagged" DNA can be attached to any support that can be coated with strepavidin. In our method, we have used strepavidin linked agarose, although magnetic beads with strepavidin attached are also available.

Once attached to the solid support, the double-strand DNA is dissociated with high pH. This strips off the DNA strand that is *not* bound to the support. Remember that only one of the strands has a biotin attached. This strand remains attached in high pH, and the other strand drifts off into the liquid phase. This single-strand DNA can be collected, concentrated and used as the template for normal Sanger sequencing.

The primary advantages of this method are that 1) any DNA that can be double strand amplified can be single-strand-sequenced, 2) only one round of amplification need be done, thereby limiting both costs and opportunity for contamination, 3) a highly conserved primer that will amplify a DNA segment from a wide variety of taxa (e.g. Kocher et al. 1989), can be biotinylated, and therefore used to rapidly collect sequence data from many species. The most serious drawback for the method is the expense of making a biotinylated primer. Some people make them themselves by incorporating Amino-link on the 5'end of their primers, and adding the biotin to this linker. Alternatively, one can purchase a biotinylated primer from many biotech companies nowadays like

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Operon, Synthetic Genetics, etc. Additionally, Biotin-linked phosphoraminodites are avalible commercially.

Ia. Producing single strand DNA using strepavidin/agarose

1. Wash Strepavidin-linked agarose beads (BRL (#5942SA)) three times in sterile 200 mM NaCl, 50 mM Tris pH7.5. Resuspend the original 5 ml suspension in 5 ml of this buffer and store in the refrigerator.
2. Add 25 µl of strepavidin-linked agarose beads to 45 µl of the double strand PCR product.
3. Allow the biotin to bind to the strepavidin by rotating this solution slowly for 1-2 hrs at room temperature.
4. Add 1ml of 1X TE and mix.
5. Microcentrifuge for 10 seconds to pellet the beads. Pour off the supernatant.
6. Denature the DNA by adding 60 µl of 0.2 M NaOH and rotating slowly for 10 minutes.
   Note: The biotinylated strand remains fixed to the agarose beads.
7. Microcentrifuge for 10 seconds and collect 70 µl of supernatant.
8. Propanol ppt with:

   70 µl of 5 M Ammonium Acetate (pH 6.8).
   140 µl of isopropanol
   1 µl of a 10 µg/µl tRNA (The tRNAdriver is necessary! Without it sequences are very faint.)

8. Incubate at -20°C for at least 15 min.
9. Spin at 4°C for 10 min in an microfuge.
10. Wash with 70% EtOH.
11. Resuspend in 7 µl of 1 x TE buffer.

Ib. Producing single strand DNA using strepavidin//magnetic beads

1. Wash Strepavidin-linked magnetic beads (Dynabeads M-280, #112.05, purchased through Robbins Scientific) three times in sterile 200 mM NaCl, 1 mM EDTA, 10 mM Tris pH7.5. (Richard's buffer). Resuspend the original 2 ml suspension in 2 ml of Richard's buffer and store in the refrigerator.
2. Add 20 µl of strepavidin-linked magnetic beads to 25 µl of the Richard's buffer, and add 45 µl of the double strand PCR product.
3. Allow the biotin to bind to the strepavidin by rotating this solution slowly for 1-2 hrs at room temperature.
4. Pull beads to bottom of tube with magnet, and remove fluid. Add 200 µl of Richards buffer.
5. Wash the beads by resuspending them, pulling them to the bottom with the magnet, and removing the fluid again.
6. Denature the DNA by adding 60 µl of 0.2 M NaOH (fresh! who knows why?)and rotating slowly for 10 minutes.
   Note: The biotinylated strand remains fixed to the magnetic beads.
7. Pull beads to bottom with magnet, and collect 70 µl of supernatant. (Don't be greedy, leave a few µl behind rather than get beads in the sample). Save the beads for solid phase sequencing.
8. Propanol ppt the supernatant with:

   70 µl of 5 M Ammonium Acetate (pH 6.8).
   140 µl of isopropanol
   1 µl of a 10 µg/µl tRNA (The tRNAdriver is necessary! Without it sequences are very faint.)

8. Incubate at -20°C for at least 1 hr.
9. Spin at 4°C for 10 min in an microfuge.
10. Wash with 70% EtOH.
11. Dry pellet, and esuspend in 7 µl of 1 x TE buffer.

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II. Primer Annealing:

Annealing and sequencing is identical to the procedure outlined in the **Sequencing from single-strand PCR products** section. Use a non-biotinylated copy of the biotinylated primer for sequencing. For solid phase sequencing of the strand attached to the beads, use the opposite primer. Follow normal procedures except that you must make sure the beads are in suspension during the extension phase of the sequencing reaction. Also DON'T put magnetic beads onto sequencing gel. ZAP!

Sequencing with Lambda Exonuclease digested PCR products:

The rationale behind generating single strand DNA by this technique is similar to template generation using a biotinyated primer. One DNA strand is "tagged" and single strand DNA is generated by distinguishing between the "tagged" and non-tagged strand. However, in this procedure, phosphate, instead of biotin, is used to label one of your PCR primers. The PCR product is digested with lambda exonuclease. The strand with the phosphate cap is chewed away, leaving behind a single-stranded template ready for sequencing. We have not tried this method. Other labs swear by it.

RUNNING A GEL

I. Pouring the gel:

A. Make up a batch of 6-8% acrylamide. For 100 ml of a 8% gel, we add 20 ml of a 40% Acrylamide stock (38.0% Acrylamide, 2.0% Bis-acrylamide) to 80 ml of a 10 M urea, 1.25 X TBE solution. We generally make up about 400 ml of this solution at a time. The solution is then filtered to remove any residual urea crystals. It is good for a couple of weeks.

B. Prepare your glass plates. Do not over silconize them. We have had some trouble in the past with the gel creeping up during a sequencing run.

C. Polymerization

1. Add 0.04g of Ammonium persulfate to 70 ml of acrylamide solution. Let the Ammonium persulfate dissolve by swirling gently—the acrylamide will not polymerize rapidly until TEMED is added.
2. Add 25.0 µl TEMED to the 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.
3. ***Pour gel immediately*!** Allow to polymerize for 30 min to an hour. Overnight is O.K. if care is taken not to dry out the well.

II. Running the gel

How long you run a gel depends on how far into the sequence you wish to read. The above sequencing protocols are designed to allow one to read to within 10 or so base pairs of the primer. Each sequencing reaction has enough volume for at least 2 sequencing runs. We typically run a short gradient gel first. This allows us to read between 200-250 base pairs from the primer. Our second run is considerably longer and allows us to read an additional 200 or so base pairs. Thus, using the above procedures and a combination of a short and a long sequencing run produces about 400-450 base pairs of information. At this point our sequencing reactions begin to fade. You can read further into gene segment by adjusting the amount of labeling mix with the sequencing reactions and further increasing the length of time that the gel runs. For further details see the sequencing pamphlet enclosed in the USBiochemicals sequencing kit.

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Short gradient gel:

1. Mount the gel to the sequencing apparatus;
2. Add 1X TBE to the top reservoir and check for leaks.
3. Add 1X TBE, 0.3M NaOAc to the bottom reservoir.

   Note: It is important to use the same TBE solution that you use to make up your Acrylamide solution to make up your running buffers.

4. Before adding samples, heat the gel up by running it for 20-30 minutes .
5. Heat samples to 95°C for 2 minutes.
6. Before loading the samples, rinse the wells. Urea will diffuse out of the gel and keep samples from entering.
7. Load 2-4 µl of samples in the order of G, A, T and C. This way of loading the gel is not arbitrary. You may be reading the complement of the message strand of DNA. By using this order, you merely flip the film over to read the message strand directly ("G"s become "C"s and "A"s become "T"s and visa versa, i.e. their complement!).
8. The gel should run between 45°C and 50°C . The power (volts x amps) determines the heating of the gel. We generally run a gel 15" X17" at between 60-70 watts and a rig 8" X 15" at between 45 and 50 watts.
9. Run the gel so that the leading dye just runs to the end.
10. If you used S^{35} in your sequencing reaction, the sequencing gel must be soaked in 5 % acetic acid and 5 % methanol solution for 15-30 minutes before drying the gel. 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.
11. 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.

Long Gel:

For longer runs, we generally run the gel without a gradient (i.e. 1X TBE in both the top and bottom reservoirs) for 3-4 hours. At that point, we change to a gradient buffering system with 1X TBE on top and 1X TBE, 0.3M NaOAc on the bottom and run for an additional 3-4 hours.

III. Problems

USB publishes a beautiful sequencing support manual, inlcuding a video that is very suitable for late night viewing. We suggest that you refer to it when trouble shooting your sequencing problems. After all, you're already paying them a fortune for those tiny little amounts of solutions.

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PCR PRIMERS

I. Making primers

Primer design is an art (but so is fingerpainting). In designing primers, keep in mind that the quality of a primer for specific amplification of desired DNA sequences depends on the length, nucleotide composition and primer-template match.

1. **Length**

Primers can be as short as 13bp and as long as 80bp. In most cases 18-24bp primers are sufficient for most purposes. The longer the primer, the higher the annealing temperature can be and the greater the specificity. However, if you are using primers that are not purified, the longer the primer, the greater the amount of non-specific primers that are present. On Applied Biosystems oligonucleotide synthesizers (e.g. PCR MATE), primers are generally made with 98% efficiency. This means that for every nucleotide added to the primers (nucleotides are added 3' to 5'), 2% of the product is not synthesized properly. If, for instance, the primer is 20bp long, then only 62% of the oligonucleotides in solution are the desired primer product. The remaining 38% are non-specific primers, approximately 2% of which are 1 bp shorter, 2% are 2 bps shorter, etc... In general, these non-specific oligonucleotides do not interfere with amplification. However, in many cases, primer artifacts (dimers) and non-specific amplification occur. If you need to make long primers, we recommend that you purify the product.

2. **Nucleotide composition**

Primers can be any sequence. The ideal primer has roughly equal numbers of each nucleotide without internal repeats or self-homology. For instance, a primer with the sequence AAATTTAAATTT may lead to mispriming and primer dimers. GC rich primers can withstand higher annealing temperatures.

3. **Primer-template match**

Specificity is obtained through maximizing sequence similarity between the primer and template. However, amplification products are obtained without perfect similarity. Single internal mismatches have little effect on PCR product yield. By contrast, single mismatches at or near the 3' end of the primer can significantly decrease amplification. Of the mismatches at the 3' end, A:G, G:A and C:C reduce yields about 100 fold, whereas A:A mismatches reduce yields 20-fold (Kwok et al. 1990). T's appear to be able to base pair with all 3 other bases.

4. **Making a primer**

When the template sequence is unknown, degenerate primers can be constructed that enhance the probability of amplification. In those cases where you are designing primers for unknown sequences, align as many homologous sequences as possible in order to identify variable positions. Look for highly conserved amino acid sequences, or conserved sequence blocks in non-coding DNA. For coding DNA, try to design your primer so as to match regions where the codons are only two-fold degenerate. This will minimize the degeneracy of the primer. Base multiplicity can be introduced when making the primer at variable positions so that a single oligonucleotide preparation can supply all the possible coding sequences that are likely to be present in the target DNA. For positions in which there is 3- or 4-fold degeneracy, deoxyinosine residues can be introduced because these base pair promiscuously. For 2-fold degenerate

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sites, purine or pyrimidine pairs can be introduced. It is also a good idea to use any available information on nucleotide bias of your target DNA to design better primers. For example, artrhropod mtDNA is highly AT rich, and 4-fold degenerate sites are about 90% occupied by A or T, so it is best to include only these two bases at 4-fold positions in primers. It is important to remember that primer degeneracy favors nonspecific amplification. In addition, the concentration of the "perfect match" primer is a function of degeneracy because the oligonucleotide preparation contains a pool of primers. For highly degenerate primers, higher concentration of primer may provide better yields.

4. **Annealing temperature**

Based on the nucleotide composition of the primer, it is simple to determine the appropriate annealing temperature, T_m: the temperature at which 50% of the primers anneal. A general rule of thumb is that A's and T's are worth 2°C and C's and G's are worth 4°C. Amplification reactions should be performed at the lowest Tm of a particular primer pair. Remember these values are for perfect matches. Imperfect matches will need a lower temperature for the first amplification.

5. **Common modifications to primers**

Restriction sites can be incorporated into the primers, thus allowing easy cloning of PCR products. Some researchers suggest adding an additional 3-5 bases to the 5' end of the primer (after the restriction site) because this greatly increases enzyme efficiency. Incorporation of amino-link bases to the 5' end allow biotintylation of primers for solid-phase sequencing and non-isotopic detection of amplified products. Recently, DuPont and ClonTech (and maybe others) have produced a biotinylated Phosphoramidite. These can be incorporated directly into your primer when it is synthesized, and produce a nicely biotinylated primer in one step. Places like Operon will do this commercially for a reasonable price.

II. Getting the primers you have made ready for their first encounter with Taq

Oligonucleotide synthsizers are lovely machines that synthesize oligonucleotides of whatever sequence you want. The oligos are synthesized 3' to 5' from the 3' nucleotide that is bound to silica beads in a column. When you get the column you need to 1) get the oligo off the silica beads and 2) strip off the protective groups that run the length of the oligo. To do this is simple.

1. Get some concentrated ammonia hydroxide. Keep it in the refrigerator. When you need to use some, take 4-5 mls out of the bottle and place in a smaller bottle.

   Note: Never open this bottle unless at room temperature as it will absorb too much water if opened cold and explode if opened warm. .....

2. Carefully open the column. You can open the column with a screwdriver. Gently tap the beads into a 1.5 ml eppindorf tube.
3. Pipette 1 ml of NH_4OH into the tube. Finger flick to suspend the beads. You can let stand or **slowly rotate** at room temperature for an hour. Pipette off the aqueous layer and put into a sterile, glass tube with a screw-on cap that screws down tight.
4. You can repeat step #3 two more times, but yields do not improve much.
5. Incubate the solution for 12-15 hrs at 55°C.
6. **Let the sample cool to room temperature, then open tube.** Dry the sample using a stream of nitrogen, argon or air. We use the simplest setup, a standard aquarium pump pushing a stream of air across the openned tubes at 55°C. Vent the room well as Ammonium hydroxide is nasty.
7. Suspend the "crud" on the bottom of the vial in 500 µl of sterile, distilled water.

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8. Make a 1/300 dilution and determine the absorbance at 260 and 280 nm using the spectrophotometer.
9. Purity of the preparation is assessed on the basis of the 260/280 ratio. Values near 1.7 are considered good.
10. To determine the concentration use the following formula. DNA at a concentration of 33 ug/ml has an absorbance of 1 OD at 260 nm. (Sorry about the formula in version 1.0, none of us could use it either).

$$\text{Concentration } (\mu M) = (A_{260} \times D \times 33{,}000) \div (N \times 330)$$

where A_{260} is the spec reading at 260nm, D is how much you diluted the primer stock (e.g. in our protocol it is 300 times), and N is the number of base pairs of the primer. **This gives you concentration in µM.** The 33,000 is the conversion for the Spec reading to µg per liter and the 330 in the denominator is the atomic mass unit for a single nucleotide.

11. Make 200 µl of a 100 µM primer solution and store the remainder at -20° or -80°C. From the 100µM solution you should make 500µl of a 10µM working primer solution.

MITOCHONDRIAL GENE PRIMERS

Here are some "global" primers for mtDNA that we use in our research. These primers are designed to be as broadly applicable across taxa as possible and therefore may provide some good lures to go fishing for sequences that can then be used to make more taxon-specific primers. The primers are all written in the 5' to 3' direction.

For each gene region, primer sequences are given and aligned with published sequences from a variety of taxa. A reference map is also included that shows the location of the primers relative to each other. H and L refer to the heavy and light strand, respectively.

Degeneracy symbols: Y = C,T; R = G,A; Z = C,G; S = C,A; Q = A,T; M = A,T,C; X = Amino link.

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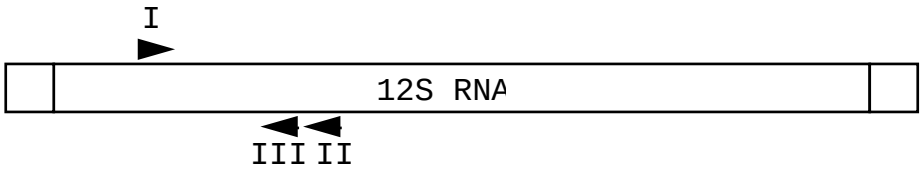
12s RNA Primers



Map Position	Primer/Taxa	Sequence	
I.	**12SA-L**	AAACTGGGATTAGATACCCCACTAT	**Position**
	Human	.....	1067
	Xenopus	.....	2486
	Urchin	....CA.....TGT...	491
	Drosophila	.....A.....T.T...	14612
II.	**12SB-H**	GAGGGTGACGGGCGGTGTGT	**Position**
	Human	.....	1478
	Xenopus	.....	2898
	Urchin	...A.....A.....	853
	Drosophila	A..A.C.....A.....	14211

Comments: Universal primers (see Kocher et al. 1989). For vertebrates it is useful to use 12SA-L with 16SA-H. This is a large fragment (≈1425 bp) that can be subject to restriction digestion or sequenced from both directions and then new primers made to walk through most of the 16S gene and the 3' part of the 12S gene. In *Drosophila*, 12SA is the L-strand primer and 12SB the H-strand primer.

Map Position	Primer/Taxa	Sequence	
I.	**12sai-L**	AAACTAGGATTAGATACCCTATTAT	**Position**
	Human	.....G.....C.C...	1067
	Urchin	....C.....G....	491
	Drosophila	.....	14588
II.	**12sbi-H**	AAGAGCGACGGGCGATGTGT	**Position**
	Human	G..G.T.....G.....	1478
	Urchin	G....T.....	855
	Drosophila	.....	14214

Comments: 12sai and 12sbi were made for cicadas (insects) by Chris Simon. They work for most crustacea (except barnacles].

III.	**12sc-H**	AAGGTGGATTTGGTAGTAAA	**Position**
	Human	.....A.C.....	1416
	Xenopus	....C.....A.C.....	2834
	Urchin	Does not exist!	?
	Drosophila	.....	14275

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I.	**12se-L**	ATTCAAAGAATTTGGCGGTA	**Position**
	Human	.C.....G.CC.....G	1154
	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.

I.	**12st-L**	GGTGGCATTTTATTTTATT--AGAGG	**Position**
	Human	..C..TGC..C..A.CCCTCT.....	1175
	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.

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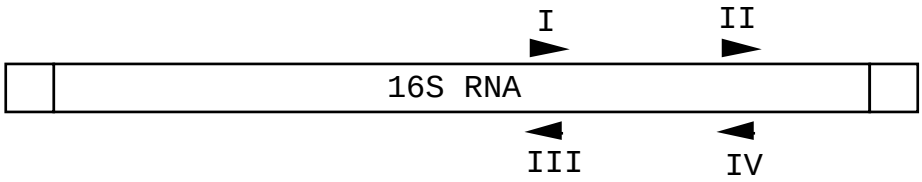
16s RNA Primers



Map Position	Primer/Taxa	Sequence	
I.	**16sar-L**	CGCCTGTTTATCAAAAACAT	**Position**
	Human	.....C.....	2510
	Xenopus	.....C..GC.T.....	3999
	Urchin	.....C.....	5092
	Drosophila	.....A.....	13398
II.	**16sb-L**	ACGTGATCTGAGTTCAGACCGG	**Position**
	Human	.....	3080
	Xenopus	.....	4572
	Urchin	.....	5682
	Drosophila	..A.....A.....	12887
III.	**16sa-H**	ATGTTTTTGATAAACAGGCG	**Position**
	Human	.....G.....	2510
	Xenopus	.....A.GC..G.....	3999
	Urchin	.....G.....	5092
	Drosophila	.....T.....	13398
IV.	**16sbr-H**	CCGGTCTGAACTCAGATCACGT	**Position**
	Human	.....	3080
	Xenopus	.....	4572
	Urchin	.....	5682
	Drosophila	.....T.....T..	12887

Comments: These 16s primers work for urchins, vertebrates, insects, 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. We have had good luck in double strand sequencing these products, but asymmetric amplifications can be very difficult.

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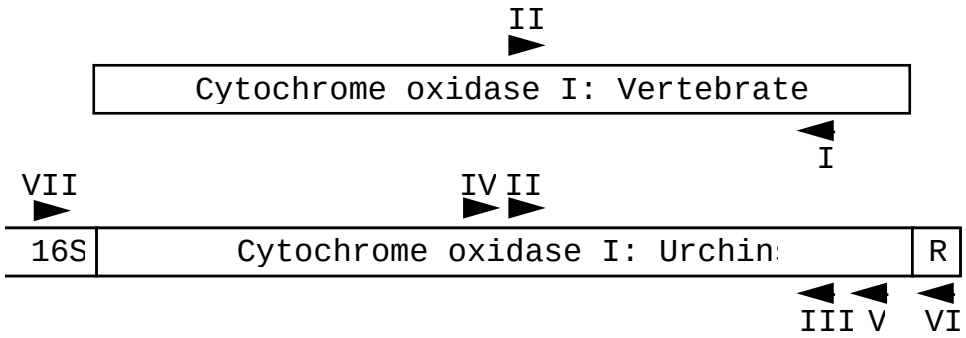
Cytochrome oxidase I Primers



Map Position	Primer/Taxa	Sequence	
III.	**CO1a-H**	AGT ATA AGC GTC TGG GTA GTC	**Position**
	Human	G.. G.. T.. A.. G..	7227
	Xenopus	T..	8720
	Urchin	T.. ... G.. ... ... A.. ...	7108
	Drosophila	... G.. ... A.. A.. ... A..	2791

Comments: Works well for a wide variety of taxa including urchins, vertebrates, crustacea and algae. Typically, we have used COIa and COIf in initial low stringency amplifications, and have used the subsequent sequence data to design taxon-specific primers.

IV.	**CO1c-L**	TC GTC TGA TCC GTC TTT GTC AC	**Position**
	Human	.. .. C.A A.. ..	6454
	Xenopus	.. ..T ... ..A ..A ..A A.. ..	7947
	Urchin	.. ..	6335
	Drosophila	.T ..A ... ..A ..A G.. A.T ..	2018

Comments: Designed for urchins, but useful for some vertebrates.

V.	**CO1d-H**	GAA CAT GAT GAA GAA GTG CAC CTT CCC	**Position**
	Human	... T.. ... AGT ... A.. G.T T.. GG.	7258
	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 sea urchins, and works erratically outside this group.

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Cytochrome oxidase I primers (cont.)

Map Position	Primer/Taxa	Sequence	
VI.	**Arg-H**	CGAAATCAGAGGTTCTCCTTAAAC	**Position**
	URCHIN	.....	7380
VII.	**16SB-L**	ACG TGA TCT GAG TTC AGA CCG G	**Position**
	HUMAN	... ...	3079
	XENOPUS	... ...	
	URCHIN	... ...	
	DROSOPHILA	... ..T ... ... .T. .	

Comments: Used for amplifiation of urchin COI. Note that the positions of the ARG tRNA . the COI, and the 16s RNA genes are different in echinoderms than other phyla.

Map Position	Primer/Taxa	Sequence	
I.	**CO1e-H**	CCA GAG ATT AGA GGG AAT CAG TG	**Position**
	Human	..T ... .A. ..G ..A	7110
	Xenopus	... .TA .A. .AC	8602
	Urchin	... ... .AG ..G ..A ..C	6992
	Drosophila	... .TA .A. .AT ... T..	2672
II.	**CO1f-L**	CCT GCA GGA GGA GGA GAY CC	**Position**
	Human	..C ..C	6569
	Xenopus	... ..C ... .T	8061
	Urchin	... ...G	6451
	Drosophila	..A ..T	2131

Comments: COIf is one of our most general protein coding primers, being useful in amplifications of every phylum tried except Cnidaria. The initial companion to COIf is usually COIa. CO1e and CO1f make superb dsDNA amplifications for vertebrates. ssDNA amplifications often smear for both primers. Sequencing is difficult with the CO1f primer, perhaps because of its small size and degeneracy. Sequencing with COIe is easier. Successful COIa-f amplifications have been obtained for dinoflagellates, sharks, lamprey, fish, sea urchins (CO1f only), spiders and shrimp. Note that CO1f is a degenerate primer.

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Cytochrome oxidase I primers (cont.)

Map Position	Primer/Taxa	Sequence	
I.	**CO1i-L**	X CTT CCT GCT TTT GGA ATA ATT TC	**Position**
	Human	- ..A ..A .GC ..C ... .T ..	6645
	Xenopus	- T.A ..A .GG ... ..C ..G ..C ..	8138
	Urchin	- T.A ..G .GA ... ..T ..G ..C ..	6526
	Drosophila	- T.A ..G .GA	2308
II.	**CO1h-H**	CGA GTG TCT ACR TCT ATA C	
	Human	..T ... .G ... ..T .	6790
	Xenopus	... ..A ... ..G .T. .G. T	8283
	Urchin	..T ..A ..A ..A ..C ..C .	6671
	Drosophila	..T ..A ..A ..G ... ..T .	2354

Comments: These primers were made for zooxanthellae.

Echinometra Probes

Map Position	Primer/Taxa	Sequence	
1.	**EP1-14l**	TCA ATT CTC CAA TG	**Position**
	Human	AGC .A. A.G A.. ..	6891
	Xenopus	GGG .CA A.T A.. ..	8384
	Urchin	..T .A.	6772
	Drosophila		2554
	EP1-10l	ATT CTC CAA T	**Position**
	Human	.A. A.G A.. .	6888
	Xenopus	.CA A.T A.. .	8381
	Urchin	.A.	6769
	Drosophila		2551
2.	**EP2-14H**	AA TAA AAA TAC AAA	**Position**
	Human	.. A.G ... G.T T..	6947
	Xenopus	.. C.. G.. .AT G..	8440
	Urchin	.. ..	6828
	Drosophila		
	EP2-10H	AA AAA TAC AA	**Position**
	Human	.G ... G.T T.	6947
	Xenopus	.. G.. .AT G.	8440
	Urchin	.. ..	6828
	Drosophila		

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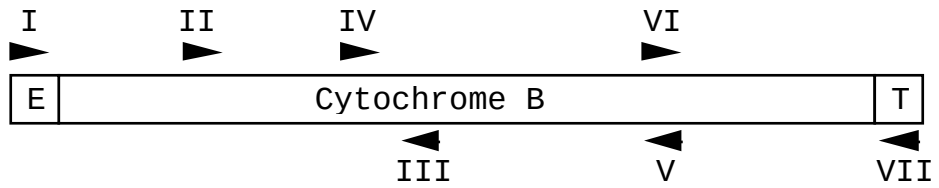
Cytochrome b Primers



Map Position	Primer/Taxa	Sequence	
I.	**GLU-L**	TGA TAT GAA AAA CCA TCG TTG	**Position**
	Human	... C.. ... ... T..	14724
	XENOPUS	... .C. ... ... C.. ...	
	CARP	... CT. ... G.. ... C.. ...	
	CHICKEN	C.G CT. ... ..G ... .T. ...	
	SHRIMP	..C AT. ... ..T G.. .T. .AT	
I.	**GLUDG-L**	TGA CTT GAA RAA CCA YCG TTG	14724

Comments: The GLU-L primer is OK but the re-designed GLUDG-L primer works much better, showing consistent success with all vertebrates tested to date, including elasmobranchs, teleost fishes, and seals.

Map Position	Primer/Taxa	Sequence	
II.	**CB1-L**	CCA TCC AAC ATC TCA GCA TGA TGA AA	**Position**
	HUMAN	... ...C	14817
	STINGRAY	... ...C A.T	
	XENOPUS	... ..A ... ..T ... T.. .T.	16321
	URCHIN	..C ... ... C.T ..C ATT ..G	14581

Comments: Universal primer (see Kocher et al. 1989). In our hands this primer works on sharks and fish. We have substituted the GluDG primer, for this, however, because GluDG to Cb2 gives a bigger fragment.

CB1a-L	ATT CTA ACT GGA CTA TTC CTT GCC	**Position**
HUMAN	..C ACC ..A ... ...A ...	14881
DROSOPHILA	... T.. ... ... T.. ..T T.A ..T	10646
URCHIN	..A T.G ... ... A.. ... ..A ..A	14644
XENOPUS	..C A.T ..A ... T.. ... T.A ..T	16385

Comments: Butterflyfish specific primer just inside the **CB1-L** primer.

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Cytochrome B primers (cont.)

Map Position	Primer/Taxa	Sequence	
III.	**CB2-H**	CCC TCA GAA TGA TAT TTG TCC TCA	**Position**
	HUMAN	... ...	15175
	STINGRAY	... ...	
	XENOPUS	... ... A.. A..	16677
	URCHIN	AG. ... A.. G.. C.. ... C.. C..	14937

Comments: Superb primer for GluDG to Cb2 amplification (see Kocher et al. 1989).

Map Position	Primer/Taxa	Sequence	
IV.	**CB4-L**	A TAT TAY GGC TCC TAC CT	**Position**
	HUMAN	. ..C ... ..A ... ... AC	15053
	XENOPUS	G ..C ... .T .T. T.	
	STINGRAY	. ..C	
	URCHIN	. ..C ... ..G ..T ..T AA	
	DROSOPHILA	T ..T ... ..A ..A ..T T.	

Comments: Works for sharks

IV.	**CB4a-L**	AAC AAA GAA ACC TGA AAY ATY GG
	STINGRAYS	..Y ... ..R ... ...
	SHARKS	T.Y ... ..R ..N ... ...
	HUMAN	T.. TC. ... ...
	FISH	C.A ... ...
	URCHIN	..G .TT ..G ... .A G.. ..

Comments: Works for elasmobranchs and fishes.

Map Position	Primer/Taxa	Sequence	
V.	**CB3-H**	GGC AAA TAG GAA RTA TCA TTC	**Position**
	HUMAN	... ... G.. A..	15560
	XENOPUS	... G..	17065
	STINGRAY	... ...	
	STURGEON	... ... G.. A..	
	URCHIN	... G.. ..A ... ... C.. ...	
	DROSOPHILA	A.. ... ..A A..	11325

Comments: Works for sharks, mammals, birds, fish, and probably all vertebrates on Earth and elsewhere.

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Cytochrome B primers (cont.)

```
CB3b-H        ATT ATC TGG GTC TCC GAA YAG GTT      Position
HUMAN         ... G.. ... ... G.. T.G G.. ..C        15490
DROSOPHILA    G.. ... ... ... ... C.. ..A A..        11260
URCHIN        T.. C.. ... ... .TT T.G GGC .CC        15253
XENOPUS       ... G.. ... ... ... T.. ... ...        16995
```

Comments: Butterflyfish specific primer just inside the **CB3-H** primer.

```
CB3a-H        C TAG GAG GTC TTT ATA GGA GAA GTA    Position
HUMAN         . G.. .GC ... ... GAT T.T .T. ...      15415
DROSOPHILA    . ..C A.T A.. C.. A.A T.T ... ...      11179
URCHIN        . ... TGT ... C.. G.T C.T ... ...      15178
XENOPUS       . ..A A.. ... ... G.. A.. ... ...      16920
```

Comments: Butterflyfish specific primer just downstream from the **CB3-H** and **CB3b-H**.

Map Position	Primer/Taxa	Sequence	
VI.	**CB3R-L**	CAT ATT AAA CCC GAA TGA TAY TT	**Position**
	RAT	..C ..C ... ..A	15560
	XENOPUS	... ...A	17065
	STINGRAY	... ...	
	URCHIN	..C ... C.G ..A ... ..G	
	DROSOPHILA	..C ... C.. ..A	11325

Comments: Works for elasmobranchs, mammals, birds, and fish.

Map Position	Primer/Taxa	Sequence	
VII.	**CB6THR-H**	CTC CAG TCT TCG RCT TAC AAG	**Position**
	HUMAN	T.T ..T ... C.. .T.	15930
	XENOPUS	... ..T	
	STURGEON	..T .GA ... ... .T.	
	SHARK	... ...	
	URCHIN	.C. TCT CTC CTG .T.	
	DROSOPHILA	A.. TTA .T. .T. .T.	

Comments: Works for elasmobranchs.

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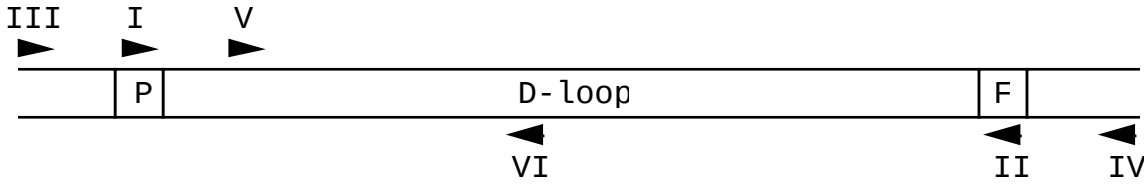
D-LOOP



Map Position	Primer/Taxa	Sequence	
I.	**PRO-L**	CTA CCT CCA ACT CCC AAA GC	**Position**
	HUMAN	.C. ..A TT. G.A	15980
	XENOPUS	.C. .TA TTG ..C	
	STURGEON	TC. ..C TT.	
	URCHIN	TAC AT. G..	
II.	**PHE-H**	TCT TCT AGG CAT TTT CAG TG	**Position**
	HUMAN	C.G ... .AA	625
	XENOPUS	..A ... CA.	
	URCHIN	C.. .TG .A.	

Comments: Works for some fish (from the Wilson lab)

Map Position	Primer/Taxa	Sequence	
V.	**CB3R-L**	CAT ATT AAA CCC GAA TGA TAT TT	**Position**
	Human	..C ..C ... ..A	15560
	XENOPUS	... ...A	
	STINGRAY	... ...	
	URCHIN	..C ... C.G ..A ... ..G	
	DROSOPHILA	..C ... C.. ..A	
IV.	**12SAR-H**	ATA GTG GGG TAT CTA ATC CCA GTT	**Position**
	HUMAN	... ...	1067
	XENOPUS	... ...	
	DROSOPHILA	... A.A ... ... .T. ...	

Comments: These two primers amplify the entire D-loop plus the flanking tRNAs and portions of the cytochrome b and 12S genes in fish and mammals.

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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. But then, this is all the excuse a good primer needs anyhow.

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.

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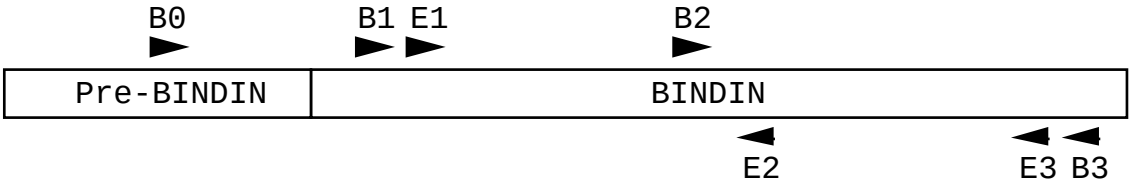
NUCLEAR GENE PRIMERS

BINDIN sperm-egg attachmemnt locus in sea urchins




```
B0            AAA AGG GCA AGT CCT CGN AAR GG
S. FRANC.     ... ... ... ... ... ..T ..G ..
S. PURP.      ... ... ... ... ... ..T ..G ..
ARBACIA       ... ..A ... ... ..G ..G ..A ..
LYTECHIN.     ... ... ... ... ... ..G ..A ..
```

Comments: Anneals in the middle of the pre-bindin in a region of high homology. We have two versions, a full length regular version, and one in which the 6 bases on the 5' end are deleted, and the degenerate position 'N' is occupied by a biotinylated spacer. There is no logic to this, it just happened this way.

```
B1            TAC CCC CAA GCA ATG AAT CC
S. FRANC.     ... ... ... ... ... ... ..
S. PURP.      ... ... ... ..C ... .G. ..
ARBACIA       ... ..A ... C.G ... ... .A
LYTECHIN.     ... ... ..G ... GGT GGA G.
```

```
B2            CAC CTC AGG CAT CAC TCC AAC CTT CTM GC
S. FRANC.     ... ... ... ... ... ... ... ... ... ..
S. PURP.      ... ... ... ... ... ... ... ... ... ..
ARBACIA       ..T ... C.C ... ... ..A ... ..C ..C ..
LYTECHIN.     ..T ... ... ... ..T .G. ... ... T.. ..
ECHINOMET.    ..T ... ... ... ... ... ... ..C T.G ..
```

```
B3            CTA CCC CTG TCG ATA ACC CTG
S. FRANC.     ... ... ... ... ... ... ...
S. PURP.      ... ... ... .A. ... ... ...
ARBACIA       T.. T.. T.. CT. .CG GTA TCC
LYTECHIN.     T.. ... T.. CAT G.. ... ...
```

```
E1            CAG CTA GCC CAA CAA GGT TA
S. FRANC.     ..A .C. ..G ..G ..G ... ..
S. PURP.      ..A .C. ..G ... ... ..G ..
ARBACIA       GGA CAG C.. GTG ... ... ..
LYTECHIN.     ..A .C. C.G ... ... AA. ..
ECHINOMET.    ... ... ... ... ... ... ..

E2            GGC GCT AAG GAC CTG TTC TCG
S. FRANC.     C.. A.. G.. ... ... ... C..
S. PURP.      C.. ... T.. ... ... ... C..
```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```
ARBACIA     T.. GG. C.. ... ... G.T ...
LYTECHIN.   ... A.. G.. ... ... ... C.T
ECHINOMET.  ... ... ... ... ... ... ...


E3          GC ATT CAC TTG TGC RCC CAT CCC TG
S. FRANC.   TG .GG A.. --- .C. ... ... ... .T
S. PURP.    C. T.G ATG .G. .C. ... ... ... .T
ARBACIA     DOES NOT ALIGN
LYTECHIN.   TT G.A AC. ... .C. A.. ... T.. .T
ECHINOMET.  .. ... ... ... ... ... ... ... ..
```


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CREATINE KINASE

```
CK6-5'     GAC CAC CTC CGA GTC ATC TCZ ATG
MOUSE      ... ... ... ..C ..G ... ... ...
FISH ... ... ... ... ... ... ... ...
URCHIN     ..T ... .CT ... ... ... ... ...

CK7-3'     CAG GTG CTC GTT CCA CAT GAA
MOUSE      ... ... ... ... ... ... ...
FISH ... ... ... ... ... ... ...
URCHIN     A.. A.. T.T ... ... ... ...
```

Comments: This pair of primers amplifies the 6th intron of creatine kinase genes. There are three loci (muscle-specific, brain-specific, and mitochondrial). In general, there are 2 or 3 (and rarely four) amplification products corresponding (presumeably) to the duplicated, triplicated (or quadrupled) genes. The amplified fragments differ in size and can be separated by gel electrophoresis, isolated and re-amplified separately. Works on elasmobranchs, teleosts, whales, birds.

```
CK1-5'     ACC ATG CCT TTC GGQ AAC ACY CA

CK2-3'     TCC TGG TTZ AGG TCR GTC TT
```

Comments: This pair of primers amplifies the 1st intron of creatine kinase genes.

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VECTOR PRIMERs

M13 Universal-Reverse GAATTCAACAGCTATGACCATG

Comments: This primer is used in vector-mediated amplification.

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A linearized map of gene order in mtDNA of three taxa. tRNA genes are shown with hatching. Relative size of the gene regions are approximations!

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

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SOLUTIONS APPENDIX

Taq Polymerase Buffer

10 mM	Tris (pH 8.3)
1.5 mM	$MgCl_2$
50 mM	KCl
0.01%	Gelatin
0.01%	NP-40
0.01%	TritonX 100

TE Buffer (pH 7.6, usually made as 100X)

10 mM	Tris
1 mM	EDTA (pH 8.0)

TBE Buffer (pH 8.3, made as 5X)

45 mM	Tris
45 mM	Boric Acid
1 mM	EDTA (pH 8.0)

TAE Buffer (pH 7.8, made as 50X)

40 mM	Tris
5.71 %	Glacial Acetic Acid
1 mM	EDTA (pH 8.0)

Dense-Dye

50 mM	EDTA
30%	Glycerol
0.25%	Bromophenol Blue
0.25%	Xylene Cyanol

dNTP mix (pH 7.0)

2.0 mM	dATP (USB 14244)
2.0 mM	dGTP(USB 14314)
2.0 mM	dCTP (USB 14279)
2.0 mM	dTTP (USB 22324)

aliquot in 0.5 ml lots in sterile tubes

Ammonium Acetate (pH 7.5)

7.5 M	Ammonium Acetate

Lysis buffer

100 mM	EDTA
25 mM	Tris pH 7.5
1%	SDS
100µg/ml	Proteinase K (added just before use)

40% Acrylamide

38%	Acrylamide
2%	Bis-acrylamide

TBE/Urea Solution

10 M	Urea
0.625X	TBE

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ABBREVIATIONS

amp - amplification
ds - Double-strand DNA
ss - Single-strand 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)

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