Alkami Quick Guide™

for PCR

A laboratory reference for the

Polymerase Chain Reaction

Volume I

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Preface

The polymerase chain reaction (PCR) is arguably the most important biotechnological innovation to date, and is rapidly becoming a standard technique in molecular biology research. Since its conception in the mid-1980's by Nobel Prize winner Kary Mullis and other scientists at Cetus Corporation, new and innovative applications for PCR have been and are being developed at an exponentially increasing rate. These new uses for PCR are literally transforming the way we do biological research, diagnostics and drug discovery.

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How often have you painstakingly searched for the information you needed to troubleshoot and optimize the complex relationships among your PCR components? Obtaining a specific bit of protocol information in the literature can be extremely time consuming and the standards used among labs vary greatly. To address these issues, we present you with a concise reference to key PCR protocol method information. This volume includes primer design tips, polymerase characteristics, PCR methods, variable ranges, enhancers, inhibitors and troubleshooting information. This material has been collected from a broad range of sources, organized and standardized so that you can more easily achieve the PCR product you need.

Recognizing the growing importance of the Internet, we have also provided you with information about online resources. This includes reviews of free online primer design software as well as a list of links for the growing number of specialized genomic databases available to you.

We hope you find this collection a valuable asset in your research.

Lab Supply Selection

Laboratory Gloves

By Laurel V. George

Donning gloves in a laboratory is so commonplace, many researchers don't stop to think twice about why gloves are necessary before they put them on. Lab technicians frequently work with chemicals like DMSO, Ethanol, Ethidium Bromide, and P32 radiation: all can burn skin or potentially alter your DNA, thereby causing cancer. Lab technicians also must be wary of sample contamination. No one wants to spend four months sequencing a piece of DNA to later find out they've just sequenced a loose skin flake that contaminated their sample! Gloves also protect researchers from being contaminated in case they're working with viruses or other pathogens. In the end, gloves save time, money, and your health. The only question is: With all the gloves on the market, which ones are best for your lab?

Latex Gloves

Latex is the kind of glove found in most laboratories. They usually come in various sizes of powdered, lightly powdered, or powder-free varieties. They fit well and are flexible, have a high resistance to cuts and tears, and aren't too costly. Latex is a natural product, namely the sap of Hevea brasiliensis, the rubber tree. The sap is a mixture of the polymer cis-1,4-polyisoprene, and hundreds of different kinds of proteins. Before latex is manufactured into a glove, chemical antioxidants and accelerators are mixed in to give added strength. It's mainly the proteins, not the added chemicals, that cause the well-known latex allergy, the effects of which can be as severe as death from shock! Because latex allergies show up only after prolonged exposure, most people won't suffer much more than irritation from their gloves before they realize they have an allergy. Latex gloves are also often powdered

with talc, which can cause microscopic cuts in your hands--adding another source of allergy and irritation.

Reflecting the various causes and degrees of allergies, many variations of latex and non-latex gloves are available. Medical-grade cornstarch powder has become the standard for most gloves, eliminating the talc problem. Unfortunately, cornstarch absorbs the protein antigens in latex responsible for the latex allergy, making the gloves even more allergenic. Many technicians choose powder-free latex gloves and avoid the powder difficulty altogether. Since latex gloves are coated with the powder before they are removed from the mold, they must be treated with chlorine before they can be considered powder free. Chlorine is also used to preserve the latex's light color; subsequently chlorine-treated gloves have a shorter shelf life (only 6 months to a year) and pose yet another health hazard to their wearers. Even though you can buy "hypoallergenic" latex gloves that have been washed and steam sterilized, the best way to avoid a latex allergy is simply to use non-latex alternatives.

Vinyl Gloves

An inexpensive alternative to latex gloves is vinyl gloves. These are almost as tear-resistant as latex and come in powdered and powder-free varieties. The chemical resistance of vinyl is about equal to that of latex, but fit and comfort aren't as good. Vinyl gloves have been proven more effective in protecting your hands from DMSO than latex, and they pose only a small allergy risk.

Nitrile Gloves

For those who have a little more money to spend, nitrile gloves are a superior non-latex choice. They have very low allergenicity and tear and puncture resistance equivalent to latex, and their fit and comfort level ranks between that of vinyl and latex. The main benefits of nitrile gloves are increased chemical resistance, strength, longevity, and sensitivity.

No matter what kind of glove you choose, a few common characteristics should be considered. Glove thickness will affect sensitivity. Some gloves are textured to increase gripping ability without reducing sensitivity. The location of the glove manufacturer will affect fit and age--and therefore dependability. A well-fitting glove will cut down on lab accidents and annoyances resulting from a baggy or circulation-cutting fit. If the gloves are made in Asia (for example), they will be cut to a narrower hand mold to fit their physique and won't fit wide North American hands as well as gloves produced here. Also, due to the chlorination process, gloves manufactured overseas will be older and will have a shorter shelf life once they arrive at the lab due to the extra shipping time. Unpowdered gloves only last for about 6 months before the chlorine starts to affect the quality of the gloves, so if they are in transit and warehouses for one to two months, that greatly affects how long you can keep the gloves around before they start to degrade.

Some manufactures also offer tapered fingertips, beaded cuffs for tear resistance, and non-cuffed nitrile gloves that increase air circulation. (The last are too expensive to throw away every time a bead of sweat appears.) For those without specific allergies, deciding whether to use powdered or unpowdered gloves is purely a matter of taste. Powder-free gloves are slightly more expensive due to the extra chlorination processing. In any case, make sure your manufacturer performs inflation- or water-testing to check for pinholes or tears; exam-quality gloves (guaranteed to keep out pathogens) must pass ASTM standards.

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PCR Microtubes

By Laurel V. George

PCR can be a very expensive process. Aside from the costs of the thermal cycler and different polymerases, plastics can play a very important role in affecting the cost of running a PCR experiment. Thermal cyclers heat the sample in order to denature the DNA, but if the heating isn't precise and accurate, the added polymerase will be destroyed as well, thus reducing the amount of usable product.

Because the effectiveness of PCR depends on the degree of temperature control you have over your sample, normal microtubes used for minicentrifuges do not function well in a thermal cycler. Minicentrifuge tubes' thick walls reduce the heat transfer from the thermal cycler to the sample. PCR-specific microtubes, on the other hand, have a special thin-walled design that promotes accurate and uniform heat transfer while simultaneously decreasing cycling times. PCR microtubes also come with specially designed caps to aid in sealing the tube during the reaction.

PCR microtubes are frequently made of polypropylene, a polymer with a smooth surface that will not bind to the enzymes in the sample, again improving product recovery. Some older tubes, especially tube grids, may be made of polycarbonate. These have structural inconsistencies leading to protein binding and loss of product. Most companies produce only polypropylene PCR plastics for this reason, but it's important to notice what the product you're interested in is composed of.

Another key feature to look for in PCR tubes is boil-proof caps. Without a secure, sealed, tight-fitting cap, product can be lost to evaporation. Thermal cyclers can be fitted with a heated lid, which increases the

sealing ability of the cap and reduces the need for sealers like an oil overlay. Tubes with domed caps are better heat conductors than the basic flat-capped variety because the top of the dome actually comes in contact with the heated thermal cycler lid. When flat-capped tubes are used, silicon pads can be fitted between the tubes and the heated lid to reduce dead airspace in between, leading to a more productive experiment.

As labs advance and accumulate more robotic technology, the amount of sample that can be run each time is increasing quickly. PCR microtubes are frequently found in .2 ml volumes and in many varieties. Grids of cycling tubes have been developed to accommodate robotics that can pipet many samples at once. Most common on the market are 96-well grids, consisting of many microtubes connected together, usually in a standard 8-by-12 format. However, 384- well grids are making their way into the market as well. Grids can be cut into any configuration needed.

The caps for the grids come in eight-cap strips that fit over a single row of tubes. Robotic pipets and tube grids greatly reduce the risk of sample contamination. Tube grids, especially skirted plates, are very stable, but they unfortunately do not fit in all standard thermal cyclers. It's best in this case to ask the supplier for a sample set so you can make sure the plastics you're interested in are compatible with your equipment.

Single rows of either 8 or 12 tubes are also available; these rows can also be used with the cap strips mentioned above. Tube strips have been known to splatter samples all over the lab if accidentally bumped the wrong way, so you have to have steady hands. For slightly more money, tube strips can be bought with individually attached caps. These are more convenient, especially if the strips are ever cut and the tubes used separately. The most economical approach to buying microtubes is getting mass quantities of individual PCR tubes.

Individual tubes come in many varieties and are more likely to have special features like boil-proof seals or calibration marks on the outside of the tube. Individual tubes with domed caps are more expensive than standard flat-capped PCR tubes, but they're more effective with respect to temperature control. Tubes without caps are also available. Some companies even have adapter tubes that enable you to spin a 0.2 ml PCR microtube in a standard 0.5 ml microcentrifuge.

A few other characteristics to look for, depending on the needs of your lab, are frosted flat-caps or frosted sides. These make labeling samples very easy. For about ten dollars more per package, PCR plastics can be gamma- irradiation sterilized. Usually, sterilized products have fewer color choices. Also make sure all tubes are made from virgin (only heated once) polypropylene and are at least tested for Dnase and RNAse, if not for human DNA contamination and PCR inhibitors as well.

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Pipetts & Tips

By Laurel V. George

Before a researcher can even contemplate running a PCR sample, they must get it into the many microtubes involved in the PCR process. As anyone who has ever worked in a molecular biology lab knows, the volumes of reactants used are measured on a scale of microliters (µl): 1/1,000,000 of a liter. Due to their small size, measuring ml samples cannot be based on human visual intuition as measuring milliliters sometimes can--especially when you're using expensive reagents. In order to sustain accuracy, you must use a calibrated pipette to measure out the microscopic quantities. Otherwise,the recorded amount of sample may be very different from the actual amount being run in the thermal cycler. This can cause experiments to fail and recorded data to be useless, and, worst of all, wastes a lot of money.

Manual Pipettes

There's a pipette for every budget and need. Manual pipettes come in single and multichannel versions and range in price from a few hundred dollars to many hundreds, depending on the model. Single-channel pipettes usually come in six varieties specific to the target volume range. Manual pipettes can be used to pipette as little as 0.1 µl or as much as 10 ml of sample. Such pipettes consist of a manual volume setting for adjusting the amount you want to draw from the main sample, a tip-ejection button, and a plunger that draws and releases the liquid. Because you use the manual plunger at the top of the pipette for pipetting and blowout (the release of liquid), one of the main problems caused by the manual pipette is hand-and-thumb stress from continual plunging. Manual pipettes have been adjusted by manufacturers over the years to help solve this problem.

One of the more economical solutions is a manual pipette resembling the original except with a trigger button located where your index finger lies. This is pressed to draw solution. These trigger pipettes are usually designed to use thin-walled, fine-tipped pipette tips, which reduce the ejection force needed. Unfortunately, such pipettes may not afford you the same control over the speed at which the liquid is drawn: this may affect the accuracy of the sample. The plunger is used to dispense the fluid and to set the volume to be drawn, as in any other manual pipette. Modern pipettes have been adjusted so that pressing the plunger will not affect the volume setting, and the volume can be adjusted easily and without snagging your gloves. Those of us who have worked with older pipettes know how frustrating it is to get your glove caught in the pipette in the middle of a tedious titration! Depending on the manufacturer, pipettes can be bought ready for either adjustable- or fixed-volume plunger attachments. Most fixed-volume attachments are color-coded and range from 0.5 µl to 5 ml. Manual pipettes, because of the lack of fancy parts and electronics, will last up to ten years longer than other versions and are a good thing to have lying around any lab, if only to be used in emergency situations.

Multichannel manual pipettes usually consist of eight channels which simultaneously draw up liquid, then quickly dispense the sample into 96-well plates. Multichannel pipettes also come in variable sample-size versions ranging anywhere from 0.5 µl to 300 ml, depending on the manufacturer. They are offered by most pipette manufacturers, but not by all.

After much repetitive usage, manual pipettes must be recalibrated. Many companies specialize in recalibration, and some manufacturers even offer this service. Most recalibration is guaranteed for up to six months.

Electronic Pipettes

Computer-controlled electronic pipettes can improve the precision of your sample because the plunger movements are automatic and controlled by the computer. This allows all samples of a certain amount to be exactly the same without influence from user variation. An electronic pipette is capable of drawing up one large sample--10 ml, for example--and then dispensing it in 1 ml samples into 10 microtubes. Because the computer controls the movements of the piston and the blowout is automatic, all the researcher has to do is press the trigger button to commence pipetting. This greatly reduces hand stress and the risk of repetitive-motion injuries.

Electronic pipettes resemble manual pipettes with the addition of a battery compartment in the handle. A small display and key pad replace the manual plunger. Unlike manual pipettes, electronic ones recalibrate themselves after every dispensing sequence, thus again decreasing the risk of measurement error. Electronic pipettes are much more expensive (prices range in the thousands of dollars) than manual pipettes and are much more complicated to use. Everything from the draw amount to the draw-up speed must be programmed in by the researcher. While electronic pipettes can quickly dispense many accurate samples from one draw-up, using one to draw a single amount to dispense right away can be more time-consuming than using a simple manual pipette for the same task.

Pipette Tips

No matter which pipette you choose, all pipettes need tips. Some pipettes are designed to fit all tips universally, while some tips are designed to fit all pipettes universally. Most tips and pipettes, however, are specifically compatible.

Filter tips are also very common. These look like normal tips but have a hydrophobic (usually) filter placed inside. This filter prevents sample vapors from rising up into the pipette and contaminating it. The filters are also useful when working with hazardous or radioactive samples because they prevent the researcher from breathing in sample vapors.

Companies also frequently provide tips for things like robotic pipette machines, or for use with very small or large volumes. Tip manufacturers will usually provide a list or chart describing which of their tips will fit pipettes made by various manufacturers. It gets very complex. Some companies provide both tips and pipettes, which makes compatibility search a little easier.

When searching out a tip manufacturer, look for one that makes tips out of virgin polypropylene. As I described in my PCR Microtube article polypropylene tips decrease sample retention in the tip after blowout and also decrease the possibility of biological contamination. A few other features to look for in tips are thin walls, which decrease ejection force, and steeply beveled tips with small openings, which increase the accuracy of the dispensed sample. Tips should be autoclaved, sterile, and DNase and RNase free.

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PCR Sealing & Storage Products

By Laurel V. George

Because thermal cyclers rotate between very high and very low temperatures when running a sample, an unprotected tube presents risks of evaporation, which could cause loss of product, and of condensation and consequent sample contamination. PCR microtube manufacturers offer caps that seal both single and microplate tubes; such caps help prevent evaporation and condensation of the liquid sample. Unfortunately, caps can be expensive, especially as adding and removing them from the tubes can cause your sample to spill and become contaminated.

Traditionally, mineral oil has been used to prevent evaporation with or without caps; a little is added to each tube before it's run, and because oil is hydrophobic, the vapors from the sample cannot break through. Wax pellets have also been used for this purpose. Both methods, but especially the oil overlay, can be time-consuming and messy. It's also difficult to retrieve product out of the tube without contaminating the sample with the foreign substance floating on top.

Much more simple, safe, and cost-efficient sealing methods have been developed for tube plates by companies specializing in PCR products; these sealing methods, as well as microtube caps, can also be used for storing your product after the experiment.

Foil and Film Adhesives

When running a PCR experiment using a 96-well plate, it's easy to imagine sealing all the tubes with one procedure. Many different kinds of foils and films are available in thin sheets, which are placed on top of the plates to seal all the tubes simultaneously. Films are flexible, clear sheets that make it possible for researchers to view the product in the tube through the film, allowing them to monitor the sample. In order to access your product, film must be peeled off, which leads to potential contamination.

Foil, on the other hand, is not clear. It is easily punctured by a pipette tip, so it need not be peeled away to remove the product (but it can be). This is a good sealer to use with robotics and when the reagents in the tube are light sensitive.

Some manufacturers offer foils and films that are sealed by a heat sealer (a special machine designed to heat-seal plates), and some offer foils and films that are sealed by a thermal cycler with a pressured, heated PCR lid (some offer both). The foils and films that are only sealed by an adhesive back aren't designed for high temperatures and should be used for storage only.

No matter which configuration you choose, the film or foil should be RNase/DNase free, autoclavable, and guaranteed to make a full hermetic seal without causing a vacuum (this causes the sample to "pop" and become contaminated due to released aerosol vapors). Also, check the temperature range for the adhesive to make sure it falls within your needed range. Non-sterile foil or film will run you about $45 for a hundred sheets, while sterilized film or foil will cost about $60 for the same quantity.

You can also use foils and films to safely store tissue cultures and biohazardous samples for a short while.

Micromats

Micromats made of rubber or silicone are available to seal 96-well plates, and unlike adhesives, they can be reused up to 50 times after

washing and decontamination. Because they aren't heat sealed, the seal isn't as tight. Micromats have been modified to have what the industry calls "dimples": protrusions in the mat which fit directly into the tubes, thus increasing sealing effectiveness.

While it may seem that the 384-well versions of sealing mats, film, and foils are appropriate for PCR, it's a good idea to read the fine print. Most of the sealing methods for 384-well plates have been specifically designed for high-throughput PCR and aren't meant to be used in a normal thermal cycler (if you can find one that uses 384-well plates). They can however, be used for long-term storage and transport.

Many accessories are available for sealing and storage methods, including heat sealers, foil strippers, and cap rollers that quickly snap rows of caps onto microtubes. Most of these are optional. Most foils and films are equipped with a simple tab that makes peeling them off an easy endeavor. They also should be available in storage boxes which are easily accessible, opened, and stacked. Because micromats can be washed, special storage devices aren't crucial to their survival and sterility. It is important, when accessing accessories, to evaluate how important and time-saving they will be to your lab.

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Primers

Primer Design Tips

Selecting the proper primer is one of the most important steps in designing your PCR experiment. You want your primer set to efficiently hybridize to your target sequence with as little hybridization as possible to other sequences that are also present in the sample.

The guidelines listed below are from a variety of origins and the ranges listed are the extreme values from all sources.

Primer lengths

Ideally, your primer should be between 18 and 30 base pairs long.

%GC content

Select primer sequences between 35% and 65% (some sources suggest 45% - 55%) G-C content. If your primer is overly G-C rich, add a string of As or Ts at the 5' end. If overly A-T rich, do the same with Gs and Cs.

Annealing Temperature (T_a)

The annealing temperature for the primer pair is generally calculated as 5°C lower than the estimated melting temperature. Differences of 4°C - 6°C between primers do not seem to affect the yield of PCR however, ideally the annealing temperature of each primer should match and be within the 55°C and 75°C range. If the T_a difference between the two primers is high, the lower T_a can be increased adding to the length of that primer at either the 3' end (this can also keep the size of the amplified locus constant) or the 5' end.

To calculate T_a for primers <= 20 bps Alkami Expert™ uses:

[4(G + C) + 2(A + T)] - 5°C

(See Suggs et al. 1981)

To calculate T_a for primers > 20 bps Alkami Expert™ uses:

62.3°C + 0.41°C (%G-C) - 500/length - 5°C

(See Bolton & McCarthy 1962)

Avoid annealing to regions of secondary structure

Primers will have difficulty annealing if they anneal to regions of secondary structure within the target that have a higher melting point than the primer.

Allow for a variety of post amplification manipulations

Restriction sites, promoter sequences and other complimentary, non-template 5' extensions may be added to primers to allow a variety of useful post amplification manipulations of your PCR product without a significant effect on the amplification itself.

100% complimentarity between primer and template is not necessary for polymerase catalyzed extension to occur. However, to insure stable annealing your primers should be as complimentary to the desired DNA sequence as possible.

Check for mispriming areas

To help prevent contaminating bands in your PCR, check the target DNA sequence to see if it is known to have mispriming areas. If you know the area around the target sequence, do a quick check of the vector sequence for regions with > 70% homology regions.

Choose your primer sequence from intronic gene regions

This is because they are divergent even in members of repeated gene families which are in tandem.

Primer Ends

Start and end primers with 1-2 purine bases.

Try to avoid Gs and Cs at the 3' ends because this may increase the chance of forming primer artifacts.

Check primers for self-complimentarity, especially at the 3' ends. If two loci are very similar (for example across species) it is useful to design the primers so that at least 1-2 bases at the 3' end are specific for the locus to be amplified.

The primer should be able to form "G/C" clamps.

Cycling conditions and buffer concentrations should be adjusted for each primer pair. This is to improve the chances for specific amplification of the desired locus, with no secondary products. If this is not possible, the sequences of the primers should be either elongated with 4-5 bases or changed entirely.

A good source of further information on how to design primers is the chapter "General Concepts For PCR Primer Design" in "PCR Primer: A Laboratory Manual" Edited by Carl W. Dieffenbach and Gabriela S. Dveksler, Cold Spring Harbor Laboratory Press, 1995.

Reviews of on-line and freeware primer design tools

By Lance Larka

The following reviews discuss a range of on-line and freeware PCR related tools. The criteria that I will be addressing include the ease of use, input/output options, interface, parameter selections, and any other special features unique to the individual tool.

The Primer Generator

http://www.med.jhu.edu/medcenter/primer/primer.cgi

The Primer Generator (TPG) is a handy tool for designing primers for Site-Directed mutagenesis. You enter the short nucleotide sequence of up to 15 bases, the desired amino acid sequence, and the maximum number of nucleotide substitutions into a simple web based form and a CGI based program generated the results. You get back a nice printable html table with all the possible primers that will produce the amino acid sequence you want. It also tells you which restriction sites will be destroyed and generated by each primer and where the cut positions occur. One notable omission from TPG is that the Tm and stability of the primers are not calculated, so a separate utility must be used to optimize your PCR protocol.

Primers!

http://www.williamstone.com/primers/javascript/

Primers! is a beautiful frame based Java program that designs primer sets for an entered nucleic acid sequence using 4 variables that you define. You can set the length of the desired primers, minimum Tm, maximum Tm, and number of ambiguous bases allowed. An extra option that is available is to restrict the regions of your entered sequence that the program will design primers for. A definite boon when you have multi-kb long sequences. I have also used this feature to design primers for primer walk sequencing.

Once you enter all the data you are presented with a color coded table of forward and reverse primers and their Tm's sorted by location. The colors make it very easy to pick out undesirable artifacts like 3' runs of As. From here you can select individual primer pairs and pull up an analysis screen that shows your original sequence, where the primers you selected are on it, the amplified sequence, detailed primer information (including 3 common T_m calculation methods), and primer/primer stability (including hairpin, primer dimer, and primer similarity). For short sequences the output is quite nice, but longer sequences generate long pages that are difficult to scroll through and/or print. It would be nice if a brief summary page would be made available. My only other complaint is that the primer/primer stability section doesn't calculate the T_m's of the hairpins, dimers, or primer/primer annealing.

Primers! has a very nice data entry error checking routine that tells you what you did wrong and suggestions for how to fix it. It also warns you when you request an obnoxious number of primers be generated, like when I accidentally set my primer range to be 4kb long and it spit back 1861 reverse primers.

For those people with 'cookie' enabled browsers you can save your variable settings and have them automatically set when you first load the page. This is a great feature when you want to clear the sequence window by reloading the page. If you already have your primer sequences in hand you can enter them on a separate page and perform the analysis portion by itself.

Something else that is being worked on is a utility to order your primers directly from within the Primers! page. It's not working yet so I can't evaluate it but I would certainly applaud this integration when it is available. All in all, this is a very nice package.

Primers! Lite

http://www.williamstone.com/primers/nojavascript/

Primers! Lite is the non-frame, non java version of Primers! The only functional difference is that there is no cookie feature and you may only enter a sequence of 1kb or less. Anything larger tends to crash the program. Please see the review of Primers! for more detail.

The GPRIME Package

http://life.anu.edu.au/molecular/software/gprime.htm

Gprime (Group Primer Selection and Analysis) is an interesting package. It is a holdover from the days when 286 processors and 2400 bps modems were the standard. It was designed to run locally and as such it is not strictly an on-line utility. It is freely downloadable as a 600kb self extracting file. GPRIME is a tool to assist researchers in designing PCR primers for closely related nucleotide sequences. It is important to note that this package only suggests likely sites for PCR primers. You must use another utility to check the other parameters of your choice primers. A further limitation of the package is that you must provide pre-aligned sequences in a specific format. While I was able to test this program in an MS-DOS window under Windows98 it took some definite tweaking and isn't the most friendly of interfaces. Further, an external program like Wordpad or Excel is required to view the output. Given all these limitations I have to recommend against using this program when other, more capable tools are available.

Additional Information about GPRIME

"This package contains two series of programs to help with the design of PCR primers for an aligned group of related nucleotide sequences. These sequences must be provided in a single file in PIR format, and this is obtained most conveniently using CLUSTAL V or W. This version will handle up to 20 sequences, each of 10000 nucleotides, and comprises:

- programs to locate primer sites shared by all sequences in the group;
- programs to locate primer sites that will separate two sub-sets of the sequences;
- programs that check for sites that have problems due to self complementarity and/or self annealing"

From the GPRIME homepage

Read more about this package in "The GPRIME package: computer programs for identifying the best regions of aligned genes to target in nucleic acid hybridisation-based diagnostic tests, and thier use with plant viruses" Adrian Gibbs, John Armstrong, Anne M. Macenzie, George F. Weiller, Journal of Virological Methods 74 (1998) 67-76

WWW GeneFisher

http://bibiserv.techfak.uni-bielefeld.de/genefisher/

GeneFisher is the type of tool that really goes for functionality and user customization. In GeneFisher, you specify the size of the PCR fragment that you want to generate off of the target sequence and it presents you with a list of forward and reverse primers that will do the trick. You start by entering either a single sequence or multiple closely related ones. And this is where the first real gem shows up. GeneFisher comes with a

built in alignment tool that feeds the results directly into the next step. You have the option of looking at a graphical representation of the alignments and the consensus sequence that is really useful for a variety of tasks, not just primer design. At this point you can reject the alignment and adjust the parameters to try again or you can continue to the primer design step.

The primer design step lets you specify a number of useful parameters including T_m range, Primer length range, PCR fragment length range, %GC composition of primer and 3` end, 3` end 'length', and 3` ending base. Acceptable default settings are provided, but there is no inline error checking of the entries you provide. For example, if you select a 100% GC content and a T_m of 1°C, the program will cheerily chug through it's algorithms trying to satisfy your impossible requirements.

The output section is particularly nice. You are provided with a list of forward and reverse primer pairs that will give you a PCR product of your desired length. You are also given the T_m difference between the two primers as well. Definitely a boon when trying to decide on a standard protocol. In addition to this listing, you can get an independent list of the forward or reverse primers to look at their individual characteristics.

Now for the problems. This is a Java based program and sometimes the Java buttons fail inexplicably. But after reloading the starting page most of these problems were resolved. You are also supposed to be able to enter Amino Acid sequences instead of nucleic acids. I could never get it to work, my browser would always 'time out' waiting for the results, no matter how short the AA sequence was. Even with these problems and limitations this is a good program. It is an excellent example of how independent utilities can be coupled effectively.

Web Primers
http://alces.med.umn.edu/webprimers.html

Web Primers is based off of the original Xprimers and is a very capable tool for graphically displaying primer sites along a target sequence. You enter a single nucleic acid sequence, desired primer T_m range, and primer length range. You are presented with an excellent GIF (or PostScript printable image) picture showing the relative positions of the selected primers, their direction, and a separate list of the primers themselves. My only complaint about this tool is that the 3` position number of the primers is not listed. Without this information, an accurate calculation of the resultant PCR fragment length is impossible. Even so, the printable nature of the output is in itself quite useful.

DOPE2 and DoPrimer are essentially the same tool. (See my DoPrimer review in this issue.) Both work well for designing PCR and sequence primers for specific DNA sequences. DoPrimer is the Java-based program for the commercial company Interactiva, and DOPE2 is the HTML testing program they used to develop it. In fact, the last time DOPE2 was updated was February 1998.

Project DOPE2 (Design of Oligonucleotide Primers)
http://dope.interactiva.de/

DOPE2 allows you to either manually paste in a DNA sequence or search the EMBL database for the entry you want and automatically launches the DOPE2 software to analyze it. You can search any of 17 organized databases by accession numbers, titles, keywords, authors, dates, and so on--basically any term you can think of to narrow the results to exactly what you want. Once you've entered or identified your sequence of interest, tell the program whether you want to generate PCR or sequence primers and enter the parameters for your oligos.

You're asked for Minimum, Optimum, and Maximum values for T_m, length, and %GC content. You are not able to specify the T_m for the Sequence oligo algorithm: the critical difference between PCR and sequence algorithms. However, you may request multiple sequence primers that are specific distances apart-- handy for designing all the oligos needed for a primer walk experiment.

The result screen

For the PCR primers, you are given the option of listing just the best pair or a summary screen of all the pairs matching your parameters. You can print either screen for later analysis. A very comprehensive on-line help file is available.

DoPrimer improves upon DOPE2 in several ways, but the only major difference between the two is that DoPrimer requires a Java-capable browser. However, if you have that capability, I strongly suggest you use DoPrimer.

DoPrimer

http://doprimer.interactiva.de

DOPE2 and DoPrimer are, for all practical purposes, the same tool for designing PCR and sequence primers for specific DNA sequences. Both work very well. DoPrimer is the Java-based program for the commercial company Interactiva, and DOPE2 is the HTML testing program they used to develop it. DoPrimer is the front-end application Interactiva added to the end of the oligo-generation process so you can automatically purchase the oligos you select. However, you can still use its primer-generation capabilities for free.

DoPrimer features

You can either manually paste in a DNA sequence or search the EMBL database for the entry you want and automatically launch the DoPrimer

software to analyze it. At this time, you can only search the Human database with accession numbers, titles, keywords, authors, dates--any term you can think of that will limit your results. Once you've entered or identified your sequence of interest, tell the program whether you want to generate PCR or sequence primers and enter the parameters for your oligos. You are asked for Minimum, Optimum, and Maximum values for T_m, length, and %GC content. Unlike DOPE2, you can enter T_m for both PCR and sequencing algorithms. You may also request multiple sequence primers that are specific distances apart. Unfortunately, the T_m isn't displayed for sequence oligos on the result screen.

The result screen

For the PCR primers, you are given the option of listing just the best pair, or a summary screen of all the pairs matching your parameters. Either screen is easily printable for later analysis. As in DOPE2, a very comprehensive on-line help file is available. From the results screen, you can automatically take your oligos to an order screen. While I didn't actually try to order a set of oligos, the system looks professional enough.

NetPrimer

http://www.premierbiosoft.com/netprimer.html

NetPrimer is good for analyzing oligos for hairpins, dimers, palindrome, base runs, and repeats of individual oligos, but not primers pairs. The result screen's print option produces a very neatly presented sheet with all the pertinent information for that oligo. The problem with this tool is inherent in its design. It's a Java applet, and it's big. If you have a fast Internet connection, you can download the applet (about 1MB) and run it online. If you don't fancy tying up your 9600 modem for a few hours during the day, you can download the self-extracting zip file (1.7MB)

overnight. You need to be running a Java-capable browser on Win 95/98/NT4.

Oligos-U-Like Primers3

http://www.path.cam.ac.uk/cgi-bin/primer3.cgi

Someone who loves having the highest degree of control possible must've designed Primers3. I have never seen a primer design tool that allows you to specify so many parameters --56, all told. Luckily, defaults are provided, and all you really need to enter is the DNA sequence. Included among this copious list of parameters are such items as Maximum Complimentarity, First Base Index, and Max End Stability. Thankfully, an excellent list of definitions and examples is provided on the same page.

The result screen

The results screen is a bit cluttered for long sequences, but it's usable. The list of results gives you the oligo sequence, its length, starting base number, T_m, %GC, and PCR product size for that pair.

Primer Selection

http://alces.med.umn.edu/websub.html

Primer Selection is the prelude to Xprimer, which in turn is the prelude to the Virtual Genome Center's Web Primers. As such, it shouldn't surprise you that Primer Selection is simple and not quite fully functional. For example, it's supposed to allow you to enter a Genebank accession number or a raw text sequence. The raw text entry doesn't work, but the Genebank accession function works just fine. After that, just enter your desired T_m--the start and end points of the PCR fragment to be generated. The program uses your exact coordinates and moves inward until the oligo T_m matches what you want--even if it's 30 bases long. The result screen is extremely minimal and not very clear.

Web Primer (Stanford)

http://genome-www2.stanford.edu/cgi-bin/SGD/web-primer

Web Primer (not to be confused with Web Primers from the Virtual Genome Center) is good for generating PCR and sequence primers. As with Primer Selection, you can enter a Genebank accession number or a raw text sequence. Both functions work--unlike Primer Selection's. When designing PCR primers, the program only looks at the very ends of the sequence text. So if you want to amplify an internal region, you must trim the ends first. You can enter the desired ranges for T_m, Primer length, %GC, and something called Primer Annealing. The last is described in the help file as a dimerize/annealing factor but isn't clearly explained, so I'm not sure what it does. You can view either the 'best' set of primer results, or the whole list of primers.

When designing sequence primers, you specify which strand to sequence (coding, non-coding, both), distance between primers, primer length, %GC composition, and that same Primer Annealing value. You are presented with a list of the oligo sequences and their location. I've used this feature to design primers for sequencing an unknown related gene to one I have the complete sequence for. This is a nice evolution from the original Primer Selection tool.

PCR Methods

Following are brief descriptions of frequently used PCR methods. Each description includes a short overview of the method, suggested ranges for selected variables, and relevant reference links. This method information should be regarded as a point of departure since each PCR reaction depends on the template, primer sequences, concentrations of the other components in the reaction, as well as your own unique laboratory conditions and experimental purposes.

Standard PCR

The polymerase chain reaction (PCR) is a technique for amplifying specific nucleic acids in vitro.

Standard PCR protocols can amplify DNA sequences less than 3000 base pairs long by a factor of about 106 for the purposes of determining sequence size, nucleotide sequence, etc.

The process was developed by Kary Mullis and is currently covered by patents owned by Hoffman LaRoche, Inc. and F. Hoffman-LaRoche Ltd.

Suggested Variable Values for Standard PCR

Reaction Volume	100 µL
Taq Polymerase	2- 2.5 U
Primers	0.1 - 1.0 µM each
dNTPs	0.2 mM each
Salt	6 - 50 mM KCl or (NH4)2SO4
Mg++	1.5 - 5.0 mM MgCl2 or MgSO4
Buffer	Tris-HCl 10 - 50 mM, pH 7.5 - 9.0
Template	102 - 105 copies
Source	"PCR Primer: A Laboratory Manual" 1995

LA PCR

Long and Accurate PCR refers to a method that results in longer and more accurate DNA extension in PCR. Routine amplification of targets from 5 kb to 40 kb have been produced by combining two thermostable polymerases one of which is a minor proofreading enzyme.

LA PCR was developed by Wayne Barnes and foreign counterparts. The patent is now owned by Takara Shuzo.

Suggested Variable Values for LA PCR

Reaction Volume	33 µL
Polymerase Klentaq	1 (a) plus 1/16 Pfu or 1/50 Deep Vent (by volume; 1/160 or 1/500 by units)
Salt	16 mM (NH4)2SO4, no KCl
Mg++	3.5 mM MgCl2
pH	9.2
Buffer	50 mM Tris
Template	2 ng lambda DNA
Cycles	20
Extension Temp	68° C
Extension Time	11-24 min, (longer at later cycles)
Source	See Long and Accurate PCR

Hot Start PCR

Hot Start PCR has been described as "a common and easy protocol to improve yield and increase specificity" ("PCR Primer: A Laboratory Manual" 1995) and as "inconvenient and can cause reproducibility and contamination problems" (Perkin-Elmer "Guide to Optimizing PCR").

During sample preparation at room temperature complexes of nonspecific primer-template may be generated. By denaturing them during the first few PCR cycles, extension of these nonspecific complexes can be prevented. With the Hot Start method a key component necessary for amplification is withheld from the reaction mix until the reaction reaches a temperature above the optimal annealing temperature of the primers. Since a key component, such as primers, polymerase, Mg++, or dNTPs, was withheld from the reaction at permissive temperatures, competing side reactions are minimized and a more specific amplification can occur.

More about Hot Start PCR

By Laura Ruth, Ph.D.

The temperature of a PCR reaction in research and applications can be a crucial experimental parameter to optimize in order to avoid amplification of non-specific bands, primer-dimer pairs or heteroduplexes. In 'hot-start' PCR at least one reaction component, which can include the polymerase, salt (KCl or MgCl2) or dNTP(s), is withheld from the reaction until the system reaches a particular temperature. The critical 'hot-start' temperature varies according to the method of delayed reaction.

The PCR reaction can be delayed in several ways. When a physical grease barrier method is used, products such as Ampligrease, petroleum jelly, or wax are used. When the polymerase is modified, it can be a modified polymerase, a polymerase plus antibody or a polymerase plus an affinity bead. A typical PCR reaction consists of a series of steps: 1) denaturation (94 C/60s) 2) priming/annealing (62-60 C/30 s), and 3) elongation (60 s). After cycling through steps 1-3 for "x" number of times, a final step of the PCR reaction is: 4) a final extension (72 C/10 min). The Ampliwax™ PCR gems (PE Applied Biosystems) are one physical barrier method to prevent the mixing of reagents until the reaction reaches 55-58 C. The ampligrease method 'hot-start' method was developed as a cheaper alternative to the ampliwax. The set-up of an ampliwax and ampligrease protocol are similar. The reaction components minus template and polymerase are mixed in the bottom of the Eppendorf tube, the wax or grease is added, melted, allowed to solidify, and then a final oil layer is added. The polymerase and template are added through the oil. The wax or grease allows the reagents to mix at around 55 C on the second melting step (Note: Not all lots of petroleum, Vaseline brand, generic USP, are suitable). A recent research report shows that a wax-tablet-based 'hot start' in a single tube RT-PCR can be used in conjunction with capillary electrophoresis to directly quantitate RNA transcripts.

There are a few choices of modified polymerase to use in 'hot-start' PCR: 1) AmpliTaq™ plus a manual 'hot-start', 2) AmpliTaq Gold™, 3) TaqStart™ Antibody, and 4) Affinity-Immobilized Taq DNA polymerase. When a modified polymerase is used in a 'hot-start' procedure, a pre-PCR heat cycle step is added to the standard PCR protocol. A perusal of literature protocols shows that the pre-heat step can occur somewhere between 92-100 C for a range of 2-20 minutes. This pre-heat step allows activation of the polymerase, which helps eliminate the polymerase acting on non-specific primer-template complexes which may form at lower temperatures. Using a 'hot-start' modified polymerase reaction eliminates the potential inconvenience of using

wax, jelly, a degradative enzyme (uracil deglycosylase), and problems of specificity, i.e. false-positives, due tube-to-tube carryover because the reaction tube is not opened. When AmpliTaq Gold™ was used in a multiplex PCR amplification of cDNA from a Hodgkin's lymphoma cell line and in amplification of short tandem repeat (STR) from genomic DNA, it showed increased sensitivity and specificity over AmpliTaq™. The use of TaqStart™ Antibody has been shown to facilitate amplification of low copy HIV DNA.

'Hot-start' PCR can be used in conjunction with other methods. 'Hot-Start' plus 'touchdown' (ramped cycle) PCR was used in the type-specific amplification of viral DNA, in a study of variation in rDNA ITS regions which show two haplotypes coexist within a single aphid genome, and in human platelet antigen genotyping using a fluorescent SSCP technique with an automatic sequencer. In the case of amplifying the platelet DNA, adding 2% v/v DMSO, and 2.5% v/v formamide were part of the optimization. However, in the amplification of viral DNA, 5% formamide did not optimize the reaction in the presence or absence of the 'hot-start' technique. Lastly, there was no difference in the sensitivity and specificity of the separate 'hot-start' and DMSO methods in typing gene polymorphisms of the human angiotensin-converting enzyme, and a triple primer method was the most effective optimization method.

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Touchdown PCR

Touchdown PCR was originally intended to simplify the complicated process of determining optimal annealing temperatures. In concept, any differences in melting temperature between correct and incorrect annealing gives a 2-fold difference in product amount per cycle (4-fold per ° C) which increases the ratio between correct and incorrect products.

Annealing takes place at approximately 15° C above the calculated T_m. During the following cycles, the annealing temperature is gradually reduced by 1-2° C until it has reached a level of approximately 5°. C below T_m.

The original reference for touchdown PCR PCR is Don, R. H., P. T. Cox, B. J. Wainwright, K. Baker, J. S. Mattick. 1991. "Touchdown PCR to circumvent spurious priming during gene amplification". Nucleic Acids Res. 19:4008

RT PCR

Reverse Transcriptase PCR is the most sensitive method available for gene expression in vitro. Irrespective of the relative quantity of the specific mRNA, it is possible to detect the RNA transcript of any gene.

An RNA template is copied onto a complimentary DNA transcript using a retroviral reverse transcriptase, followed by amplification of the cDNA using PCR. Theoretically, only one RNA molecule needs to be completely intact between the two primer binding sites. Though the results from a degraded sample will not be quantitative, the relative amount of one RNA molecule can be compared to another RNA molecule in the same sample. To be determined accurately, the absolute abundance of a specific transcript by RT-PCR requires the use of competitive PCR RT-PCR techniques.

Suggested Variable Values #1 for RT-PCR

Variable	Values
Reaction Volume	100 µL
Polymerase	2-5 U/ µL Taq
Primers	10 µM each
dNTPs	10 mM mix*
Mg++	50 mM MgCl2
Buffer	200 mM Tris-HCl pH 8.4, 500 mM KCl
Source	See RT PCR Protocol, Using Reverse Transcriptase

* 10 mM each dATP, dGTP, dCTP, and dTTP at neutral pH.

Suggested Variable Values #2 for RT-PCR

Polymerase	2.5 U/100 µL Taq
Primers	0.2 each
Template	1 µg/100 µL
dNTPs	0.2 mM each
Mg++	1.5 mM MgCl2
Source	See General Protocols for PCR: PCR Amplification of cDNA

Reverse-Transcriptase (RT) PCR

By Laura Ruth, Ph.D.

RT-PCR is one of the methods used by itself or as part of a series of experiments which can be used to amplify and quantify RNA by producing cDNA from mRNA. Several questions should be answered to determine if RT-PCR is the best experimental technique to use or if other methods are better experimental options. The questions which should be addressed are:

1) Is the availability of cells or tissues for mRNA extraction and analysis limited,
2) Is quantitative information regarding the level of mRNA expression necessary, and
3) Is it important to identify the cells or gene of interest? When the amount of material is limited, RT-PCR is a better choice than a Northern blot or RNase protection assay. However, in situ hybridization or in situ RT-PCR is a better option when sample preservation is desired

A basic RT-PCR protocol includes isolation of RNA, initiation or priming of cDNA reaction, reverse transcription of RNA to produce cDNA, and PCR amplification of cDNA. As any experiment will vary between reagents and scientists, a link on the Alkami web site can be used as a

reference for sample RT-PCR protocols. In addition, there is more than one way to isolate RNA for RT-PCR from biopsy samples and blood. The various mRNA extraction methods include acid guanidinium thiocyanate and phenol-chloroform, TRIzol Reagent plus DNase treatment, and the cationic surfactant Catrimox. There are several reverse transcriptases to choose from, which include, Moloney murine leukemia virus (MMLV) RT, avian myeloblastosis virus (AMV) RT, thermostable reverse transcriptases from Thermus thermophilus and Thermus flavus, and finally two RNase H- mutants of MMLV (SuperScript and SuperScript II). The choice of enzyme can be based on the need for efficiency, i.e. using one enzyme to amplify both cDNA from RNA and DNA from cDNA, and the length of cDNA one wants to produce from the mRNA. Lastly, there are three basic primer strategies which can be used: gene-specific priming (GSP), oligo-dT priming, and random hexamer priming. Using GSP primers yields the most accurate and sensitive results.

There are many research and clinical applications of RT-PCR sited in the literature. Some specific examples of RT-PCR include detection of a splice site in X-linked hydrocephalus, tracking bcr-abl mRNA during ribozyme therapy, functional and molecular analysis of glutamate-gated channels at the single level, and analysis of differential gene expression in the kidney (DDRT-PCR). A combination of RT-PCR and PCR technology has been used to detect cancer cells in biopsies of solid tissue, lymph nodes, bone marrow, peripheral blood, and other body fluids. When RT-PCR is used to monitor metastatic cancer the results may not be clearly interpreted for a variety of reasons: patient selection criteria, sample collection and processing, choice of primers, use of proper controls, contamination-minimizing techniques, multiple samples from each patient must be standardized, and the need for more information about the biological markers of the various cancers. Developing proper internal controls for quality assurance and

fluorogenic primers for more sensitive detection may help RT-PCR in general and in the particular area of metastatic cancer diagnosis.

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In Situ PCR

Suggested Variable Values for In Situ PCR

Polymerase	0.15 U/µl (3% v/v) Amplitaq
Primers	1 µM each primer, 10 µM stock solution
dNTPs	200 µM dNTPs, 10 mM stock solution
Salt	50 mM KCl
Mg++	1.5 mM MgCl2
Buffer	10 mM Tris-HCl, **pH** 8.3
Source	See An In Situ PCR Cookbook

In situ PCR

By Laura Ruth, Ph.D

Classical in situ hybridization uses a labeled DNA probe to detect a minimum of 10 copies of DNA/cell. Amazingly, by coupling the technique of hybridization with PCR (Polymerase Chain Reaction), i.e. in situ PCR, the sensitivity of hybridization is increased 10-fold. One can detect as little as 1 copy of specific DNA in a background of 1 ug cellular DNA. When using in situ PCR, it is possible to investigate viral DNA, single copy genes and gene rearrangements, or viral RNA and gene transcripts (when incorporated with RT [Reverse Transcriptase] PCR). In contrast to a southern blot hybridization, the cell or tissue is preserved using in situ PCR, thus the percentage of a given cell type that contains the target of interest can be measured.

An in situ PCR protocol typically consists of four steps: preparation/fixation of cells or tissue, semi-permeabilizing cell membranes, amplification with PCR, and detection. Tissue samples and cell from cultures are typically fixed using a 10% buffered formalin. The tissue samples are then embedded in paraffin, while the cells are

washed and resuspended in diethylpyrocarbonate (DEPC) water. At this point either sample can be placed on a silane-coated slide, and the paraffin embedded sample will be further treated with xylene and ethanol to remove the paraffin. The cell membranes are then permeabilized using pepsin, trypsin, or proteinase K. Pepsin or trypsin is preferred because the reagents are less stable than proteinase K and their activity is inhibited by an increase in pH during the wash step. It is important to note that the proper protease digestion is highly dependent on the time of formalin fixation. The protease is removed/inactivated by washing the slide in DEPC followed by 100% ethanol. A 'hot-start' PCR technique is typically used with in situ PCR. The reaction solution added to the slide usually consists of PCR buffer, $MgCl_2$, dNTP solution, 2% bovine serum albumin (BSA), digoxigenin dUTP solution, primer 1 and primer 2 , water, and Taq DNA polymerase.

The thermocycler and sample are pre-heated before running an in situ PCR protocol. One should note that in situ PCR requires 15 cycles, while PCR in situ hybridization requires 35 cycles. After amplification, the slides are washed in a series of buffers and salt, treated with digoxigenin antibody, and followed by a chromagen for detection of the probe. The major difference between in situ PCR and PCR in situ hybridization is the use of either digoxigenin or biotin labeled nucleotides for in situ PCR, and omission of the hybridization step. In situ PCR is much quicker and simpler than PCR in situ hybridization. However, PCR in situ hybridization works better on frozen tissues or cytospins that have not been heated.

A last piece of technical information which may be useful for the bench scientist is a list of the companies which make equipment for in situ PCR: Biometra, Hybaid, Lab-Line Instruments, MJ Research, PE Applied Biosystems, Robbins Scientific, St. Johns Associates, Stratagene, and Techne. In situ PCR experiments are preferably performed on slides which fit into a slide thermocycling block specific to each type of machine.

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Quantitative PCR

by Investigen Writers

Early on, PCR promised the ability to determine the amount of target DNA present in a sample--a very enticing goal. Regular PCR determines whether the target is present. Quantitative PCR determines how much is present: Is it at a level that needs action, or is the level so low as to be irrelevant?

Quantitative PCR should be simple. Theoretically, the amount of end product doubles with each amplification cycle. Simplistically the number of amplicons doubles with each amplification cycle. However, this makes several false assumptions, the worst of which is that reaction occurs at 100 percent efficiency in all amplification cycles. Inhibitors alter this efficiency. Additionally, later amplification cycles of a typical PCR reaction have very low efficiencies compared to earlier cycles--the PCR reaction has plateaued.

For quantitative PCR, one addresses this efficiency problem with an internal control: a competitor amplicon. Hence, this type of reaction is known as quantitative competitive PCR (QC-PCR). The internal control improves the reliability of the quantitative results by providing a means to monitor and correct for the efficiency of the PCR reaction. This works best if the nature and quantity of inhibitors equally affect target and control amplicons.

The ideal control amplicon is usually an artificial control, amplified with the same primer pair as the real target. It is of similar length and has a similar base pair composition to the target amplicon. However, it is important that some feature of the control distinguishes it from the target. If both amplicons are equally affected by inhibitors, use the same

primers, and are of similar size and composition, they are expected to be amplified at the same efficiency.

Once the control amplicon is created, it is quantitated, a known amount of control is spiked into the sample, and amplifications run for a fixed number of cycles. After the PCR reaction is complete, the products get quantitated to determine the ratio of target to control.

QC-PCR has numerous drawbacks. The main difficulty is creating and optimizing the control amplicon. However, even when this obstacle is overcome, the procedure has a limited dynamic range. The target-to-control ratio should be between 1:10 to 10:1 for reliable results. Additionally, there needs to be a method for the accurate quantitation of the end products. This can be time consuming and prevents high-throughput applications. Finally, and not uniquely to QC-PCR, contamination is always a threat. These drawbacks have severely hampered the development and use of quantitative PCR.

Despite these limitations, quantitative PCR has great appeal. Additional methods, however, may allow PCR to fulfill its early promise of achievable quantitative results.

PCR Variables

There is no single factor or shortcut that will always produce maximum **specificity** (selective reactivity), **efficiency** (yield), and **fidelity** (accuracy) in your PCR reactions. Usually a combination of variable modifications is necessary.

Each variable can be modified to improve your results in these three domains. However, since these components are interdependent it is not always possible to improve one without sacrificing another. The following variable descriptions include information about which modifications influence specificity, efficiency, and/or fidelity.

Primers

Standard Range: 0.1 - 1.0 µM.

However, 0.2 - 0.5 µM should work for most cases. Note that your primer concentration should be titrated together with the dNTPs.

Improve specificity: A reduction in primer and dNTP concentration can result in a dramatic improvement of specificity. High concentrations can encourage nonspecific annealing and product formation, as well as formation of primer dimers where primers anneal to themselves and not the template.

Reduce the primer/template ratio so that it's as low as practical and still within the given range. A high ratio is also likely to generate non-specific amplification products and primer-dimers.

Improve efficiency: Increase the primer/template ratio. Reduce primer concentration if there is a substantial surplus of primer.

Polymerases

Standard Range: 1 - 5 Units/µL

The actual definition of a "Unit" may vary among suppliers

Improve specificity: Reduce concentration within the suggested range. The concentration of polymerase is a critical factor in determining the stringency of a PCR. A high concentration not only reduces specificity but also incurs unnecessary costs.

Improve fidelity: Use a high fidelity polymerase. See Methods and reagents - Fidelity of DNA polymerases for PCR.

Templates

Standard Ranges: 10^2 - 10^5 copies.

The quantity of template in a reaction should be measured by the number of target sequence copies, not by weight. In general, if you lower the DNA template concentration you should also lower the concentration of DNA polymerase (within the recommended range).

Improve specificity: Increase the amount of template DNA - analyses that use small amounts of template DNA are especially prone to PCR-induced artifacts.

Reduce the primer/template ratio. A high ratio is prone to generate unspecific amplification products and primer-dimers.

Improve efficiency: Increase the primer/template ratio.

Template concentrations will vary depending on the type of sequences to be amplified but in general, the efficiency of PCR is greater for smaller sized template DNA. Molecular weight can be reduced by mechanical shearing and/or rare restriction enzyme digestion of genomic DNA prior to PCR for high molecular weight templates, such as undigested eukaryotic genomic DNA.

Deoxynucleoside Triphosphates (dNTPs)

Standard Range: 20 -200 µM

dNTP concentration should be titrated together with the primers. If one dNTP is at a higher concentration it will be preferentially incorporated for this reason, the four dNTP concentrations (dATP, dCTP, dTTP, dGTP) should be the same so that accurate incorporation takes place. In addition, it is important to keep the four dNTP concentrations above the estimated Km of each dNTP (10 µM - 15 µM).

Improve specificity: Reduce dNTP concentration but note that the Mg++ concentration should be lowered in an equimolar proportion. This is because the main source of phosphate groups in a reaction is the dNTPs so any change in their concentration affects the concentration of available Mg++ since Mg++ form a soluble complex with dNTPs.

Improve efficiency: For a large template sequence, increase the amount of dNTP.

Improve fidelity: Lower dNTP concentrations but note that the Mg++ concentration should be lowered in an equimolar proportion.

Magnesium ions

Standard Range: 0.5 - 10 mM

Free Mg++ ions should exceed that of total dNTP concentration by 0.5 mM - 3.0 mM. The main source of phosphate groups in a reaction is the dNTPs so any change in their concentration affects the concentration of available Mg++ since Mg++ form a soluble complex with dNTPs.

Mg++ has been shown to be a superior divalent cation compared to Mn++ and Ca++ and will bind to the template DNA, dNTPs, primers, and polymerase. Its concentration affects product specificity, primer annealing, the formation of primer-dimer artifacts, melting temperature, and enzyme activity and fidelity. Thus, an important optimization strategy is to titrate your magnesium concentration for each DNA template, dNTP, primer and/or polymerase concentration change - especially if large products (>1 kb) are being amplified.

Improve specificity: Decrease concentration. Note that inadequate Mg++ concentration can result in lower efficiency/yields.

Improve efficiency: Increase concentration. However, excess Mg++ tends to cause nonspecific reactions and smeared electrophoretic bands, lowering specificity.

Preincubation Temperatures and Times

Standard Range: 92°-96° C, 30 secs - 10 mins

In the absence of enzyme, preincubation should inactivate harmful proteases or nucleases in the sample. Preincubation can also insure complete denaturation of complex starting templates, as in the case of genomic DNA.

Melting Temperatures and Times

Standard Range: 68°C - 75°C, 30 - 120 secs

The melting temperature is defined as the point at which the transition from double-stranded to single-stranded DNA is 50% complete. Calculating the melting temperature for your PCR reaction can be more of an art than a science. Factors which will influence your temperature are the total mass and concentration of the sample including the oil or wax vapor barrier. There have been a number of proposed calculation methods, which provide only an estimation, however, the optimal temperature for your experiment needs to be determined empirically.

The time needed will rely on the ramp time of your particular thermocycler and on whether the thermocycler calculates the expired time on the basis of the temperature in the sample or of the thermoblock.

To calculate T_m for primers <= 20 bps Alkami Expert™ uses:

[4°C (G + C) + 2°C (A + T)]

(See Suggs et al. 1981)

To calculate T_m for primers > 20 bps Alkami Expert(tm) uses:

62.3°C + 0.41°C (%G-C) - 500/length

(See Bolton & McCarthy 1962)

Temperature - Annealing/Hybridization

Standard Range: 37°C - 65°C, 10 - 120 secs.

During annealing, the primers are rapidly hybridized. Differences in the T_m possessed by different DNA polymerases can change the effective primer annealing temperature.

Annealing times for LA PCR can be approximated with the formula:

60 secs + (2.5 secs/100 bases) = approximate annealing time

Improve specificity: Increase the annealing temperature (increments of 2°-5° C are recommended) since it reduces the possibilities of non-specific priming and therefore nonspecific product formation. Reduce annealing times since very long annealing times normally do not improve yield, but rather produce an increase in spurious priming and thus greater amounts of nonspecific PCR products.

Temperature - Extension/Polymerization

Standard Range: 72°C, 60 -120 secs.

The extension rate of commonly used DNA polymerases is at least 50 nucleotides per second so you can keep extension times shorter than 15 seconds for PCR products less than 400 bp long. Some dNTPs are already inserted during the annealing phase, in particular, if the annealing phases are run at relatively high temperatures. Very long PCR products need correspondingly longer extension times and it may also be advisable to run longer extension times in each PCR cycle by using a delay function in order to compensate for the increase of viscosity in the sample.

Estimating extension times for LA PCR

Extension times for LA PCR can be approximated with the formula:

60 secs + (2.5 sec/100 bases) = approximate extension time

Cycles

Standard Range: 25-50 cycles

Typically, a PCR cycle consists of three steps: denaturization, annealing, and extension. However, depending on the polymerase and thermocycler being used, the number of steps needed per cycle can be reduced to two. Optimum yield for a given number of cycles can be approximated using the formula:

2n X initial number of copies

n = number of cycles

There is an exponential phase of amplification in PCR until the number of product copies reaches approximately 1012. After this point the accumulation of product amplification generally drops off dramatically and the product stops accumulating exponentially. The number of cycles needed to reach this point can be approximated with the formula:

$$N_f = N_o(1 + Y)n$$

Where N_f is the final number of the double stranded target sequence

N_o is the original number of target copies

Y is the efficiency per cycle of the polymerase

n is the number of cycles

Several conditions can effect the plateau:

- The utilization of substrates, either primers or dNTPs.
- The stability of the reactants.
- End product inhibition.
- Competition for reactants by nonspecific products or primer-dimers.
- Reannealing of product at higher concentrations which prevents the extension process.
- Incomplete denaturation at higher product concentration.

Increasing the number of cycles does not increase specificity or efficiency of your PCR. Because the plateau effect encourages nonspecific amplification.

Improve specificity: Reduce number of cycles. Reduce cycle segment lengths.

Improve efficiency: For amplifying large fragments (> 1 kb) increase the duration of each thermal step.

Reaction Volumes

Standard Range: 20 - 100 µL in 0.5 mL microcentrifuge tubes.

5 µL reactions have also been successful. Large volume samples will be inefficiently heated and cooled, while smaller reaction volumes will change temperature more quickly but generate less product yield. The geometry of the reaction mix tube, thickness of the tube wall, and the particular thermocycler (some models have a slow response to the heat block so that wells near the periphery are slow to heat up) are factors which influence the time needed for each of the cycle steps.

In addition, some thermocyclers are calibrated by block temperature, not sample temperature. Others are calibrated by the sample temperature in which case you can use thin walled tubes & cut the sizes down.

Contributed by Denise Rubens, National Cancer Institute, Frederick, MD

Improve efficiency: Increase your reaction volume (as well as your incubation times to insure adequate thermal equilibrium). Use an oil cap to reduce evaporation and internal condensation (for a 100 µL reaction volume).

PCR Polymerases

Each thermophilic polymerase has unique characteristics, such as pH and salt optima, that affect the efficacy of your PCR protocol. As applications of PCR become increasingly sophisticated and specific, the special needs of your experiments can utilize these distinctive properties.

A high fidelity polymerase typically demonstrates 3' to 5' exonuclease activity, that is, a "proofreading" ability where the polymerase can correct misincorporated nucleotides in the strand of DNA being synthesized. Several optimized polymerase mixtures have been developed which combine a standard polymerase, such as Taq, with a small amount of a high fidelity polymerases, such as Pfu, Vent, and Deep Vent.

A significant problem with PCR is the lack of fidelity which occurs under various conditions with different polymerases. Polymerase errors can take place during five distinct activities of the extension phase: the binding of the dNTP by the polymerase, the rate of phosphodiester bond formation; the rate of pyrophosphate release; the continuation of extension after a misincorporation; and the degree of 3'-5' exonuclease proofreading activity.

There are several reasons for the variation of error rates in addition to those involved in the laxity of the polymerase. One potential cause could be physical damage to the DNA resulting in misincorporated bases, gaps and/or crossover products. The condition of the template can also affect fidelity. This is something that is not generally considered when error rates are determined and compared.

The error rates for the various polymerases are defined by errors per nucleotide. Several studies have determined that polymerase error rates can range between 2.1×10^{-4} and 1.6×10^{-6} errors per nucleotide per extension.

Additionally, the types of errors that occur during the PCR are dependent on the particular DNA polymerase. For example, among high-fidelity (proofreading) polymerases, there is a tendency to degrade single-stranded DNA primer one base at a time from the 3' end during the course of an experiment. As a result, some of the positions on the primer could be degraded past the positions containing the desired base changes before the primer anneals to the template. Note that longer primers have a higher initial degradation rate than shorter primers.

The following DNA polymerase characteristics were collected from a variety of sources and thus under different conditions. They are meant only as guidelines for tuning your PCR protocols.

Taq (Thermus aquaticus)

Taq has a relatively high error rate since it does not have the 3' -> 5' exonuclease proofreading function. However, the error rate of Taq polymerase has been reduced by a factor of 2.8 (from 2 x 10-4 to 7.2 x 10-5 by modifying reaction conditions (Ling et. al. 1991).

The most common mutations observed with the use of Taq are AT to GC transitions (Keohavong and Thilly 1989). It is also highly likely to generate deletion mutations if the template DNA has the potential to form secondary structures (Cariello et al. 1991).

Notes on Taq

- Don't store Taq diluted
- Keep it stored in the buffer it's in
- Pure Taq in its tube is active at room temperature.
- Taq is shipped at room temperature, but people are told that if they receive it and forget to put it in the freezer, it's still ok to use it.
- The dNTP's are probably more fragile than the Taq enzyme.
- Keep Taq on ice whenever possible, especially when combined with other components.
- Taq will extend the primer at room temperature.

Contributed by Denise Rubens, National Cancer Institute, Frederick, MD.

Suggested PCR Coreagents and Known Characteristics

dNTPs: 40 µM - 200 µM
Mg++: 1 mM - 10 mM
Salt: 50 mM KCl
pH: 8.0 - 8.8
Buffer: 10 mM Tris-HCl, pH 8.3

Additives: BSA, NP-40, Tween 20

Error Rates:

2.4 x 10-5 frameshift mutations/bp (Tindall and Kunkel, 1988)
1.1 x 10-4 errors/bp (Tindall and Kunkel, 1988)
2.1 x 10-4 errors/bp (Keohavang and Thilly, 1989)
7.2 x 10-5 errors/bp (Ling et al., 1991)
8.9 x 10-5 errors/bp (Cariello et al., 1991)
2.0 x 10-5 errors/bp (Lundberg et al., 1991)
1.1 x 10-4 errors/bp (Barnes, 1992)
2.0 x 10-4 errors/bp

Efficiency/Cycle: 36% - 88%

Sources:

Perkin Elmer PCR Reagents catalog, "PCR Primer: A Laboratory Manual", C.W. Dieffenbach & G.S. Dveksler 1995, error rates compiled by Eric First (erfi@eel.sunset.se), Dunning et al., 1988, Keohavong & Thilly 1989

KlenTaq (Thermus aquaticus, N-terminal deletion mutant)

Klentaq is a 5'-exo-minus, N-terminal deletion of Taq DNA polymerase. It's optimal range of Mg++ concentration is broader than most enzymes so that is easier to optimize other reaction conditions.

Suggested PCR Coreagents and Known Characteristics

Error Rates:

5.1 x 10-5 errors/bp (Barnes, 1992)

Sources:

Clonetech PCR Enzyme Systems brochure, error rates compiled by Eric First (erfi@eel.sunset.se)

Stoffel Fragment

(Carboxy-terminal 544 amino acids of Taq DNA polymerase)

Suggested PCR Coreagents and Known Characteristics

dNTPs: 40 - 200 µM each

Mg++: 2 - 10 mM

Salt: 10 mM KCl

pH: 7.0 - 7.5

Buffer: 10 mM Tris-HCl, pH 8.3

Additives: BSA, Tween 20

Sources:

"PCR Primer: A Laboratory Manual", C.W. Dieffenbach & G.S. Dveksler 1995, Clonetech 1997 PCR

Enzyme Systems Catolog

Tth (Thermus thermophilus)

Tth polymerase can efficiently reverse transcribe RNA (up to 1000 bp long) in the presense of Mn++ ions, and, synthesize DNA from a DNA template with Mg++ ions. Additionally, since Tth shows RT activity at high temperatures, it can beused to overcome problems typically associated with the high degree of secondary structure common in RNA molecules.

Suggested PCR Coreagents and Known Characteristics

Mg++: 1.5 mM

Salt: 90 mM KCl

pH: 9 at 25 ° C

Buffer: 10 mM Tris-HCl, pH 8.3

Additives: glycerol, Tween 20 has potent activity with Mn++

Extension: 75° C

Sources:

"PCR Primer: A Laboratory Manual", C.W. Dieffenbach & G.S. Dveksler 1995, B. Frey, C. (Boehringer Mannheim Corp), Getting Started in PCR: Properties of Common PCR Enzymes, Ruttiman et al. 1985

Pfu (Pyrococcus furiosus)

Pfu is a high fidelity polymerase, is very thermostable, and has a very low error rate. The use of Pfu polymerase with PCR reactions results in blunt ended PCR products. Pfu requires a minimum extension time of 1.5 - 2 minutes/kb of amplified template.

Suggested PCR Coreagents and Known Characteristics

dNTPs: 100 µM - 250 µM

Mg++: 1.5 mM

Salt: 10 mM KCl, 6 mM (NH4)2SO4

pH: 8.2 - 8.4

Buffer:

20 mM Tris-HCl, pH 8.2 or 10 mM Tris-HCl, pH 8.8

Additives: BSA, Triton X-100

Error Rates:

1.6 x -6 errors/base (Lundberg et al., 1991)

7 x 10 -7 errors/base (P. Andre - unpublished)

1.3 X 10-6 errors/base (Cline et al., 1996)

Efficiency/Cycle: 60%

Sources:

"PCR Primer: A Laboratory Manual", C.W. Dieffenbach & G.S. Dveksler 1995, "The Scientist" p. 16-17 Jan 5 1997, Nielson, K.B., Costa, G.L., Braman, J., "Optimization of PCR Using Pfu DNA Polymerase", "Stratagies" Vol 24, error rates compiled by Eric First (erfi@eel.sunset.se) and Cha & Thilly 1995

Vent (Thermococcus litoralis)

Vent polymerase has the 3' -> 5' exonuclease proofreading function and thus has relitively higher fidelity than other polymerases such as Taq. Note that primer extension longer than 2 kb almost always require Mg++ levels higher than 2 mM. There is no correlation between Mg++ levels and primer extensions lengths shorter than 2 kb. This polymerase will not extend if inosine or deoxyuridine is present in the template strand.

Suggested PCR Coreagents and Known Characteristics

dNTPs: 200 µM - 400 µM

Mg++: 1.5 mM - 7.5 mM

Salt: 10 mM KCl, 10 mM (NH4)2SO4

pH: 8.5

Buffer: 20 mM Tris-HCl, pH 8.8

Additives: Triton X-100

Error Rates:

2.4 x 10-5 errors/bp (Cariello et al., 1991)

4.5 x 10-5 errors/bp (Ling et al., 1991)

5.7 x 10-5 errors/bp (Matilla et al., 1991)

Efficiency/Cycle: 70%

Sources:

"PCR Primer: A Laboratory Manual", C.W. Dieffenbach & G.S. Dveksler 1995, NEB Technical Bulletin 10/2/95, error rates compiled by Eric First (erfi@eel.sunset.se)

Deep Vent (Pyrococcus species GB-D)

Deep Vent is an extremely stable enzyme at both high and low temperatures and tolerates a wide range of cosolvents such as formamide and DMSO. It yields very long primer extensions, the longest product to date (4/22/96) is 14 kb. According to New England Biolabs, Inc., Deep Vent shows four times more exonuclease activity than Vent DNA polymerase and 5 - 15 times more activity than Taq. Note that primer extension longer than 2 kb almost always require Mg++ levels higher than 2 mM. There is no correlation between Mg++ levels and primer extensions lengths shorter than 2 kb. This polymerase will not extend if inosine or deoxyuridine is present in the template strand.

Suggested PCR Coreagents and Known Characteristics

Mg++: 2 mM - 6 mM

Salt: 10 mM KCl, 10 mM (NH4)2SO4

Buffer: 20 mM Tris-HCl, pH 8.8

Additives: Triton X-100

Error Rates:

New England Biolabs claims fidelity is approximately two times higher than that of Vent (2.4 - 5.7 x 10-5 errors/bp)

Sources:

"PCR Primer: A Laboratory Manual", C.W. Dieffenbach & G.S. Dveksler 1995, NEB Technical Bulletin 10/2/95, NEB Technical Bulletin 4/22/96

UlTma (Thermotoga maritima)

UlTma is a high fidelity DNA polymerase which provides high yields of PCR products for templates <= 3 kb long. It produces blunt ended PCR products suitable for cloning and gene expression.

Suggested PCR Coreagents and Known Characteristics

dNTPs:	40 µM
Mg++:	1.5 - 2.0 mM
Salt:	10 - 35 mM KCl
pH:	8.3 - 9.2
Buffer:	10 mM Tris-HCl, pH 8.8
Additives:	Tween 20
Extension:	72° C - 80° C

Sources:

"PCR Primer: A Laboratory Manual", C.W. Dieffenbach & G.S. Dveksler 1995, "The Scientist" p. 16-17 Jan 5 1997, PE Feature Chart for PCR Enzymes

PCR Troubleshooting

Where appropriate, these troubleshooting categories are subdivided into groups of components and conditions.

Non-Specific Product Yields

Reaction specificity can be checked by using a negative control to check for spurious background bands.

Possible Causes: Components

- Primer concentration is too high.
- Primer degeneracy is too high.
- Nested primers are required.
- New primers are required. Some primers are immune to optimization and it's possible that your primers are good matches to other sites in addition to the desired template. See Primer Design.
- Template denaturation efficiency is too low.
- Mg++ concentration is too high. Try decreasing the Mg++ concentration by 0.2 mM increments.
- dNTP concentration is too high.
- Polymerase concentration too high.
- pH is suboptimal. Check at higher and lower levels.

Possible Causes: Conditions

- Annealing temperature is too low, causing mispriming. Try using a much higher annealing temperatures, especially in the first few cycles.
- Cycles periods are too long.
- Too many cycles. Compare the number of bands and their relative amounts after fewer cycles. There may be excessive

template if proportionally more of the intended product is present at the earlier cycles.

- Ramp speed is too slow. Check your thermocycler specifications.

- Inhibitors are present and/or concentrations are too high. See About Inhibitors
- Enhancers are required. See About Enhancers.
- Contaminants are present. See How to Reduce Contamination.
- Hot Start or Touchdown PCR is required.

Little or No Product Yield

Possible Causes: Components

- Primer concentration is too low.
- Primer concentrations not balanced. Make sure primers are present in equal concentrations.
- New primers are required. Some primers are immune to optimization. See Primer Design.
- Nested primers are required. Reamplify dilutions (1:10 to 1:1000) of the first reaction using nested primers.
- Contaminated primer. See Rescuing Contaminated PCR Primers and Wayward PCR Primers.
- Template concentration is too low. Use a higher concentration of template.
- Template concentration is too high. Excessive template can inhibit the reaction by binding all the primers.
- Template is degraded. Check the integrity of the template by electrophoresis after incubation.
- Template: Target sequence is not present in target DNA. Redesign your experiment or try other sources of target DNA.

- Mg++ concentration is too low. This may compromise enzyme activity so try increasing the concentration by 0.2 mM increments.
- dNTP concentration is too low. Normally concentrations between 20 - 200 µM are optimal.
- dNTPs degraded. Keep nucleotides frozen in aliquots, thaw quickly and keep on ice once thawed. Avoid multiple freeze/thaw cycles.
- pH of the reaction buffer is too high.
- Reaction mixture is incomplete or degraded. Do a control check with positive control DNA.
- Buffer isn't diluted enough. Add more water.

Possible Causes: Conditions

- Denaturing temperature is suboptimal. Try extending the time and/or increasing the temperature of the initial denaturation step prior to cycling (5 minutes at 95° C is standard).
- Annealing temperature is too high. Start at 10° C below calculated optimal annealing temperature.
- Suboptimal extension time. Increase by 1minute increments, especially for LA PCR.
- Inhibitors are present. See Enhancers & Inhibitors in this Guide.
- Enhancers needed. Some reactions may amplify only in the presence of additives. See Enhancers & Inhibitors in this Guide.
- Mineral oil problem. The reaction must be overlaid with high-quality, nuclease-free light mineral oil. Do not use autoclaved mineral oil.
- Reaction tubes are contaminated. tubes eliminates contaminants that inhibit amplification. See also How to Reduce Contamination.

- Too few cycles. Try doing 10 additional cycles at a constan annealing temperature (i.e. 55° C) and recheck.
- Thermal cycler didn't cycle. Check to see if the thermal cycler was actually turned on.
- Thermal cycler was programmed incorrectly. Check to see if times and temperatures are correct.
- Thermal cycler temperatures are too low in some positions. Do a set of control reactions to determine if certain positions give low yields.
- Thermal cycler top was left open.

Multiple Product Yields or High Molecular-Weight Smear is Observed

Possible Causes: Components

- Primer concentration is too high.
- New primers are required. Some primers are immune to optimization. See Primer Design.
- Nested primers are required.
- Template: Check the target DNA sequence to see if there are known mispriming areas. If you know the area around the target sequence, do a quick check of the vector sequence for regions of near 70% or above homology.
- Template: Band purification (target bands can be removed from gels) followed by reamplification is required.
- Template is degraded.
- Template concentration is too high.
- Mg++ concentration is too high.
- Polymerase concentration is too high.

Possible Causes: Conditions

- Denaturation temperature is too low.

- Annealing temperature is too low.
- Annealing incubation times are too long (check your thermocycler ramp time).
- Extension incubation times are too long (check your thermocycler ramp time).
- Two temperature PCR protocol is required.
- Hot Start or Touchdown PCR is required.
- Too many cycles.
- Contamination by another target RNA/DNA. See How to Reduce Contamination.

Primer-Dimers

Possible Causes: Components

- The 3' ends of the primers are complimentary.
- Primers need to be longer.
- Primer concentration is too high.
- Target template concentration is too low.

Possible Causes: Conditions

- Annealing temperature is suboptimal.
- Too many cycles.
- Product is the Wrong Size
- Possible Causes: Components
- The primers have homology with a repetitive DNA sequence in the template. The presence of 'amplifiable' DNA in the sample can be tested with primers for an alternative target.
- The published size for expected products is only a rough estimate. Check the basis for the size estimation.

Result Plausibility: Repeat PCRs with primers from an independent but related sequence. Set-up, thermal cycling, and reaction sensitivity can

be tested with negative and positive controls to check that PCR parameters are suitable and that expected product yields are obtained.

About Inhibitors

You can test for inhibition from endogenous substances known in the sample by spiking a control reaction with a known amplifiable target and its respective primers. Re-extraction, ethanol precipitation, and/or centrifugal ultrafiltration may correct the problem" See the chapter by K.H. Roux, "Optimization and Troubleshooting in PCR" in "PCR Primer: A Laboratory Manual" 1995

- chloroform
- ionic detergents (such as SDS and Sarkosyl)
- phenol
- heparin
- xylene cyanol
- bromphenol blue
- inosine
- deoxyuridine

About Enhancers

- DMSO (dimethylsulfoxide) : 1% - 10% (v/v)
- By changing the T_m of the primer-template hybridization reaction, DMSO can enhance specificity. However, add a bit more Taq since it is inhibited to some extent by the DMSO.
- *Posted by David Croan - david.croan@agal.gov.au in NWFSC Molecular Biology Forums*

- **Glycerol:** 5% - 20% (v/v)

Enhances specificity by changing the T_m of the primer-template hybridization reaction and extends the resistance of Taq to heat destruction. Different templates and different thermal cycling

schemes require different concentrations of glycerol for optimal amplifications.

- **DMSO and glycerol**

Recommended by a number of authors to improve amplification efficiency and specificity of PCR. However, in the multiplex reaction these enhancers gave conflicting results. For example, 5% DMSO improved the amplification of some products, decreased the amount of others and some loci were not affected at all. The same observations were made with 5% glycerol.

- **Bovine Serum Albumin (BSA):**

100 µg/ml - 1 ug/ul, 0.01% - 0.1% (w/v)

The addition of albumin to tissue DNA samples increases the amount of DNA generated by neutralizing many deleterious factors found in tissue samples which can inhibit PCR. Concentrations of up to 0.8 µg/µL may increase the efficiency of a PCR reaction much more than either DMSO or glycerol.

- **Formamide:** 1.25% - 10% (v/v).

In regions of high G/C content, specificity is reduced and multiple bands are observed when PCR products are separated on an agarose gel. Formamide enhances specificity and yield by changing the T_m of the primer-template hybridization reaction and lowers enzyme resistance to heat destruction. However, formamide can also be inhibitory to DNA polymerases so it needs to be tested at various concentrations to determine its optimal concentration levels.

- **PEG (polyethylene glycol) 6000:** 5% - 15% (w/v)
- **Spermidine**:

Reduces non-specific reactions between the polymerase and DNA.

- Tris-HCl: 10 mM - 67 mM, pH 8.2 - 9.0
- KCl: 25 mM - 50 mM
- MgCl2: 1.5 - 5.0 mM
- Gelatin: 0.01% - 0.1% (w/v)
- Non-ionic detergents
- Tween 20: 0.05% (v/v)
- Triton-X-100: 0.01% (v/v)
- Tetramethyl ammonium bromide
- TMANO (trimethylamine N-oxide)
- Betaine

How to Reduce Contamination

Possible Sources of Contamination: Components

Reagents

- Purified restriction fragment of target sequence
- Plasmid DNA that contains target sequence
- Post PCR contamination from the handling of PCR products.

Possible Sources of Contamination: Conditions

- Biological samples (e.g. patients, animal, etc.)
- Sample collection methods
- Lab Staff
- Lab environment
- Liquid nitrogen/ice
- Tissue homogenizer
- Gloves
- Pipetts/pipette tips
- Reaction tubes/glassware
- Recombinant or biological products (e.g. gelatin, bovine serum albumin, restriction enzymes)
- Hood or fume-cupboard filters

- Centrifuges/centrifuge tubes
- Thermal cycler, heating blocks, water baths
- UV transilluminator
- Electrophoresis apparatus
- Dot-blot apparatus
- Razor and/or microtome blades
- Microcentrifuges
- Concentrators and vacuum bottles
- Gel apparatus or UV light box
- Dry ice/ethanol or water baths

Techniques Used to Prevent Contamination

DNA Polymerase Contaminants (specifically Taq)

- Aliquot your reagents. Reagents stored as aliquots minimize repeated samplings. Contamination of reagents with target DNA can be checked by doing your PCR in the absence of exogenously added DNA.
- Add non-sample components to the reaction mixture before adding your sample DNA and cap each tube before proceeding to the next sample.
- Derive single- and double-stranded DNA with chemical adducts, such as isopsoralen.
- For prevention of contamination by dUDP-labeled PCR products, the use of uracil DNA-glycosylase (UNG) and deoxyuridine triphosphate (dTTP) substituted for thymidine triphosphate (dTTP).

Conditions

- Physical separation of the individual parts of the PCR into sample preparation, pre-PCR, and post-PCR locations.

- The use of UV light. This technique is limited because it can not destroy all types of contamination.
- Use positive displacement pipettors (filtered tips), pipettors with disposable tips and plungers.
- Change your gloves frequently.
- Uncap tubes carefully to prevent aerosols.
- Minimize sample handling
- Reliability of results and occurrence of sporadic contaminations can be checked by running the PCR protocol twice.

PCR Inhibitors & Enhancers

General/Intro, Feces, Formalin-Fixed Paraffin Sample

By Laura Ruth, Ph.D.

Optimization of PCR reactions from crude samples, such as feces, cerebrospinal fluid, blood, food, shellfish, soil, urine, saliva, formalin fixed, biopsies, respiratory, pus, and sputum, by various extraction methods to get rid of PCR inhibitors or adding enhancers to improve the sensitivity of the amplification can seem like a bit of magic because the same method is not universal.

There are a variety of standard and unique purification clean-up methods which can be used to get rid of PCR inhibitors in a variety of samples. When no amplification of a sample and amplification of a control occurs, then some of the first clean-up techniques to use include extraction (chloroform:iso-amyl alcohol 49:1 or ether), ethanol precipitation or centrifugal ultrafiltration.

When the standard PCR clean-up methods fail, alternative extraction methods must be found to eliminate PCR inhibitors and enhance the reaction. Fecal samples often inhibit PCR reactions. Shames et al. (1995) developed the method of isolating the bacteria from the fecal material using polyvinylpyrollidone (PVPP) at 4 C and then using a Chelex 100 treatment step. Although the PVPP isolation step can be performed at room temperature, it seems that the PCR reaction is less than optimal which may be due to an increased release of PCR inhibitors at higher temperatures. The amount of Tth polymerase and polymerase enhancer (Perfect Match, Stratagene) used on the PVPP isolated DNA was increased (3.2 vs 2.0-2.5 U, 1.25 vs. 1.0 U).

Another interesting technical note in this particular extraction method is the comparison of Tth and Taq polymerases. It is known that Tth polymerase has a decreased sensitivity compared to Taq polymerase in the presence of a PCR enhancer, such as the single-stranded-DNA-binding protein Perfect Match (Stratagene). However, when the MgCl2 concentration in the 10x reaction buffer was increased from 15 to 30 mM, there was a significant increase in sensitivity and no decrease in the specificity of the PCR. The excess concentration of $MgCl_2$ may saturate chelators which are present in the feces and leave enough $MgCl_2$ for the PCR reaction to run normally. The Tth polymerase was able to detect the E. coli DNA in the five H. hepaticus-positive fecal samples, while the Taq polymerase was only able to detect two. The use of bovine serum albumin is the last PCR reaction component to note in this particular method.

Isolating DNA from formalin-fixed, paraffin-embedded tissues can also be technically challenging with the presence of PCR inhibitors. Specifically, when Lampertico et al. were detecting hepatitis B virus (HBV) from liver tissues of patients with hepatocellular carcinoma, smaller than anticipated yields of HBV were obtained. However, when the resuspended desiccated pellet was boiled for 30 minutes and then centrifuged through a 1.0 ml Sephadex G50 mini column, a PCR inhibitor was removed.

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Feces, Norwalk Virus, Rotavirus, Urine

By Laura Ruth, PhD.

Feces

Previously I discussed one fecal extraction protocol to eliminate PCR inhibitors. But if you compare several fecal extraction methods, you'll realize that the methods can be applied to other crude or clinical samples. The three fecal sample protocols I will review here are:

(1) The magnetic immuno polymerase chain-reaction assay for detection of salmonellae (Widjojoatmodjo, M.N. et al., 1992)

(2) Detection of Norwalk virus in stool (Jiang, X.I., et al., 1992)

(3) Removal of inhibitory substances from human fecal specimens for detection of group A rotaviruses (Wilde, J., et al., 1990)

Magnetic Immuno Polymerase

Typically, fecal clinical specimens are diluted to decrease the concentration of bile salts (sodium glycocholate and sodium taurocholate) and bilirubin, which inhibit PCR at 50 ug/ml. However, dilution results in fewer bacteria, sacrificing the sensitivity of the PCR. Magnetic immuno PCR assay (MIPA) combines specific extraction of bacteria by specific monoclonal antibodies (MAbs) with primer-specific PCR amplification, allowing for bacteria enrichment and a more concentrated sample.

Serogroup-specific MAbs directed against serogroup A to E strains of the salmonellae, which account for 95% of the strains causing infections. Each Mab is incubated with Magnisort M magnetic chromium dioxide particles (Dupont). Stool specimens are diluted twentyfold and vortexed. After the specimens settle, 100 ul of supernatant is added to the mix of magnetic MAbs A-E. The antibodies are retrieved using magnetic force (MPC-96; Dynal). The bacterial sample is retrieved from the antibody by incubation and centrifugation; then it's used for PCR. Adding Chelex 100 during the boiling step with IMS-pretreated feces enhances amplification and can further increase the sample amplification.

Norwalk Virus

It can also be technically challenging to detect the RNA of a virus--such as the Norwalk virus--in stool using RT-PCR followed by PCR. In this case, the fecal sample is suspended in water (10 to 50 percent) and extracted once with an equal volume of 1,1,2-tricholor-1,2,2-trifluoroethane. The aqueous virus phase is precipitated with a 8% polyethylene glycol (PEG) 6000 (BDH Chemicals), 0.4 M NaCl solution. After resuspending and digesting the pellet using SDS, EDTA, and proteinase K, cetyltrimethylammonium bromide (CTAB -cationic detergent; Sigma) and sodium chloride were added to a final concentration of 1.25% CTAB and 0.45 M NaCl. The mixture is incubated at 56ºC for 30 minutes, then extracted with an equal volume of phenol-choloroform and reextracted with an equal volume of chloroform.

The RNA in the sample is then precipitated with 2.5 volumes of ethanol and used for RT-PCR. The CTAB RNA preparation method is shown to be more effective than dialysis, heating, phenol-chloroform extraction, and oligo (dT) cellulose chromatography methods, and it needs to be compared to hydroxyapatite and CF11 chromatography methods in the future. It's also important to note that in order to prevent cross-contamination, the authors found it necessary to use a separate room for the preparation of reagents and samples for testing.

Removal of inhibitory substances

While detection of Norwalk viral RNA utilized the PEG/CTAB procedure, extraction of rotavirus RNA used chromatographic cellulose fiber (CF11) powder in a series of rapid washing and elution steps, which allowed detection of less than 1 pg of genomic rotavirus RNA. In this case, fecal samples are diluted 1:1 with 0.01 M phosphate buffered saline, pH 7.4, containing 0.01 M CaCl2 and 0.01 M $MgCl_2$, vortexed, settled, and supernatant collected for further analysis.

A 100 ul sample is mixed with 200 ul extraction buffer (O.2 M glycine, 0.1 M Na_2HPO_4, 0.6 M NaCl, 1% SDS, pH=9.5), vortexed, and extracted with equal volume of water-saturated phenol and then a mix of phenol/chloroform-isoamyl alcohol (24:24:1). The aqueous phase is mixed in an Eppendorf tube with ethanol to bring the final solution to 15%. 30 mg of CF11 cellulose is added to aqueous ethanol mix, vortexed, and mixed at 4C for 90 minutes. The sample is then pelleted and washed several times with a sodium, EDTA, Tris-HCl, and ethanol solution. The fourth wash did not include ethanol in order to elute the RNA from the CF11. The RNA is precipitated from the final supernatant with ethanol and 0.3 M sodium acetate, pH=5.4.

Urine

Cytomegalovirus

Another body fluid used as a source of PCR material is urine. It is extremely useful if only small sample volumes (0.1-5 ul) are necessary for the PCR reaction. Yamaguchi (et al., 1992) found that extraction of cytomegalovirus DNA by either a PEG precipitation or glass powder (which has been used before to recover viral DNA and RNA from blood) were two efficient methods for sample retrieval. In the PEG protocol, urine is mixed 1:1:0.5 with 20% PEG 6K (Wako Pure Chemical Industries) and 2M NaCl, then incubated and centrifuged to recover the pellet.

Using the glass powder method, urine is mixed 1:1:0.07:0.2 with 6M guanidine thiocyanic acid, 2M NaCl, and glass powder suspension (DNA PREP, Asahi Glass, Co.). The mixture is incubated, centrifuged, and washed with an ethanol, Tris-HCl, and NaCl solution. Finally, the DNA is eluted from the glass powder with distilled water. Although both protocols seem equally effective in the quantity and quality of DNA obtained from urine, the glass powder method is slightly shorter at 1 hour compared to the PEG treatment, which requires at least 2 hours.

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Respiratory Fluid, Blood, Shellfish

By Laura Ruth, Ph.D.

You can find a vast number of PCR inhibitor and enhancer recipes in the literature, but here are a few more protocols for a variety of clinical samples where it can be technically challenging to obtain a detectable PCR signal.

Respiratory/sputum

The PCR detection of Mycobacterium tuberculosis in clinical specimens like respiratory fluid is often inhibited. Although the heme group in blood is known to inhibit PCR, no one knows the exact identity of other endogenous inhibitors in the samples. Re-extraction procedures for removing the inhibitors are tedious and don't work 100 percent of the time. Forbes and Hicks performed an initial experiment of adding BACTEC 12B broth to sputum samples and were able to detect the Mycobacterium tuberculosis in samples where the PCR assay had been previously inhibited. A rigorous study of the individual components of the BACTEC 12B medium--which includes PANTA reagent (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin), reconstituting fluid, 0.2% glycerol, 0.05% Tween 80, and 0.05% bovine serum albumin (BSA)--revealed that only adding the 0.05% BSA resulted in amplification of a previously inhibited sample. The study showed a concentration range of 0.00038 to 0.1% BSA to be an effective enhancer.

Blood culture

Amplification of 16S rRNA from inoculated blood-culture media failed many times even after standard extraction methods (phenol-chloroform, wash/centrifuge, QIAmp column purification) were repeated. By systematically testing blood-culture media from different manufacturers--including BACTEC aerobic medium, anaerobic medium, and mycobacterial media 13A and 12B (Becton Dickson)--and BacT Alert aerobic and anaerobic media (Organon Teknica), the only product which did not inhibit the PCR reaction was BACTEC 12B. BACTEC 12B doesn't contain the inhibitor sodium polyetholesulfonate (SPS). SPS is added to blood-culture media for its anticoagulant and anitcomplementary activities, which increases the level of growth of most microbes. SPS can be monitored spectrophotometrically with an absorption peak at 284 nm. SPS is chemically similar to DNA. Both are high-molecular weight polyanions, which are soluble in water and insoluble in alcohols. Therefore, it's not surprising that SPS copurifies with DNA in many solvent systems.

All the DNA samples purified from blood cultures using standard methods--such as phenol-chloroform, QIAmp silica column adsorption, and Isoquick--have a single absorption peak at 284 nm, which suggests the presence of SPS in the sample. Removal of SPS and purification of DNA for PCR amplification is achieved using an organic extraction method with lysis buffer, which consists of guanidine hydrochloride in Tris buffer as the aqueous phase and benzyl alcohol as the organic phase. The addition of guanidine hydrochloride in the extraction process is critical in promoting the partition of SPS into the benzyl alcohol phase, while the DNA remains associated with the aqueous phase. Guanidine isothiocyanate--which replaces guanidine hydrochloride in the Isoquick extraction method--is an inferior chaotropic agent.

Shellfish

It's important to detect viruses in shellfish in order to prevent disease transmission. Enterovirus is one microbial pathogen that causes gastroenteritis. Lees et. al, 1994, developed a method for detecting enteroviruses in shellfish, which can be applied to detecting other microbial pathogens such as hepatitis A, Norwalk virus and small, round-structured viruses (SRSVs). Blending, sonicating, and centrifuging 25 g of oyster and mussel flesh in 0.05 M glycine buffer pH = 9.0-9.5 yields a supernatant, which is then re-pHed to 7.2-7.4 and mixed with polyethylene glycol (PEG) 6000 to a final concentration of 8% (w/v). The PEG solution is then subject to detoxification by resuspension in Na2HPO4 pH=9.0-9.5, sonicated 2x, and centrifuged. Then the supernatant is pHed to 7.4. Next, the supernatant is extracted 5x with equal volume of 1,1,2-trichloro,2,2,1-trifluoroethane (Freon TF) and concentrated for further uses. Nucleic acid (NA) extraction continues by mixing the shellfish concentrate with glass powder matrix (BIO 101, La Jolla), 6.6 M guanidine isothiocyanate (GITC), and 1.7% Triton X-100. The RNA bound to the glass powder is then pelleted, washed 2x with 5M GITC,1x with ice-cold 70% EtOH, and 1x with ice-cold acetone. The RNA is then eluted from the air-dried pellet with T.E. buffer, precipitated with 3M NaAc, and finally 2 volumes EtOH.

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Body Fluids and Food

By Laura Ruth, Ph.D.

Body Fluids: Blood, Plasma, and Placenta

Whole blood, peripheral blood mononuclear cells (PBMCs), or plasma samples gathered for clinical research are collected in the presence of an anticoagulant such as heparin, EDTA, acid citrate dextrose (ACD), or potassium oxalate. Blood samples collected in the presence of heparin can yield decreased amounts of cellular DNA. Holodniy et al., 1991, measured the inhibitory effect of heparin on PCR by using an enzyme-linked affinity assay for PCR product detection and quantitation of a variety of samples: cellular DNA from whole blood and washed PBMCs; cellular RNA from washed PBMCs; and cell-free RNA from HIV-infected plasma or serum. "The HIV gag sequence was amplified from three sources:

1. cellular DNA
2. reverse-transcribed RNA obtained from HIV-infected U1 cells from HIV seronegative whole blood, and
3. HIV seropositive blood.

All three samples were collected in the presence of each anticoagulant, sodium heparin, ACD, EDTA, and potassium oxalate. Results showed reduced amounts of the HIV gag PCR product in the presence of heparin [50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.4 mM MgCl2, 0.02% gelatin, 1 mM dNTP, 50 pmol primer, 2.5 U Amplitaq].

Holodniy et al., 1991 obtained similar results in the amplification of the HLA-DQ sequence from placental DNA [1ug placental DNA, +/- 10x Taq (25 U), +/- 7.4U heparinase II, 1x PCR Buffer]. The addition of less than 0.05 U of heparin per reaction suppressed detection of the HLA-DQ sequence by PCR. Preincubation of the HIV samples with heparinase II

increased the PCR product. Like the HIV samples, preincubation of the placental DNA sample with heparinase II also reversed some of the PCR inhibition. In addition, a tenfold increase of Taq polymerase (25 U/reaction) in the placental DNA PCR reaction reversed some inhibition. However, increasing the KCl concentration or template primer did not seem to do so.

Food

The bacteria Listeria monocytogenes has been found as a cause of disease in humans and animals for over 50 years and has been documented as a public health problem in common foods such as cheese, milk, and meat. These foods contain compounds that hinder PCR reactions. Although the inhibitors in foods such as yogurt, pasteurized milk, ice cream, soft cheese, smoked salmon, beef, and pork seem to be unidentified, there are methods which can help minimize false negative PCR results --i.e., no signal due to inhibition.

According to Wang, et al., 1992, and Wernars, et al., 1991, soft cheese contains compounds which tend to inhibit PCR. Wernars, et al., showed that using phenol-chloroform or ion-exchange Qiagen column purification methods helped eliminate inhibitory compounds in the soft-cheese DNA samples. Wang, et al., 1992, was able to use a rapid polymerase chain-reaction method: shorter denaturing time, shorter annealing time, a rapid transition, and an increase in the number of cycles to increase the sensitivity of the reaction in many food products such as poultry, sausage, and yogurt. The rapid PCR method, however, did not eliminate the inhibition of PCR by soft cheese. Other authors, including Niederhauser, et al., 1992, and Thomas, et al., 1991, found that using an enriched medium when culturing the bacteria increases the DNA yield. Niederhauser, et al., also found that sometimes centrifuging the bacteria before lysis can increase yield and reduce the amount of enrichment culturing time.

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Plants & Soil

By Laura Ruth, Ph.D.

Plants

Since polysaccharides are common inhibitors in PCR reactions that use DNA from plant tissues, Demeke and Adams (1992) thought it would be interesting to investigate the effects of both neutral and acidic polysaccharides -as well as buffer additives--on PCR amplification.

Common plant DNA purification methods can remove proteins, but they're not always effective for removing polysaccharides. To test the inhibitory nature of a variety of polysaccharides, Demeke and Adams purchased polysaccharides from Sigma and purified DNA from fresh spinach (Spinaciaolearacea L.) using a hot CTAB protocol. A ratio of 500:1 (w/w) polysaccharide/DNA was first used in the PCR reaction [50 mM KCl, 10 mMTris-HCl (pH 9), 1.5 mM MgCl2, 0.01% gelatin and 0.1% Triton X-100, 0.2 mMof each deoxynucleoside triphosphates (dNTPs), 0.4 mM primers, 3 ng spinach DNA, 2 units Promega Taq DNA polymerase]. If the polysaccharide was found to be inhibitory, they would lower the amount (100:1, 50:1, 5:1).DMSO (5%), formamide (5%), glycerol (5%, 9%, 13%), polyethylene glycol(PEG) 400 (5%), Bovine serum albumin (BSA) 2ug/ml, and Tween 20 (0.25%,0.5%, 1.0%, 2.0%) were added to the PCR buffer to attempt reversal of PCR inhibition. The PCR products were analyzed by electrophoresis in 1.5%agarose gels with EtBr staining for detection.

Some of the neutral polysaccharides, which were of interest to Demekes and Adams to test, included arabinogalactan,dextran, gum guar, gum locust bean, inulin, mannan, and starch. Amazingly,none of them proved PCR inhibitive. Two of the acidic polysaccharides, dextran sulfate and gum ghatti, inhibited PCR amplification. Gum ghatti was only

inhibitory at the polysaccharide ratio of 500:1 and no lower ratios. The inhibitory effect was uniquely reversed by the addition of 0.5% Tween 20. In contrast, dextran sulfate was inhibitory at 500:1,100:1, and 50:1. The dextran sulfate/DNA 50:1 ratio inhibitory effect can be reversed with Tween 20 (0.25% and 0.5%), DMSO (5%), or PEG 400 (5%). As a control reaction, Demeke and Adams reconfirmed that 0.1% SDS inhibits PCR, which can then be reversed with the addition of Tween 20. Although a previous study by Do and Adams demonstrated that carrageenan, gum karaya, pectin, and xylan inhibited a restriction enzyme digestion, these particular acidic polysaccharides seem not to inhibit PCR amplification. Demeke and Adams tried to rationalize why one acidic polysaccharide might be inhibitory due to sulfate moieties and/or sugar residue, but the rationalization does not yield a clear pattern.

Soil

Amplifying DNA from soil and sediment samples is useful for monitoring the presence of genetically engineered microorganisms, and for both the detoxification and biodegradation of genes in the environment. Soil and sediment samples can contain humic substances or other compounds--e.g.,iron--that inhibit PCR. Tsai & Olson, 1992, tested a variety of concentrations of humic extract (Aldrich) on the PCR reaction using"rapidly extracted" seeded soil DNA [1X PCR Buffer, 300 nm primer, 200 uMdNTP, 2.5 U AmpliTaq]. They found that as little as 10 ng/100 ul PCR reaction inhibited PCR either by inhibiting the polymerase activity or the primer binding. Although this can be somewhat reversed by dilution, it reduces the detection limit.

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Special PCR Topics

Why Aren't PCR-Based Diagnostics Main Stream?

By Michael S. Zwick, Ph.D.

The polymerase chain reaction reaction (PCR) has been greatly refined since its advent 14 years ago. Advances in PCR include the development of reliable thermal cyclers and thermal stable polymerases. Just as important, and in parallel, is the much-reduced cost of DNA sequencing. Sequencing a gene used to be an entire Ph.D. thesis, but today the technique is much simplified. Now, sequence data streams daily into large genetic databases that allow researchers to identify novel genetic regions that can be used as target primer sites for detection of a specific organism. This is the precise strategy our company, Investigen, uses to develop PCR-based diagnostic tools for organism detection.

PCR is a standard and proven technology. Most genetic labs regard PCR as routine. But in the realm of microbial identification, particularly in the clinical field, PCR-based diagnostics have been slow to catch on. The reasons for this can be attribute to four areas: the clinical lab, regulations, licensing and data.

Most labs around the world rely on culturing methodologies for microbial identification. Considered the "work horse" of microbiology, most culturing techniques have remained the same for decades. This is not a negative; culture-based tests work very well. Advances include standardization of tests and media, reduction in test size and automation. The introduction of other diagnostic methods is difficult when labs consider culturing the golden standard. In theory PCR-based diagnostics compare favorably and in some cases are superior to

culturing based diagnostics. So why are PCR-based diagnostics not more common? The underlying reason is likely that there are very few FDA-approved PCR-based diagnostic assays available. Thus, labs are much less likely to introduce a new diagnostic method into the lab arena when so few tests can be conducted using the PCR format. It just isn't cost effective.

PCR-based diagnostic methods compare favorably with culture based diagnostic methods. So why are there so few approved PCR-based diagnostic assays? The costs associated with obtaining regulatory approval should be no greater for a PCR-based diagnostic assay than for an antigen-based diagnostic assay. The Federal Drug Administration (FDA) alone has placed stringent stipulations and caveats on such testing methods despite their external only use. Some PCR-based tests despite having the same methodologies as other approved commercial tests are taking up to 5 years for approval. Recently however, approval times have been declining as the demand for PCR based testing increases.

A large obstacle to developing a PCR-based diagnostic assay is the cost of licensing the technology from other companies. It's no secret that PCR is a patented technology. Many of the primers used in a diagnostic kit are also patented. To use patented technology for monetary gain an arrangement must be made with the organization or individual that holds the patent. This arrangement usually involves royalties and up-front costs. The large sums of up-front money have certainly restricted the development and introduction of new PCR-based diagnostics.

Finally, the development of a PCR-based assay requires sequence information. It is only recently that sequence libraries have accumulated enough data that researchers can develop specific PCR-based assays without having to do the sequencing themselves. Assay development will continue to improve as more data becomes available.

Despite these obstacles molecular diagnostics is clearly the way of future.

PCR-Based Detection Kits Part I

Advantages and Disadvantages

By Michael S. Zwick, PhD

Previously I discussed the lack of PCR-based diagnostic kits in clinical settings. Reasons ranged from a lack of acceptance by the clinical lab to the many sub-licensing contracts required for a company to sell a PCR-based diagnostic kit.

This month I'll explain why I feel PCR should be the method of choice for pathogen detection. Don't get me wrong, PCR isn't perfect. But in most aspects of pathogen detection it is advantageous. Especially when used as a preliminary screen. I also don't want to imply that it should be used as the only means of detection/diagnosis in all cases. It, like any tool, should be used in conjunction with other diagnostic methods if possible.

Listed below are the attributes which, in my view, make PCR an attractive technology for pathogen detection. Where applicable, I have tried to include the drawbacks of using PCR compared to other detection methods.

First, PCR is fast. The entire process of DNA extraction, amplification, and detection generally takes less than six hours. The process may take longer if the reaction utilizes nested primer sets. For some samples, such as those originating from feces or cuticle, several additional steps may be involved in the DNA extraction. This can extend the total detection time to 14 hours.

Shorter detection times may be achieved by using commercially available nucleic acid extraction kits, having a robot do the extraction, optimizing the PCR protocol, or by using faster thermal cycler technology. With an optimal specimen, fast extraction and a light cycler thermal cycler the total detection time could be as little as 2 hours. However, six to eight hours is typical.

This is a tremendous improvement over the 2-5 days it takes for most culture-based methods. This is also faster than some antibody detection tests, which require culture enrichment for accurate results. And, while there are commercially available antigen-based detection kits that are more rapid than PCR-based methods, most of these assays can only be used on a very limited spectrum of organisms.

Second, PCR does not require culturing. This is intimately tied to the speed of PCR-based detection. Since PCR is extremely sensitive (as few as 10 cfus per sample has been reported), culturing is generally not necessary. Many of the currently available PCR detection kits recommend selective enrichment, especially when samples are obtained from sources that contain high quantities of PCR inhibitors. Culturing can be avoided by using a nucleic acid extraction method that yields PCR-quality DNA and by optimizing the PCR reaction. Culturing may be required when detecting very low concentrations of a pathogen in a large volume of sample, or if the organism is pathogenic below the threshold of PCR sensitivity (i.e. when detecting S. typhi). In any event, the need for culturing is rare and by removing the culturing step, critical time is saved.

More important than saving time, the elimination of the need for culturing allows for the rapid detection of organisms that are difficult or impossible to culture. This would include organisms such as certain viruses, fungi, anaerobes and mycoplasma.

Third, PCR is cost-effective. When compared to the costs associated with culturing (tech time and materials), or other antibody-based assays (high assay costs), PCR-based diagnostics are very inexpensive.

When one considers culturing of a microbe, few consider time as an associated cost, even though this is probably the most costly aspect of culturing-based methods. Most samples have to be cultured and re-cultured for proper identification. This requires a fair amount of hands-on time (adding expense to the assay), and also increases the risk of contamination.

In contrast, most antibody assays generally require little hands-on time but the assays themselves are expensive.

The components of the PCR reaction (primers, enzyme, dNTPs and buffer) cost less than forty cents per assay, and the time involved to set up the reaction, including nucleic acid isolation, is generally under an hour. This, of course, depends on the sample, technician, and the technique used to isolate the nucleic acids. Since PCR takes less time overall to set up, it costs less to conduct.

PCR does, however, require a thermal cycler, UV source and some type of image capture device, thus the initial costs of setting up a PCR lab can be high. If a lab only conducts a low volume of testing then the initial expenses that go along with establishing PCR capability may not be justified. But, since most diagnostics labs already have most of this equipment, there is generally little cost associated with adapting PCR as a diagnostic tool.

PCR-Based Detection Kits Part II

Advantages and Disadvantages

By Michael S. Zwick, Ph.D.

In my preceding article, I discussed three reasons why PCR is particularly useful, i.e., that it is fast, doesn't require culturing, and is cost-effective. Today I will discuss some additional rationale for considering the use of PCR in the clinical setting.

First, PCR is not antigen-based.

Antibody-based detection kits (ELISA and others) work either by detecting 1) human antibodies directed against a pathogen, or 2) surface antigens on a pathogen. Human antibody-detection kits thus require the presence of an immune response, and an elevated antibody titer, before they can correctly identify the presence of the antigen. In the case of a recent infection, a test of this sort will fail to detect human antibodies to the pathogen, and this may be falsely interpreted to mean that there is no infection. In reality, the patient is infected, but not enough time has elapsed to develop a titer high enough for detection. This is a common problem with HIV antibody-based detection. This type of test also has trouble detecting the presence of pathogens in immuno-compromised patients for much the same reason. Conversely, antibody-based tests have also been known to give false positive results.

Probably the greatest problem with antibody-based tests, however, is their inability to detect new pathogen sub-types with altered surface antigens, leading to false negatives. (PCR-based assays can encounter similar problems due to changes, or mutations, in the DNA, however, PCR-based assays can be designed to minimize these types of false negatives.)

Despite these drawbacks, antigen-based test kits are a very important means of detection, and in most cases are faster than PCR-based assays.

Second, assays can be easily customized to suite a particular need.

By simply using different sets of target-specific primers, kits can be rapidly designed for many detection applications.

Third, PCR is an easy to use, time proven technique.

Samples for pathogen detection can be routinely processed in less than 10 minutes using commercially-available nucleic acid extraction products. Once processed, samples are added to a reaction mix and run on a thermal cycler. Upon completion, samples can be read on an automated system or by gel electrophoresis.

A few PCR assays will soon be available that are virtually 100% automated. Machines will do everything from sample preparation to analysis of the results. Such systems will initially be very pricey and probably only accommodate a single manufacturer's platform. This will make the initial units unattractive for most clinical labs but, as the price comes down, will ultimately make automated PCR diagnostics practical.

This concludes my discussion on PCR-based diagnostics. I hope I have provided you with a general overview of the advantages of PCR-based assays, have given you some insight into the current state of the technology, and have addressed some of the issues which are slowing the process of incorporation of this technology into the clinical lab setting. Within the constraints of this column, it is impossible for me to address every issue regarding PCR-based diagnostics. Every type of diagnostic tool has its pros and cons, and most have an important role in diagnostics. None are foolproof. Any diagnosis should be confirmed by a second, independent method. PCR has a place as either the primary diagnostic tool, or as a means of confirmation. It is an excellent technology, and deserves to be moved from the research to the clinical setting.

FISH, PRINS & Cytogenics: Do You Have A Light?

By Dr. Laura Ruth, PhD

Lighting up chromosomes with fluorescent probes is a cytogenetic imaging method used in FISH (Fluorescent in situ hybridization) and PRINS (Primed in situ hybridization). FISH technology combines DNA hybridization with immunofluorescence which has allowed new research and clinical avenues in gene mapping, chromosomal and gene abnormalities, gene expression, genome organization, as well as identification of infectious diseases and investigation of viral integration sites in mitotic (metaphase) and interphase cells. The haptens or fluorescent molecules used in FISH have a better to signal to noise ratio compared to the traditional radioactive probes. The development of new fluorescent molecules and multicolor FISH protocols enables the simultaneous visualization of multiple chromosomal targets in different colors. PCR protocols are part of the variety of methods used to generate the 'chromosome paints', i.e. probes, in 'chromosome painting': 1) flow cytometry of chromosomes followed by cloning to create chromosome-specific DNA library and/or by DNA amplification using PCR strategies, 2) isolation of a number of chromosomes or subchromosomal segments by micromanipulation/microdissection followed by universal PCR amplification, 3) PCR amplifying specific sequences from somatic-cell hybrid DNA, such as a target which can be prepared by inter-Alu-PCR, and 4) pooling of individual fragments cloned and mapped to a chromosome of interest. FISH probe length varies between 200 - 1000 bp. On a routine basis, FISH images are inspected by human eye. There are, however, machines which have been engineered to automate the detection of metaphase chromosomes (MetaSystems Group, Inc. and Applied Imaging), and there is research continuing in this area to improve the accuracy of the machines (LLNL system, Lawrence Livermore National Laboratory).

There have been many developments of FISH methodology. Micro-FISH is a technique to generate band specific probes. Chromosomal DNA, such as from metaphase chromosomes, is dissected from regions of interest, PCRed, and propagated in bacterial vectors using universal primers. In-cell RT-PCR can be used to identify chimeric mRNA as in chronic myeloid leukemia (CML). FICTION (fluorescence immunophenotyping and interphase cytogenetics as a tool for investigation of neoplasms) is used in lineage analysis of cancers, such as myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML), and identifying sex and chromosomal aneuploidies in fetal nucleated cells isolated from maternal circulation. Fiber-FISH is performed on extended chromatin of interphase nuclei. The 'halo' one sees can be used for high resolution gene mapping, which is useful in clinical disorders with subtle DNA rearrangments. Lastly, comparative genomic hybridization (CGH) is extremely useful in tumor genetics where the ratio of color differences in probes bound to tumoringenic cell chromosomes indicate either loss of heterozygosity or oncogene amplifications.

PRINS (Primed in situ DNA synthesis) is a combination of FISH and PCR and therefore, eliminates the need for pre-labeled probes. Oligonucleotide primers can be annealed to both interphase and metaphase cells which are then extended by using Taq polymerase in the presence of labeled nucleotides. (A flat block/slide holder is used in the PCR machine). PRINS requires only a short amount of time, e.g. <2 hours, and is better at detecting small DNA targets, like chromosomal breakpoints. PRINS can be substituted for traditional FISH studies, however, heating the cells to elevated PCR temperatures of 96 C can be a problem. The PRINS technique has been further derivatized to develop SPRINS (self primed in situ DNA synthesis). The SPRINS technique combines PRINS and nick translation in situ, which is sited to

be useful in detecting the distribution and methylation of CpG islands. Click HERE for related links

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Clinical Applications of Dot Blots

Hybridizing With a Reason

By Laura Ruth, Ph.D.

Dot blots and reverse dot blots are techniques where PCR products are hybridized with allele specific oligonucleotides (ASOs) for the purpose of scanning for mutations, detecting RNA, cancer, or pathogens. In the dot blot technique, PCR products are immobilized and denatured onto a membrane, such as nitrocellulose or nylon, followed by specific hybridization conditions with ASOs. The ASO is labeled with either a radio label, flurophore or biotin which are detected, respectively, by autoradiography or fluorescence after irradiating at a particular UV wavelength and linking to an avidin- or streptavidin-enzyme complex. (a colorimetric or chemiluminescent signal). False negatives can be ruled out by running an aliquot of the PCR reaction mixture on an electrophoresis gel.

In contrast to the dot blot, the reverse dot blot enables the analysis of several alleles in a single test. The technique can be considered an inverted dot blot. ASOs with poly(dT) tails are immobilized on to a membrane by UV irradiation. A PCRed probe, which is biotinylated or radiolabeled, is hybridized to the ASO. Using the reverse dot blot technique, many probes can be screened against specific ASOs. For both the dot blot and the reverse dot blot, the T_m for each immobilized ASO will be allele-dependent and therefore each filter can only have bound ASOs with similar T_m. ASOs usually contain about 20 nucleotides if they are around 50% GC in content. Obviously, both the T_m and specificity to a particular probe of an ASO will be altered by changing the length of an ASO.

A survey of recent literature shows a variety of applications for both the dot blot and the reverse dot blot. Types of samples which can be detected using a dot blot are CMV DNA in urine, mycobacterium tuberculosis complex in cattle, mercury resistant plasmids in soil bacteria, helicobacter infection in pet cats, and phylogenetic analysis and indentification in activated sludge. In comparison, the types of samples which can be detected using a reverse dot blot are: K-ras mutations in stools and tissues, hepatitis RNA in oyster meat, RNA from paraffin blocked histological samples, T-cell a and b chain variable region, and lung cancer carcinoembryonic antigen mRNA. The hybridization protocol for each application varies, but in general there is a pre-hybridization and then hybridization step. The membrane is typically soaked in an EDTA, Na2[n1]HPO4, 7% SDS, and sonicated salmon sperm or calf thymus DNA solution for 1 hour at 60 C. The labeled probe or ASO is denatured with NaOH and then neutralized with HCl. The hybridization step consists of a series of 'low to high stringency' conditions where 'low stringency' is obtained at 20 C with 2 X SSC (or SSPE)/0.1% SDS and 'very high stringency' is performed at 60 C with 0.1 X SSC(SSPE)/0.1% SDS. Proper negative and positive controls should be included in the samples for the purpose of monitoring and interpreting the results of the hybridization.

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Appendix A – Units and Formulas

Unit Conversions

Multiples & submultiples	Prefixes	Symbols
10^{12}	tera	T
10^{9}	giga	G
10^{6}	mega	M
10^{3}	kilo	k
10^{2}	hecto	h
10	deca	da
10^{-1}	deci	d
10^{-2}	centi	c
10^{-3}	milli	m
10^{-6}	micro	µ
10^{-9}	nano	n
10^{-12}	pico	p
10^{-15}	femto	f
10^{-18}	atto	a

Weight Conversions

1 µg = 10^{-6} g

1 ng = 10^{-9} g

1 pg = 10^{-12} g

1 fg = 10^{-15} g

Spectrographic Conversions

$1A_{260}$ unit of double-stranded DNA = 50 µg/ml solution

$1A_{260}$ unit of single stranded DNA = 33 µg/ml solution

$1A_{260}$ unit of single stranded RNA= 40 µg/ml solution

Micromolar extinction coefficient

ε, at 264 nm

Where ε = OD_{264} units in a solution of 1 µmole dissolved in 1 liter, that is

ε = OD_{264} units of 1 µmole

ε = {(8.8 X *n*T) + (7.3 X *n*C) + (11.7 X *n*G) + (15.4 X *n*A)} X 0.9

Calculating the Molecular Weight of DNA

MW =

(249 X *n*A) + (240 x *n*T) + (265 X *n*G) + ((225 X *n*C) + (63 X *n* – 1) + 2

DNA Molar Conversions

1 µg of 1,000 bp DNA = 1.52 pmol (3.03 of ends)

1 pmol of 1,000 bp DNA = 0.66 µg

1 µg of pBR322 DNA = 0.36 pmol DNA

1 pmol of pBR322 DNA = 2.8 µg

For dsDNA:

To convert pmol to µg:

pmol X N X 660 pg/pmol X 1µg/10^6 pg = µg

To convert µg to pmol:

µg X 10^6 pg/µg X pmol/660 pg X 1/N = pmol

where N is the number of nucleotide pairs and 660 pmol/pg is the average MW of a nucleotide pair

For ssDNA:

To convert pmol to µg:

pmol X N X 330 pg/pmol X 1µg/10^6 pg = µg

To convert µg to pmol:

µg X 10^6 pg/µg X pmol/330 pg X 1/N = pmol

where N is the number of nucleotide pairs and 330 pmol/pg is the average MW of a nucleotide pair.

Biophysical Data for Deoxynucleotide Triphosphates

Component	MW (Daltons)	Molar Extinction Coefficient and Peak Absorbance Wavelength (pH 7.0)
dATP	491	15,200 at 259 nm
dCTP	467	9,300 at 271 nm
dGTP	507	13,700 at 253 nm
dTTP	482	9,600 at 267 nm

Appendix B - The Genetic Code

Second Position of Codon

First Position	T	C	A	G	Third Position
T	TTT Phe [F]	TCT Ser [S]	TAT Tyr [Y]	TGT Cys [C]	T
	TTC Phe [F]	TCC Ser [S]	TAC Tyr [Y]	TGC Cys [C]	C
	TTA Leu [L]	TCA Ser [S]	TAA *Ter* [end]	TGA *Ter [end]*	A
	TTG Leu [L]	TCG Ser [S]	TAG *Ter* [end]	TGG Trp [W	G
C	CTT Leu [L]	CCT Pro [P]	CAT His [H]	CGT Arg [R]	T
	CTC Leu [L]	CCC Pro [P]	CAC His [H]	CGC Arg [R]	C
	CTA Leu [L]	CCA Pro [P]	CAA Gln [Q]	CGA Arg [R]	A
	CTG Leu [L]	CCG Pro [P]	CAG Gln [Q]	CGG Arg [R]	G
A	ATT Ile [I]	ACT Thr [T]	AAT Asn [N]	AGT Ser [S]	T
	ATC Ile [I]	ACC Thr [T]	AAC Asn [N]	AGC Ser [S]	C
	ATA Ile [I]	ACA Thr [T]	AAA Lys [K]	AGA Arg [R]	A
	ATG Met [M]	ACG Thr [T]	AAG Lys [K]	AGG Arg [R]	G
G	GTT Val [V]	GCT Ala [A]	GAT Asp [D]	GGT Gly [G]	T
	GTC Val [V]	GCC Ala [A]	GAC Asp [D]	GGC Gly [G]	C
	GTA Val [V]	GCA Ala [A]	GAA Glu [E]	GGA Gly [G]	A
	GTG Val [V]	GCG Ala [A]	GAG Glu [E]	GGG Gly [G]	G

Appendix C - OnLine Genomic Databases

Databases at the National Agricultural Library:

- **Plant Genome**
http://probe.nalusda.gov:8000/alldbs.html#genome

- **Livestock Animal Genome**
http://probe.nalusda.gov:8000/alldbs.html#animal

- **Other Organisms Genome**
http://probe.nalusda.gov:8000/alldbs.html#other

- **Plant Reference**
http://probe.nalusda.gov:8000/alldbs.html#reference

- **Insect Reference**
http://probe.nalusda.gov:8000/alldbs.html#insect

- **ACEDB Documentation Library**
http://probe.nalusda.gov:8000/acedocs/index.html

- **FTP site**
ftp://probe.nal.usda.gov/pub/"

- **Rodent Genome Databases**
http://www.hgmp.mrc.ac.uk/GenomeWeb/rodent-gen-db.html

- **Other Vertebrate Genome Databases**
http://www.hgmp.mrc.ac.uk/GenomeWeb/vert-gen-db.html

- **Nematode Genome Databases**
http://www.hgmp.mrc.ac.uk/GenomeWeb/nematode-gen-db.html

- **Insect Genome Databases**
http://www.hgmp.mrc.ac.uk/GenomeWeb/insect-gen-db.html

- **Protozoon Genome Databases**
http://www.hgmp.mrc.ac.uk/GenomeWeb/protozoon-gen-db.html

- **Other Invertebrate Genome Databases**
http://www.hgmp.mrc.ac.uk/GenomeWeb/invert-gen-db.html

- **Plant Genome Databases**
http://www.hgmp.mrc.ac.uk/GenomeWeb/plant-gen-db.html

- **Fungal Genome Databases**
http://www.hgmp.mrc.ac.uk/GenomeWeb/fungal-gen-db.html

- **Prokaryotic Genome Databases**
http://www.hgmp.mrc.ac.uk/GenomeWeb/prokaryote-gen-db.html

- **Organellar Genome Databases**
http://www.hgmp.mrc.ac.uk/GenomeWeb/organelle-gen-db.html

- **Comparative Genome Databases**
http://www.hgmp.mrc.ac.uk/GenomeWeb/comp-gen-db.html

- **Taxonomic Databases**
http://www.hgmp.mrc.ac.uk/GenomeWeb/taxon-gen-db.html

- **Regulation & Cellular-Level Databases**
http://www.hgmp.mrc.ac.uk/GenomeWeb/cell-gen-db.html

Human Genome Databases

- Online Mendelian Inheritance in Man

http://www.hgmp.mrc.ac.uk/omim/
"This database is a catalog of human genes and genetic disorders authored and edited by Dr. Victor A. McKusick and colleagues at Johns Hopkins University and elsewhere…OMIM is intended for use primarily by physicians and other professionals concerned with genetic disorders, by genetics researchers, and by advanced students in science and medicine." – *Homepage cited*

- **GDB (Genome DataBase)**
http://www.hgmp.mrc.ac.uk/gdb/gdbtop.html
"An international collaboration in support of the Human Genome Project" – *Homepage cited*

- **GeneCards - integrated biomedical genetic information**
http://bioinformatics.weizmann.ac.il/cards/
"GeneCards is a database of human genes, their products and their involvement in diseases. It offers concise information about the functions of all human genes that have an approved, as well as selected others [gene listing]. It is especially useful for those who are searching for information about large sets of genes or proteins, e.g. for scientists working in functional genomics and proteomics." – *Homepage cited*

- **OMIM gene map**
http://www.ncbi.nlm.nih.gov/Omim/searchmap.html
"The OMIM gene map presents the cytogenetic map location of disease genes and other expressed genes described in OMIM. See the OMIM Morbid Map for a list of disease genes organized by disease. For more refined maps of genes and DNA segments, consult the NCBI Entrez Genomes division and the Genome Data Base." – *Homepage cited*

- **The HuGeMap database**
http://www.infobiogen.fr/services/Hugemap/
"HuGeMap is interconnected to the radiation hybrid gene map database RHdb, maintained at EBI. This interconnection is based on CORBA servers that have been implemented at Infobiogen and EBI, and that share the same IDL (see the Object Management Group for an introduction to CORBA)." – *Homepage cited*

- **GenAtlas**
http://bisance.citi2.fr/GENATLAS/
"A catalog of three types of object : 9396 genes, 20009 markers and 1971 phenotypes" – *Homepage cited*

- **Genome Navigator: Whitehead/MIT**
http://www.mpimg-berlin-dahlem.mpg.de/~andy/GN/mithumanrh/
"This is an attempt to put together an interactive genome viewing/browsing facility using external data. In this particular case, the source of data for objects depicted on the map is Whitehead/MIT, data release 11, October 1996." – *Homepage cited*

- **The Genome Channel**
http://compbio.ornl.gov/tools/channel/

- **Genome Catalog**
http://genome.ornl.gov/GCat/species.shtml
"An HTML browsing and querying interface with summary and detail data about annotation organized around chromosome, contigs, submitted GenBank clones, GenBank annotated genes, GRAIL-EXP gene models, GENSCAN gene models, STS markers, and other features." – *Homepage cited*

- **HGBASE - human genic bi-allelic sequences - SNPs**
http://hgbase.interactiva.de/
"HGBASE (Human Genic Bi-Allelic SEquences) is a database of intra-genic (promoter to end of transcription) sequence polymorphisms." – *Homepage cited*

- **Single Nucleotide Polymorphisms in the Human Genome**
http://www.ibc.wustl.edu/SNP/

"This website is designed to provide the human genetics community with access to single nucleotide polymorphism (SNPs) that have been developed as genetic markers on the human genome. The site is organized by chromosomes and cytogenetic location. Each SNP has PCR primer and conditions associated with it." – *Homepage cited*

♦ **dbSNP A Database of Single Nucleotide Polymorphisms**
http://www.ncbi.nlm.nih.gov/SNP/
"A key aspect of research in genetics is associating sequence variations with heritable phenotypes. The most common variations are single nucleotide polymorphisms (SNPs), which occur approximately once every 100 to 300 bases. Because SNPs are expected to facilitate large-scale association genetics studies, there has recently been great interest in SNP discovery and detection."

"In collaboration with the National Human Genome Research Institute, The National Center for Biotechnology Information has established the dbSNP database to serve as a central repository for both single base nucleotide subsitutions and short deletion and insertion polymorphisms." – *Homepage cited*

♦ **Human SNP Database**
http://www-genome.wi.mit.edu/SNP/human/

♦ **STS-Based Map of the Human Genome**
http://www-genome.wi.mit.edu/cgi-bin/contig/phys_map Whitehead/MIT
"The genetic maps displayed at this site now reflect the final Genethon genetic linkage map of the genome published in Nature. The reference for the genetic map is: Dib, C., et al (1996). A comprehensive genetic map of the human genome based on 5,264 microsatellites. Nature, 380: 152-154." – *Homepage cited*

♦ **Transcript Map of the Human Genome**
http://www.ncbi.nlm.nih.gov/Science96/
A Gene Map of the Human Genome

♦ **Human Telomere Information**
http://www.genlink.wustl.edu/teldb/human.html

♦ **The Genetic Location Database (LDB)**
http://cedar.genetics.soton.ac.uk/public_html/
"The genetic location database (LDB) gives locations for expressed sequences and polymorphic markers. Locations are obtained by integrating data of different types (genetic linkage maps, radiation hybrid maps, physical maps, cytogenetic data and mouse homology) and constructing a single 'summary' map. The data are obtained from internet, published sources and from data and maps generated within the Wessex Human Genetics Institute. The component maps and all other data are available from this site." – *Homepage cited*

♦ **The dysmorphic human and mouse homology database**
http://www.hgmp.mrc.ac.uk/DHMHD/dysmorph.html
"This application consists of three separate databases of human and mouse malformation syndromes together with a database of mouse/human syntenic regions. The mouse and human malformation databases are linked together through the chromosome synteny database. The purpose of the system is to allow retrieval of syndromes according to detailed phenotypic descriptions and to be able to carry out homology searches for the purpose of gene mapping." – *Homepage cited*

♦ **BodyMap - Anatomical Expression Database of Human Genes**
http://www.imcb.osaka-u.ac.jp/bodymap/welcome.html

♦ **CEPH-Genethon integrated map**
http://www.cephb.fr/ceph-genethon-map.html

♦ **CEPH Genotype database**
http://www.cephb.fr/cephdb/
"The Centre d'Etude du Polymorphisme Humain (CEPH) maintains a database of genotypes for all genetic markers that have been typed in the reference families for linkage mapping of the human chromosomes (Genomics 6: 575-577, 1990; Science, 265: 2049-2054, 1994)." – *Homepage cited*

♦ **Cooperative Human Linkage Center (CHLC)**
http://lpg.nci.nih.gov/CHLC/
"The goal of the Cooperative Human Linkage Center was to develop statistically rigorous, high heterozygosity genetic maps of the human genome that are greatly enriched for the presence of easy-to-use PCR-formatted microsatellite markers." – *Homepage cited*

♦ **GeneMap '98 - The International RH Mapping Consortium Map**
http://www.ebi.ac.uk/RHdb/
"RHdb is a database of raw data used in constructing radiation hybrid maps. This includes STS data, scores, experimental conditions, and extensive cross references." – *Homepage cited*

♦ **dbEST Expressed Sequence Tag Database**
http://www.ncbi.nlm.nih.gov/dbEST/index.html

♦ **UniGene - Unique Human Gene Sequence Collection**
http://www.ncbi.nlm.nih.gov/Schuler/UniGene/

"UniGene is an experimental system for automatically partitioning GenBank sequences into a non-redundant set of gene-oriented clusters. Each UniGene cluster contains sequences that represent a unique gene, as well as related information such as the tissue types in which the gene has been expressed and map location." – *Homepage cited*

♦ **dbSTS Sequence Tagged Site Database**
http://www.ncbi.nlm.nih.gov/dbSTS/index.html
"dbSTS is an NCBI resource that contains sequence and mapping data on short genomic landmark sequences or Sequence Tagged Sites (Olson et al., 1989)." – *Homepage cited*

♦ **Whitehead Institute/MIT Genome Center**
http://www-genome.wi.mit.edu/
"This is a World-Wide Web server run by the Center for Genome Research at the Whitehead Institute for Biomedical Research in Cambridge, Massachusetts, USA. It contains information on map and sequence releases, software and people at the Genome Center." – *Homepage cited*

♦ **V BASE: A Directory of Human Immunoglobulin V Genes**
http://www.mrc-cpe.cam.ac.uk/imt-doc/public/INTRO.html
The database of human antibody genes

♦ **Human CpG Island database**
http://biomaster.uio.no/cpgisle.html
"CpG islands are short and dispersed regions of unmethylated DNA with a high frequency of CpG dinucleotides relative to the bulk genome. Although the cytosines in the CpG dinucleotides in mammalian DNA are highly methylated, a small fraction of the genome contains very stable methylated CpGs in all tissues." – *Homepage cited*

♦ **Human population genetics database (Genography)**
http://human.stanford.edu/

♦ **The IMGT/HLA Database**
http://www.anthonynolan.com/HIG/
"The IMGT/HLA Database is part of the international ImMunoGeneTics IMGT project and provides a specialist sequence databases for sequences of the human major histocompatibility (HLA). This includes all official sequences for the WHO HLA Nomenclature Committee For Factors of the HLA System." – *Homepage cited*

Other Databases

♦ **Terry Gaasterland's running list of genome sequencing projects**
http://www-fp.mcs.anl.gov/~gaasterland/genomes.html

♦ **Oligonucleotide Probe Database: OPD**
http://www.cme.msu.edu/OPD/
"OPD is a hyperlinked database of oligonucleotide sequences and associated experimental information which may be useful to scientists interested in using oligonucleotide probes in their research. "– *Homepage cited*

♦ **Flybase**
http://fly.ebi.ac.uk:7081/
A Database of the Drosophila Genome

♦ **SWISS-PROT**
http://www.expasy.ch/sprot/
"SWISS-PROT is a curated protein sequence database which strives to provide a high level of annotations (such as the description of the function of a protein, its domains structure, post-translational modifications, variants, etc), a minimal level of redundancy and high level of integration with other databases." – *Homepage cited*

♦ **Transcription Regulatory Regions Database**
http://www.bionet.nsc.ru/trrd/

♦ **TRANSFAC - The Transcription Factor Database**
http://transfac.bionet.nsc.ru/transfac/
The TRANSFAC database is free for non-commercial use.

Other Useful Links

♦ **Atlas of Genetics and Cytogenetics in Oncology and Haematology**
http://www.infobiogen.fr/services/chromcancer/

♦ **Gene Family Nomenclature**
http://www.gene.ucl.ac.uk/nomenclature/genefamily.shtml

Appendix D - PCR Product Suppliers

The following list is only a sample of the suppliers offering PCR products that you may need for your lab. Following this list are additional links to online sites which offer large databases of distributors that may be more appropriate for your particular needs.

Reagents & Kits

Amersham Pharmacia Biotech
Web Address: www.apbiotech.com
E-Address: apbcsus@am.apbiotech.com
Phone: 1-800-526-3593
Fax: 1-877-295-8102
Address: Amersham Pharmacia Biotech, Inc
800 Continental Avenue
Piscataway, NJ 08855-1327
USA

5 Prime ' 3 Prime, Inc
Web Address: www.5prime.com
E-Address: info@5prime.com
Phone: 1-800-533-5703
Fax: 1-303-440-0835
Address: 5 Prime ' 3 Prime, Inc.
5603 Arapahoe Ave
Boulder, CO 80303-1332
USA

Invitrogen
Web Address: www.invitrogen.com
E-Address: See **Web Address:** www.invitrogen.com/sales/index.html for the e-mail address of a local sales representative.
Phone: 1-800-955-6288
Fax: 1-760-603-7201
Address: Invitrogen Corporation
1600 Faraday Avenue
Carlsbad, CA 92008
USA

MBI Fermentas
Web Address: www.fermentas.com
E-Address: info@fermentas.com
Phone: 1-800-340-9026
Fax: 1-800-472-8322
Address: MBI Fermentas, Inc.
Suite 29
4240 Ridge Lea Road
Amherst, NY14226 USA

Promega
Web Address: www.promega.com
E-Address: litreq@promega.com
Phone: 1-800-356-9526
Fax: 1-800-356-1970
Address: Promega Corporation
2800 Woods Hollow Road
Madison, WI 53711-5399
USA

Thermal Cyclers

Denville Scientific
Call for Catalog
Phone: 1-800-453-0385
Fax: 1-908-757-7551
Address: Denville Scientifc, Inc.
PO Box 4588
Metuchen, NJ 08840-4588
USA

Eppendorf
Web Address: www.eppendorfsci.com
E-Address: eppendorf@eppendorfsci.com
Phone: 1-800-421-9988
Fax: 1-516-876-8599
Address: Eppendorf Scientific, Inc.
One Cantiague Road
Westbury, NY 11590-0207
USA

PE Applied Biosystems
Web Address: www.perkin-elmer.com/peaitran.nsf/index.html
E-Address: webmatser@perkin-elmer.com
Phone: 1-800-345-5224
Fax: 1-415-572-2743
Address: PE Applied Biosystems
850 Lincoln Centre Drive
Foster City, CA 94404
USA

Techne
Web Address: www.techneuk.com
E-Address: techneusa@worldnet.att.net
Phone: 1-800-225-9243
Fax: 1-609-987-8177
Address: Techne Incorporated
743 Alexander Road
Princeton, NJ 08540-6328
USA

Centrifuges and Shakers

Brinkman Laboratory Products
Web Address: www.brinkmann.com
E-Address: info@brinkmann.com
Phone: 1-800-645-3050
Fax: 1-516-334-7500
Address: Brinkman Instruments, Inc.
One Cantiague Road
PO Box1019
Westbury, NY 11590-0207
USA

Daigger®
Web Address: www.daigger.com
E-Address: diagger@diagger.com
Phone: 1-800-645-3050
Fax: 1-516-833476-7506
Address: A. Daigger & Company, Inc.
675 Heathrow Drive
Lincolnshire, IL 60069-4206
USA

Denville Scientific
Phone: 1-800-453-0385
Fax: 1-908-757-7551
Address: Denville Scientifc, Inc.
PO Box 4588
Metuchen, NJ 08840-4588
USA

Fiberlite Centrifuge, Inc
Web Address: www.piramoon.com
E-Address: mp@piramoon.com
Phone: 1-408-988-1103
Fax: 1-408-988-1196
Address: Fiberlite Centrifuge, Inc.
422-B Aldo Avenue
Santa Clara, CA 95054
USA

PGC Scientifics
Web Address: www.pgcscientifics.com
E-Address: pgcsi@juno.com
Phone: 1-800-424-3300
Fax: 1-800-626-1112
Address: PGC Scientifics
PO Box 7277
Gaithersburg, MD 20989-7277
USA

Plastics (including tubes, holding racks and well plates)

Axygen
Web Address: www.axygen.com
E-Address: info@axygen.com
Phone: 1-510-494-8900
Fax: 1-510-494-0700
Address: AxyGen Scientific, Inc.
33170 Central Avenue
Union City, CA 94587
USA

Becton Dickinson Labware
Web Address: www.bdl.bd.com/labware
E-Address: mail@cbpi.com
Phone: 1-888-237-2762
Fax: 1-800-847-2220
Address: Becton Dickinson Labware
1 Becton Drive

Franklin Lakes, NJ 07417-1886
USA

Continental Laboratory Products, Inc
Web Address: www.conlab.com
E-Address: mail@conlab.com
Phone: 1-800-456-7741
Fax: 1-619-549-7865
Address: Continental Laboratory Products, Inc.
9240 Mira Este Court
San Diego, CA 92126
USA

PGC Scientifics
Web Address: www.pgcscientifics.com
E-Address: pgcsi@juno.com
Phone: 1-800-424-3300
Fax: 1-800-626-1112
Address: PGC Scientifics
PO Box 7277
Gaithersburg, MD 20989-7277
USA

United Scientific Products, Inc.
Web Address: www.uspinc.com
E-Address: customerservice@uspinc.com
Phone: 1-800-382-3082
Fax: 1-510-382-0118
Address: United Scientific Products, Inc.
750 Whitney Street
San Leandro, CA 94577
USA

Pipettes and Tips

Brantech Scientific
Web Address: www.brandtech.com
E-Address: products@brandtech.com
Phone: 1-888-LAB-BRAND
Fax: 1-860-767-2563
Address: Brandtech Scientific
25 Middlesex Turnpike
Essex, CT 06426-1476
USA

Brinkman Laboratory Products
Web Address: www.brinkmann.com
E-Address: info@brinkmann.com
Phone: 1-800-645-3050
Fax: 1-516-334-7500
Address: Brinkman Instruments, Inc.
One Cantiague Road
PO Box1019
Westbury, NY 11590-0207
USA

Daigger®
Call for Catolog
Web Address: www.daigger.com
E-Address: diagger@diagger.com
Phone: 1-800-645-3050
Fax: 1-516-833476-7506
Address: A. Daigger & Company, Inc.
675 Heathrow Drive
Lincolnshire, IL 60069-4206
USA

PGC Scientifics
Web Address: www.pgcscientifics.com
E-Address: pgcsi@juno.com
Phone: 1-800-424-3300
Fax: 1-800-626-1112
Address: PGC Scientifics
PO Box 7277
Gaithersburg, MD 20989-7277
USA

Rainin
Web Address: www.rainin.com
E-Address: pipets@rainin.com
Phone: 1-800-4-RAININ (800-472-4646)
Fax: 1-510-652-8876
Address: Rainin Instrument Company, Inc.
5400 Hollis Street
Emeryville, CA 94608-2508
USA

Pipette Calibration

American Pipette Calibration
Web Address: www.webchester.com/apc/index.html
E-Address: apc@webchester.com
Phone: 1-888-405-6066
Address: American Pipette Calibration
17 Le Moyne Drive
Beaufort, SC 29902
USA

Continental Laboratory Products, Inc.
Web Address: www.conlab.com
E-Address: mail@conlab.com
Phone: 1-800-456-7741
Fax: 1-619-549-7865
Address: Continental Laboratory Products, Inc.
9240 Mira Este Court
San Diego, CA 92126
USA

Scientific Calibration
Web Address: www.scicial.com
E-Address: pipette@att.net
Phone: 1-800-331-7462
Address: Southern Instrument Maintenance Co
260 Hamlett Spring Road
Cave City, AR 72521-9805
USA

Southern Instrument Maintenance Co
Web Address: www.pipetterepair.com
E-Address: pipette@att.net
Phone: 1-800-331-7462
Address: Southern Instrument Maintenance Co
260 Hamlett Spring Road
Cave City, AR 72521-9805
USA

Zero Sigma
Web Address: home.navisoft.com/zerosigma/index.htm
E-Address: zerosigma@aol.com
Phone: 1-800-4-PIPETS
Fax: 1-800-747-4872

Gloves

Continental Laboratory Products, Inc.
Web Address: www.conlab.com
E-Address: mail@conlab.com
Phone: 1-800-456-7741
Fax: 1-619-549-7865
Address: Continental Laboratory Products, Inc.
9240 Mira Este Court
San Diego, CA 92126
USA

Daigger®
Web Address: www.daigger.com
E-Address: diagger@diagger.com
Phone: 1-800-645-3050
Fax: 1-516-834-7500
Address: A. Daigger & Company, Inc.
675 Heathrow Drive
Lincolnshire, IL 60069-4206
USA

Denville Scientific
Call for Catalog
Phone: 1-800-453-0385
Fax: 1-908-757-7551
Address: Denville Scientifc, Inc.
PO Box 4588
Metuchen, NJ 08840-4588
USA

Microflex
Web Address: www.life-assist.com/mflex/mflex.html
Phone: 1-800-876-6866
Fax: 1-800-876-6632
Address: Microflex
PO Box 32000
Reno, NV 89533-2000
USA

United Scientific Products, Inc.
Web Address: www.uspinc.com
E-Address: customerservice@uspinc.com
Phone: 1-800-382-3082
Fax: 1-510-382-0118
Address: United Scientific Products, Inc.
750 Whitney Street
San Leandro, CA 94577
USA

Gel Electrophoresis

Continental Laboratory Products, Inc.
Web Address: www.conlab.com
E-Address: mail@conlab.com
Phone: 1-800-456-7741
Fax: 1-619-549-7865
Address: Continental Laboratory Products, Inc.
9240 Mira Este Court
San Diego, CA 92126
USA

Daigger®
Web Address: www.daigger.com
E-Address: diagger@diagger.com
Phone: 1-800-645-3050
Fax: 1-516-834-7500
Address: A. Daigger & Company, Inc.
675 Heathrow Drive
Lincolnshire, IL 60069-4206
USA

Denville Scientific
Call for Catalog
Phone: 1-800-453-0385
Fax: 1-908-757-7551
Address: Denville Scientifc, Inc.
PO Box 4588
Metuchen, NJ 08840-4588
USA

PGC Scientifics
Web Address: www.pgcscientifics.com
E-Address: pgcsi@juno.com
Phone: 1-800-424-3300
Fax: 1-800-626-1112
Address: PGC Scientifics
PO Box 7277
Gaithersburg, MD 20989-7277
USA

Novex
Web Address: www.novex.com
E-Address: nvxmktg@novex.com
Phone: 1-800-403-5024
Address: NOVEX
11040 Roselle Street
San Diego, CA 92121
USA

Lab Safety

Continental Laboratory Products, Inc.
Web Address: www.conlab.com
E-Address: mail@conlab.com
Phone: 1-800-456-7741
Fax: 1-619-549-7865
Address: Continental Laboratory Products, Inc.
9240 Mira Este Court
San Diego, CA 92126
USA

Denville Scientific
Call for Catalog
Phone: 1-800-453-0385
Fax: 1-908-757-7551
Address: Denville Scientifc, Inc.
PO Box 4588
Metuchen, NJ 08840-4588
USA

Daigger®
Web Address: www.daigger.com
E-Address: diagger@diagger.com
Phone: 1-800-645-3050
Fax: 1-516-334-7500
Address: A. Daigger & Company, Inc.
675 Heathrow Drive
Lincolnshire, IL 60069-4206
USA

Lab Safety Supply
Web Address: www.labsafety.com
E-Address: webmaster@labsafety.com
Phone: 1-800-356-0783
Fax: 1-800-543-9910
Address: Lab Safety Supply Inc.
PO Box1368
Janesville WI 53547-1368
USA

Thomas Scientific
Web Address: www.thomassci.com
E-Address: value@thomassci.com
Phone: 1-800-345-2100
Fax: 1-609-467-3087
Address: Thomas Scientific
PO Box 99
Swedesboro, NJ 08085
USA

Labware (glassware, hotplates, titrators etc.)

Becton Dickinson Labware
Web Address: www.bdl.bd.com/labware
E-Address: mail@cbpi.com
Phone: 1-888-237-2762
Fax: 1-800-847-2220
Address: Becton Dickinson Labware
1 Becton Drive
Franklin Lakes, NJ 07417-1886
USA

Brinkman Laboratory Products
Web Address: www.brinkmann.com
E-Address: info@brinkmann.com
Phone: 1-800-645-3050
Fax: 1-516-334-7500
Address: Brinkman Instruments, Inc.
One Cantiague Road
PO Box1019
Westbury, NY 11590-0207
USA

Daigger®
Web Address: www.daigger.com
E-Address: diagger@diagger.com
Phone: 1-800-645-3050
Fax: 1-516-833476-7506
Address: A. Daigger & Company, Inc.
675 Heathrow Drive
Lincolnshire, IL 60069-4206
USA

Nalgeneâ Labware
Web Address: www.nalgenunc.com
E-Address: nnics@nalgenunc.com
Phone: 1-800-NALGE CS
Fax: 1-800-NALGENE
Address: Nalge Nunc International
PO Box 20365
Rochester, NY 14602-0365
USA

PGC Scientifics
Web Address: www.pgcscientific.com
E-Address: pgcsi@juno.com
Phone: 1-800-424-3300
Fax: 1-800-626-1112
Address: PGC Scientifics
PO Box 7277
Gaithersburg, MD 20989-7277
USA

Appendix E - Online Biosuppliers

Following are links to sites where you can locate and order online from a wide range of biological suppliers. The list of companies offering these services is growing and changing rapidly. Some information providers for the biosciences, such as BioSpace.com (www.biospace.com) and BioMedNet (www.biomednet.com) are adding these ecommerce services to their sites in addition to their other bioscience information resource offerings. The following is for your information only. We don't endorse any particular provider.

In alphabetical order

Anderson Unicom Group
http://www.atcg.com/
"The WebAlong, our comparative electronic catalog, lists detailed product information on over 625,000 LifeScience, MRO and Office Supply products from more than 2,500 suppliers." - *AUG Home Page*

BioSupplyNet
http://www.biosupplynet.com/
"The authority on where to find biomedical research supplies and more..." - *BioSupplynet Home Page*

Glen Research
http://www.glenres.com/
"Glen Research still offers far and away the widest selection of minor base phosphoramidites, modifiers and reagents for labelling oligonucleotides during the synthesis." - *Glen Research Web Site*

LabDeals.com
http://www.labdeals.com/
"Find great deals on surplus and discount scientific products and supplies." - *LabDeals.com Home Page*

BioMedical Products Online
http://www.bioprodmag.com/
"Tools and Techniques for Life Science Researchers"

Chemdex.com: Biological & Chemical Reagents
http://www.chemdex.com/
Chemdex Corporation is the leading provider of business-to-business e-commerce solutions for the life sciences industry. Only Chemdex offers a complete solution consisting of an extensive online marketplace, powerful purchasing functionality tailored to the unique business requirements of each customer, and comprehensive service and support. "Order reagents online from over 90 suppliers with the click of a mouse. Save time by ordering from your personal catalog and by using Chemdex's world class customer service." - *Chemdex.com Home Page*

Laboratory Network Auction
http://www2.laboratorynetwork.com/content/industrydeals/PageContent.asp
"Laboratory Network has created a unique forum for buying and selling new or surplus industrial products at competitive prices: The Online Auction!" - *Laboratory Network Home Page*

SciQuest
http://www.sciquest.com/
"Our comprehensive online catalog features thousands of products from hundreds of suppliers. We're continually adding more products, suppliers, and useful information to satisfy your purchasing needs. We're building SciQuest.com to be the first choice for all your laboratory product needs." - *SciQuest Homepage*

Appendix F – Contributors

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Dr. Laura Ruth received her bachelors degree in chemistry from the Massachusetts Institute of Technology (MIT), a Ph.D. in Biological Chemistry at the University of Pennsylvania, and completed an NIH Post-Doctoral Fellowship in crystalography and medical genetics at UCLA & Cedere-Sinai Medical Center. Her research experience includes projects at Syntex, Institute for Cancer & Developmental Biology; Stanford University, Departments of Neurobiology & Chemistry; and the Lawrence Berkeley Laboratory, Department of Molecular & Cell Biology.

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Editor
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Ms. Stewart is founder and CEO of Alkami Biosystems, Inc.

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