Introduction to PCR

The polymerase chain reaction (PCR), the repetitive bidirectional DNA synthesis via primer extension of a region of nucleic acid, is simple in design and can be applied in seemingly endless ways. Because PCR is so much more than just mixing reagents in a tube and running a machine, this section outlines the best ways to utilize the available laboratory space in which PCR procedures are set up and includes protocols on how to avoid PCR contamination. In addition to discussing where to perform clean PCR, this section also describes how to get started with a standard PCR protocol.

The components of a standard PCR protocol using *Taq* DNA polymerase are as follows:

10× Enzyme-specific reaction buffer:
- 10–50 mM Tris-HCl, pH 7.5–9.0
- 6–50 mM KCl or $(NH_4)_2SO_4$
- 1.5–5.0 mM $MgCl_2$ or $MgSO_4$

0.2 mM of each dATP, dGTP, dCTP, and dTTP
0.1–1.0 μM of each oligonucleotide primer
2.0–2.5 units of a thermostable DNA polymerase
Nucleic acid template, 10^2–10^5 copies
Distilled water to 100 μl

PCR amplification of a template requires two oligonucleotide primers, the four deoxynucleotide triphosphates (dNTPs), magnesium ions in molar excess of the dNTPs, and a thermostable DNA polymerase to perform DNA synthesis. The quantities of oligonucleotide primers, dNTPs, and magnesium may vary for each specific application. Because PCR tends to be an empirical technology, there are differences

in recommended conditions from protocol to protocol throughout this book. These recommended conditions may need to be optimized for different DNA sequences and oligonucleotide primers.

"A Standard PCR Protocol: Rapid Isolation of DNA and PCR Assay for β-Globin" provides an example of a basic DNA-PCR protocol, whereas "Specificity, Efficiency, and Fidelity of the PCR" addresses the effects of varying the components of the reaction mix on product yield and enzyme fidelity. Different parameters can be adjusted if the PCR yield is suboptimal; these parameters, as well as the possible addition of co-solvents, are discussed in "Optimization and Troubleshooting in PCR."

A common and easy protocol to improve product yield and increase specificity in the amplification is the hot start technique. There are a number of recommended methods for this, including uracil-*N*-glycosylase and dUTP, a wax barrier, a wax bead impregnated with magnesium, or an anti-*Taq* DNA polymerase monoclonal antibody. With all of these methods, after heating the reaction to 92°C for the first time, all the reaction components mix, and DNA synthesis occurs only from accurately hybridized primers. The use of hot start is detailed in "Setting Up a PCR Laboratory" and "Enzymatic Control of Carryover Contamination in PCR."

The commercially available thermostable DNA polymerases have different pH optima as well as different salt requirements. The recommended buffers, reverse transcriptase, and exonucleolytic activities, as well as the manufacturers of some of the more popular thermostable DNA polymerases, are shown in Table 1 (pages 4, 5).

An additional variable to consider is the final volume of the reaction. PCR requires rapid changes of temperature, which are accomplished by the thermal cycler. As a general rule, reactions are usually between 20 and 100 μl. Large-volume samples will be inefficiently heated and cooled, while small-volume reactions render insufficient product for manipulation and analysis.

Three distinct events must occur during a PCR cycle: (1) denaturation of the template, (2) primer annealing, and (3) DNA synthesis by a thermostable polymerase.

1. DNA denaturation occurs when the reaction is heated to 92–96°C. The time required to denature the DNA depends on its complexity, the geometry of the tube, the thermal cycler, and the volume of the reaction. For DNA sequences that have a high G+C content, the addition of glycerol, longer denaturation times, and the use of nucleotide analogs have been reported to improve the yield of the PCR.
2. After denaturation, the oligonucleotide primers hybridize to their complementary single-stranded target sequences. The temperature of this step varies from 37°C to 65°C, depending on the homology of the primers for the target sequence as well as the base composi-

tion of the oligonucleotides. Primers are present at a significantly greater concentration than the target DNA, and are shorter in length; as a result, they hybridize to their complementary sequences at an annealing rate several orders of magnitude faster than the target DNA duplex can reanneal.

3. The last step is the extension of the oligonucleotide primer by a thermostable polymerase. Traditionally, this portion of the cycle is carried out at 72°C. The time required to copy the template fully depends on the length of the PCR product. Depending on the PCR thermal cycler being used, it is feasible in certain circumstances to use two-step PCR rather than the traditional three steps as discussed in "Specificity, Efficiency, and Fidelity of the PCR."

The most serious issue with the widespread use of PCR is the contamination of reactions with target nucleic acids. This can occur during steps prior to the actual amplification reaction. "Enzymatic Control of Carryover Contamination in PCR" and "Ultraviolet Irradiation of Surfaces to Reduce PCR Contamination" provide specific protocols to reduce contamination, and "Setting Up a PCR Laboratory" discusses how to integrate these methods into good laboratory practices and improved laboratory design.

One of the significant breakthroughs in PCR is the ability to amplify DNA segments of up to 45 kb efficiently. This occurs through the use of a combination of two thermostable DNA polymerases, one with proofreading activity and one lacking this function. As discussed in "Long-distance PCR," the successful amplification of very long PCR products requires the optimization of buffer conditions as well as of the temperatures and length of each part of the cycle.

The quantity and quality of the starting material for amplification—the template nucleic acid—is of central importance in PCR. The size of the PCR product that can be amplified is dependent on both the reaction conditions and the template quality.

Sample preparation is discussed in detail in Section 2. One point that is occasionally overlooked when setting up a PCR is the quantity or copy number of target sequences added. The quantity of template in a reaction should be measured by the number of copies of the target sequence present, not by weight. The presence of excess template can prevent successful amplification.

The appropriate handling of both the template nucleic acid and the resulting PCR products is required to obtain meaningful data from a PCR. For this reason, we have stressed the importance of methods to control both sample- and PCR product-derived contamination. Without these precautions, and the methods to validate and assess reaction sensitivity and specificity that are detailed in this section, PCR can become more of a bane than a boon to your research effort.

Table 1 Thermostable Polymerases, Buffers, Activities, and Availability

Enzyme and supplier[a]	Exo activity[b]	Buffer/pH (RT)[c]	Salt	Divalent cation[c]	Additional additives[d]
Taq DNA polymerase[B,L,M,N,P,T] *Thermus aquaticus*	5	10 mM Tris-HCl, pH 8.3	50 mM KCl	1.5–5.0 mM $MgCl_2$	BSA, NP-40, Tween 20
Stoffel fragment[P] (Carboxy-terminal 544 amino acids of *Taq* DNA polymerase)	NO	10 mM Tris-HCl, pH 8.3	10 mM KCl	2.0–10.0 mM $MgCl_2$	BSA, Tween 20
UlTma DNA polymerase[P] *Thermotoga maritima*	5	10 mM Tris-HCl, pH 8.8	10 mM KCl	1.5–5.0 mM $MgCl_2$	Tween 20
Tth DNA polymerase[B,E,P,T] *Thermus thermophilus*	5	10 mM Tris-HCl, pH 8.3	90 mM KCl	1.0–2.0 mM $MnCl_2$, reverse transcriptase activity 2.0–4.0 mM $MgCl_2$, DNA synthesis EGTA used to chelate the Mn^{++} ion	glycerol, Tween 20 has potent RT activity with Mn^{++}
Pfu DNA polymerase[S] *Pyrococcus furiosus* (native)	3	20 mM Tris-HCl, pH 8.2	10 mM KCl 6 mM $(NH_4)_2SO_4$	1.5–2.5 mM $MgCl_2$	BSA, Triton X-100
Pfu DNA polymerase[S] (recombinant and Exo^- forms)	3 exo^- = NO	20 mM Tris-HCl, pH 7.5	–	8.0 mM $MgCl_2$	BSA

Vent, DeepVent[N] *Thermococcus litoralis, Pyrococcus GB-D*	5 $Exo^- =$ NO	20 mM Tris-HCl, pH 8.8	10 mM KCl 10 mM $(NH_4)_2SO_4$	1.5–5.0 mM $MgSO_4$	Triton X-100
Tli DNA polymerase[M] *Thermococcus litoralis*	5	10 mM Tris-HCl, pH 9.0	50 mM KCl	1.5–5.0 mM $MgCl_2$	Triton X-100
Hot Tub DNA polymerase[H] *Thermus ubiquitus*	NO	50 mM Tris-HCl, pH 9.0	20 mM $(NH_4)_2SO_4$	0.7–2.0 mM $MgCl_2$	
Tfl DNA polymerase[E,M] *Thermus flavus*	5	50 mM Tris-HCl, pH 9.0 or 20 mM Tris-acetate, pH 9.0	20 mM $(NH_4)_2SO_4$ 70 mM K-acetate[M]	1.5–5.0 mM $MgCl_2$ for DNA synthesis 1.5–5.0 mM $MnSO_4$ for reverse transcriptase activity	0.5% Tween 20, has potent RT activity with Mn^{++}
Pwo DNA polymerase[B] *Pyrococcus woesei*	3	10 mM Tris-HCl, pH 8.85	20 mM $(NH_4)_2SO_4$	1.5–4.0 mM $MgSO_4$	BSA, cannot use dUTP
Tbr DNA polymerase[A,F] *Thermus brockianus*	5	10 mM Tris-HCl, pH 8.8	50 mM KCl	1.5–5.0 mM $MgCl_2$	Triton X-100

[a]Name of enzyme and microorganism from which the enzyme was isolated or cloned. Included as a superscript are the suppliers. Complete information about suppliers is provided in the Appendix. [A]AmRESCO, [B]Boehringer Mannheim, [C]CLONTECH, [E]Epicentre Technologies, [F]Finnzymes OY, [H]Amersham, [L]Life Technologies, Inc., [N]New England Biolabs, [M]Promega Corp., [P]Perkin Elmer, Applied Biosystems Division, [S]Stratagene, [T]TaKaRa(PanVara).

[b]Exonuclease activity: 3′-5′ exo = 3, 5′-3′ exo = 5, no exonuclease activity = NO.

[c]The buffer, salt, and divalent cation conditions used for routine amplifications.

[d]Additional additives and comments about specific enzymes.

Setting Up a PCR Laboratory

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INTRODUCTION

Because of the nature of PCR, it is critical that the only DNA that enters the reaction is the template added by the investigator. Thus, PCR must be performed in a DNA-free, clean environment. The issue of contamination and the cleanliness required to perform contamination-free PCR have been compared to the good microbiological techniques used for handling pathogens (J. Sninsky, pers. comm.). The major difference here is that the "biohazard" infects the PCR, not the researcher. This chapter provides guidance for the establishment and maintenance of a clean environment for any PCR-based assay system, regardless of the number of samples being processed. These suggestions work best if implemented before a contamination problem occurs; if contamination is already a problem, strategies for handling this situation are also provided.

As the use of PCR grows in areas such as clinical diagnosis of genetic diseases (Wang et al. 1992) or the monitoring of viral burden in patients receiving antiretroviral therapy (Piatak et al. 1993), rational guidelines for installing a PCR facility and monitoring for contamination will need to be advanced. This discussion deals with the establishment of two types of laboratories—those performing contamination-sensitive PCR assays, such as measurement of quantities of target sequences in a sample, and those that are using PCR as a contamination-insensitive molecular biology tool, as in mutagenesis of a DNA clone.

The purpose of planning ahead in considering the design, location, and execution of PCR in discrete areas is to avoid contamination of the new PCR assays with old PCR products, molecular clones, or

sample-to-sample contamination. To date, four approaches have been devised to prevent contamination. The first is the physical separation of the individual parts of the PCR into sample preparation, pre-PCR, and post-PCR locations (Kwok and Higuchi 1989). This approach should be a central part of any contamination control strategy and can be scaled to suit the needs of the investigator. The physical separation of parts of the PCR process requires some additional space, money, and supplies to equip and maintain a larger infrastructure. However, these components alone are not foolproof, because good laboratory practice is still required for the prevention of sample-to-sample contamination. The second method, the use of uracil DNA-glycosylase (UNG) and deoxyuridine triphosphate (dUTP) substituted for thymidine triphosphate (dTTP), is effective only against contamination with dUTP-labeled PCR products (Longo et al. 1990). The third method, the use of UV light, is effective against all types of contamination. However, this approach is limited because it cannot destroy all of the contamination; UV light only reduces the contamination by several logs, and it is less effective if the DNA fragment is less than 300 bp (Sarkar and Sommer 1990, 1991). The final method is the derivatization of single- and double-stranded DNA with chemical adducts, such as isopsoralen. These adducts prevent the contaminating DNA from serving as a substrate in the reaction (Cimino et al. 1991). The implementation and use of UNG and UV contamination control systems are described in subsequent chapters (Hartley and Rashtchian; Cone and Fairfax) in this section. With these caveats in mind, we suggest the following guidelines be considered when establishing a PCR laboratory.

ESTABLISHMENT OF A PCR LABORATORY

To perform PCR for the repetitive detection of a specific sequence, three distinct areas are required. The specific technical operations and reagents for each one are detailed below. There is new interest in using PCR for the quantitative detection of target sequences, such as human immunodeficiency virus (HIV) (Piatak et al. 1993; Mulder et al. 1994). As the interest in quantitation of specific RNAs and the importance of measurements of viral burden by RNA-PCR grow, there is an increasing need for contamination-free, reliable PCR.

Sample Preparation Area

This room is specific for sample preparation only. The following special precautions should be taken in the preparation and handling of the reagents to be used in nucleic acid extraction:

1. PCR products or DNA clones containing the sequence to be amplified cannot be handled in this room.

2. Tissue cultures, tissue specimens, and serum samples are all brought into the sample preparation room and processed for the extraction of DNA or RNA, depending on the application.

3. Tools used in sample processing should not be used for general molecular cloning or manipulation of the target sequence.

4. DNA samples should be manipulated with specialized barrier or positive-displacement pipettes, which prevent the carryover of aerosols created during pipetting.

5. Large volumes should be pipetted with individually wrapped, sterile, disposable pipettes.

6. Aerosols should be minimized by briefly centrifuging the tubes prior to opening; also, tubes should not be popped open, which creates an aerosol.

7. Lab coats and gloves should be worn at all times, and gloves should be changed frequently, particularly between each step of the purification process. Lab coats should be dedicated to the sample preparation area and washed frequently.

The method chosen for the purification of template can have a significant impact on the risk of contamination. In general, the simpler the method that gives reliable results, the better, because less sample manipulation will be required. Always use freshly prepared or properly stored unused reagents and buffers for nucleic acid extraction. Do not use reagents that have been exposed previously to other samples.

If your laboratory or institution does not have the space for a specific region for sample preparation, consider making arrangements with colleagues to borrow space and the necessary supplies for sample preparation. This arrangement should only be made with laboratories that have never performed molecular cloning with any of the sequences you are interested in amplifying. Although other laboratories are surely contaminated with DNA, this is irrelevant if your primer sets will not amplify their molecular clones. This ad hoc approach can work for both the sample preparation and pre-PCR areas.

Sample Preparation and RNA-PCR

The extra steps associated with RNA-PCR require additional sample handling and, therefore, there is increased chance of sample-to-

sample contamination. To avoid this problem, the reverse transcription step can be performed in the sample preparation area. The use of UNG with RNA-PCR to prevent contamination has also been reported (Pang et al. 1992), and this method is described by Hartley and Rashtchian in a later chapter in this section. This approach is particularly valid if the reverse transcription is performed with random hexamer primers rather than with the specific antisense oligonucleotide primer. In this method, all of the RNA present in the sample is converted to cDNA with relatively equal efficiency. Because no single, specific product is being produced, it is less likely to become a source of contamination.

The reverse transcription reaction should be terminated by boiling the reaction, which kills the reverse transcriptase and denatures the RNA:DNA duplex. Alternatively, the RNA strand can be eliminated by RNase H treatment or base hydrolysis. For many applications, r*Tth* polymerase can be used as an alternative to traditional two-step reverse transcriptase and *Taq* polymerase (Myers and Gelfand 1991). The advantages of using r*Tth* are significant: (1) there is a single buffer system; (2) dUTP and UNG can be incorporated in the reverse transcription reaction; and (3) there is improved specificity of the reaction resulting from less mispriming because the cDNA synthesis reaction is performed at a higher temperature with r*Tth*. For laboratories performing large numbers of assays on RNA samples, the reduced handling and improved contamination control make the r*Tth* system an attractive alternative to the two-enzyme RNA-PCR systems.

Pre-PCR Area

An area devoted to the preparation of the individual reactions is essential. This area must be maintained clean and free of all sources of contamination from molecular cloning and sample preparation. Requirements for the pre-PCR area are reagents and equipment; specifically, positive-displacement pipettes that are dedicated to the pre-PCR area.

Each laboratory or department must make a decision as to whether or not synthesis of the primers and probes for its assays will be performed internally or prepared externally. If the synthesis capabilities exist internally, both the synthesis and the subsequent purification of the primers must take place in an area removed from post-PCR activities, sample preparation, and standard molecular biology. Any of the above-mentioned activities can lead to the inadvertent contamination of primers with DNA that will be impossible to remove and could cause spurious results. Again, the pipettors used for handling of primers should be dedicated to this purpose.

Handling of Reagents in the PCR Laboratory

Specific consideration should be given to the preparation and maintenance of clean PCR components.

1. All of the solutions used should be prepared free from contaminating nucleic acids and/or nucleases (both DNases and RNases). To ensure that the nucleic acid preparations routinely amplify properly, always use the highest-quality components for each solution. This should prevent problems due to the introduction of heavy metal ions, nucleases, or other unspecified contaminants. Gloves should be worn at all times when preparing reagents, handling samples, setting up reactions, and performing the subsequent detection of the amplified product.

2. The water used in all PCR reagents should be the highest quality—freshly distilled/deionized, filtered using a 0.22-micron filter, and autoclaved. We have found that USP-certified water that has been filtered and autoclaved is also sufficient for use in PCR. Contamination may also be avoided by using a new bottle for each set of experiments. Routine analysis of the water from the source tap should be performed to determine the conductivity of the water, as well as the possible contamination of the water supply with bacteria or fungi. Never assume that the supply is clean. Significant bacterial contamination has been detected even in house-distilled or -deionized water systems that employ UV sterilization. It is important to remember that bacteria, fungi, and algae grow in water storage systems (i.e., plastic water jugs); therefore, to minimize the chance of contamination of the water used for reagent preparation, always use freshly collected and processed water.

3. The addition of antimicrobials such as sodium azide is recommended for any reagents that will be stored from 20°C to 25°C. The inclusion of 0.025% sodium azide in the amplification reagents or sample preparation reagents does not inhibit the amplification reaction.

4. All reagents should be made up in large volumes. Test to determine if the reagent performs satisfactorily, and then aliquot the reagent into single-usage volumes for storage. Using aliquots of a proven reagent (stored under the appropriate conditions) establishes consistency from experiment to experiment.

5. Disposable, sterile bottles and tubes should be used for all reagents and sample preparation procedures. Glassware that is washed and autoclaved in a common-use laboratory kitchen is a potential source of contaminating nucleic acids. This is particularly true

when many molecular biology laboratories share a large centralized wash room.

6. Newly made reagents should be tested before they are used to prepare new specimens. If possible, try to keep aliquots of a few samples that can be used either to demonstrate a performance standard with a given set of reagents, or, if the system is prone to difficulties, to keep a representative problematic specimen to check reagent performance.

7. The pipettes used in sample preparation and pre-PCR should be carefully stored when not in use. Storage in airtight, self-sealing bags is an effective way to keep pipettes free from contamination.

Lab coats cannot leave the pre-PCR area. Also, the movement of personnel must be carefully thought out. When experiments are planned or in progress, researchers should not move at all from the "dirty" molecular biology rooms into the "clean" sample preparation and pre-PCR rooms. It is also best if a different investigator is responsible for the analysis of the results in the post-PCR area. If one individual is responsible for the entire assay, then that person should move unidirectionally from pre- to post-PCR.

Construction of PCR Mixtures in the Pre-PCR Area

The pre-PCR area should contain storage for the reagents needed for PCR.

1. Ready-to-use "master mix" solutions can be prepared, aliquoted, and stored at either −20°C or 4°C. (dNTPs at the lower concentrations used for PCR buffers are stable at −20°C for months.) These are useful if the laboratory is involved in the amplification of one or a few specific sequences. These master mix solutions have all but one of the necessary components for amplification to occur (i.e., no Mg^{++} or no enzyme). Either a 10X or 2X final concentration can be prepared and stored for easy, convenient use. This allows experiment-to-experiment consistency and removes a significant chance for the introduction of experimental error by miscalculation or a pipetting error when preparing complex reaction mixtures. The possibility for inadvertent contamination of the PCR mixture is eliminated if these reagents are aliquoted into single-use tubes using a clean pipettor, with or without wax sealing.

2. If your laboratory uses multiple primer sets so that construction of single-use reaction mixtures containing all the reagents is not cost-

effective, consider aliquoting and storing the individual components of the PCR in daily-use sizes. This method provides a degree of protection should an inadvertent contamination of one of the stock reagents occur. However, the master mix must be carefully constructed and aliquoted each time a PCR is performed. In this situation, you should consider the use of wax to seal in the reagents and to provide for a hot start.

3. As a rule, you should have a panel of negative, weak, and strong positive control samples to assay the efficiency and cleanliness of the sample preparation and pre-PCR processes. In addition, you may wish to validate the final sample preparation buffer by using a known weak positive to demonstrate that there is no inhibition of amplification.

4. The negative controls that are run alongside each set of samples should be constructed to assay for sample-to-sample contamination as well as for contamination with PCR products. The negative controls should include all reagents used except the input nucleic acid.

5. When positive controls are to be performed, the quantity of nucleic acid being manipulated should be minimized for two reasons. First, a limited number of copies of the target sequence (10^1–10^4) should be included in the reaction to serve as a valid control. Second, by minimizing the amount of DNA, there is less chance of contamination of other samples by DNA aerosol.

6. Because control reactions are essential, the properties of the control template should be considered. The control template should have identical amplification properties to the natural target. In the past, this has meant that many investigators used a cloned version of the natural template, making it very difficult to track contamination of PCR assays with the positive control. By constructing a control that is a different size from the natural target and yet maintains the amplification efficiency of the natural target, the problem with contamination with the positive control can be monitored.

Options for Contamination Control

A powerful enzymatic method for elimination of one form of contamination—the use of UNG—has been devised (Longo et al. 1990). This technique efficiently eliminates contamination arising from PCR products. An alternative method of contamination control is the use of UV light. This method does not completely eliminate the contamina-

tion problem, but reduces it by several orders of magnitude. However, UV can effectively control contamination arising from molecular clones that are present in the laboratory. Thus, UV serves as a prophylactic treatment of reagents if low-level contamination is a problem. Both of these approaches to contamination control should be considered. The integration of these procedures is described in this section.

If you are using PCR only in a contamination-insensitive manner, it is not critical to maintain sample preparation and pre-PCR areas. Often the substrate in the PCR consists of cloned sequences used at 10^4 to 10^6 copies per reaction. However, most laboratories often perform a mixture of contamination-sensitive and -insensitive PCR. Reagents found in all PCR procedures, such as nucleotides and *Taq* DNA polymerase, need to be aliquoted or purchased in sufficient quantities to ensure that there are adequate supplies for the contamination-sensitive reactions. Reagents can always be used from the pre-PCR area for contamination-insensitive reactions; however, once used in the standard molecular cloning lab, they must not be returned to the pre-PCR area. Reagents for PCR sequencing should also be stored separately from other PCR components.

Location of the PCR Machine

Where the PCR machine should reside seems like a trivial issue. However, if the machine is to be used for multiple applications, including ones that are contamination-insensitive, the machine should be located in a room where PCR products will be handled. This decision is a trade-off. You must consider the fact that standard hot start techniques (the addition of a key ingredient to each reaction at the start of the first cycle) cannot be performed on a PCR machine in a post-PCR area. The use of Ampliwax PCR Gems (Perkin-Elmer) to separate the reaction into two parts or the use of the monoclonal antibody to *Taq* polymerase (TaqStart antibody, CLONTECH) provides different systems for hot start without opening or manipulating the samples once they are placed in the machine. An alternative hot start method is the use of UNG as follows: After the standard UNG incubation, include a single 2-minute incubation at 50°C prior to the first denaturation cycle (Kinard and Spadoro, pers. comm.). This provides sufficient time for UNG to degrade all the mispriming events that have been initiated in the reaction.

Post-PCR Area

After the PCR assays have been performed, the samples need to be analyzed and the data interpreted. An area should be set aside specifically for the post-reaction manipulation of samples. It is imperative

that all reagents, disposables, and equipment used in post-PCR activities be dedicated solely to this purpose. Never use equipment or reagents from this area of the laboratory in any pre-PCR activity.

The major source of contamination seen in PCR laboratories is the DNA obtained as product from previous PCR procedures; this arises from the microaerosols that are generated during the pipetting and manipulation of the PCR samples. Although these aerosols cause no problems if they are confined to the post-PCR area, they can cause havoc if they travel to the pre-PCR area on a pair of gloves, in a pipettor, on an investigator, or on a lab coat. If the laboratory has opted not to use the UNG and dUTP system to control PCR contamination, it is extremely critical that there be no mixing of reagents and personnel between the post-PCR and the pre-PCR areas. Therefore, the traffic flow in the laboratory should be unidirectional, always moving from "clean" to "dirty."

Within the post-PCR area, the tools for analysis should be dedicated to post-PCR use. If the laboratory is using the 96-well ELISA format for analysis, the specialized equipment can be shared with protein-based or cell culture-based assays but not with anything involved in pre-PCR. If analysis is to be performed by gel electrophoresis, then be sure that all the necessary equipment is located in standard molecular biology areas where contamination-sensitive PCR will not be performed.

From a laboratory management perspective, the division of labor for research projects is critical. However, unless a laboratory is involved in the high-volume throughput of hundreds of samples per week, it is not possible to have laboratory personnel dedicated solely to working in the pre-PCR area. Thus, investigators should plan to perform the clean work first; then, when that is completed, move on to perform the standard molecular biology from which PCR contamination can arise. The establishment of "clean days" and "dirty days" can be considered. On clean days, sample preparation and pre-PCR experiments are performed, and no one in the lab can perform studies that are prone to generate contaminating aerosols, such as plasmid DNA isolation, manipulation of phage, or analysis of PCR products.

The Perspective of the Small Laboratory

For a laboratory that is studying the structure, function, and expression of a particular gene, the questions of use of PCR, planning for a pre-PCR area, and maintaining contamination-free areas require careful thought. If the laboratory is considering establishing a PCR assay to detect a sequence that has been manipulated extensively within the laboratory, several critical changes need to occur. First, new equipment must be purchased, primarily micropipettors and positive-

displacement devices that will be dedicated to a pre-PCR area. Next, sites where plasmid and phage clones of the sequence of interest have not been manipulated in the laboratory must be identified. Often a sample preparation/pre-PCR area can be as little as 3–4 linear feet of bench space, a single drawer, and less than one cubic foot of freezer (–20°C) space. Ideally, available space meeting these requirements should be on a separate floor from the home laboratory. If such space is not available during normal working hours, it is possible that the space is available early in the morning or in the evening. It is preferable to perform the sample preparation and pre-PCR procedures early in the day, before your colleagues unknowingly begin aerosolizing DNA all over the lab.

Anticipation of contamination, acknowledging that it can happen, and acting to prevent it are important first steps to trouble-free PCR. If contamination has not yet been seen in the PCR, there are two possibilities–it is not there or you have not looked hard enough.

Dr. John Sninsky has proposed that PCR contamination be considered as a form of infection. If standard sterile techniques that would be applied to tissue culture or microbiological manipulations are applied to PCR, then the risk of contamination is greatly reduced. Above all else, common sense should prevail.

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A Standard PCR Protocol: Rapid Isolation of DNA and PCR Assay for β-Globin

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INTRODUCTION

This chapter describes a standard PCR protocol starting from DNA. This protocol contains all the essentials of a basic PCR protocol: sample preparation, master mix for the PCR, appropriate positive and negative controls, and a reliable detection system. Each of these facets of PCR is dealt with in more detail throughout this book. This protocol describes a simple and effective method to extract the total DNA complement from peripheral blood mononuclear cells (PBMC). The copy number of β-globin DNA is determined by quantitative PCR and represents the number of cell equivalents in a specific DNA sample. This technique is useful for standardizing numbers of cell equivalents from sample to sample as well as from assay to assay.

REAGENTS

Proteinase K (GIBCO/BRL, cat. no. 5530UA): Resuspended at 10 mg/ml, made fresh, held on ice, and used within 10 minutes

DLB (detergent lysis buffer):
10 mM Tris-HCl, pH 7.5
2.5 mM $MgCl_2$
0.45% Triton X-100
0.45% Tween 20

PROTOCOL

Preparation of Cellular DNA

1. Aliquot 0.5–1.0 × 10^6 PBMC into sterile 1.5-ml microfuge tubes. This protocol assumes that some or all of the specimens are HIV-infected.

Note: HIV-infected cells are handled in a biohazard level 2+ (BL2+) facility in Class II Biosafety hoods.

2. Centrifuge tubes at 1500 rpm for 15 minutes at room temperature.

3. Carefully siphon off the supernatant using clean, disposable micropipette tips. Discard tips and supernatant into containers with a virucidal solution.

4. Resuspend the pellet and lyse the cells with 100 μl of DLB.

5. Add 1.2 μl of fresh proteinase K.

6. Cap tubes and spray their exteriors with 70% ethanol.

7. Remove the samples from the hood and place them in a 60°C water bath or heat block for 1 hour. If the water bath is used, the water level should only cover the volume of the lysate. DO NOT ALLOW THE TUBES TO SUBMERGE.

8. If the tubes contain biohazardous material, spray them down with 70% ethanol. It is now safe to remove the samples from the BL2+ facility, and the remainder of the work may be carried out on a BL2 bench top.

9. Transfer the tubes to a 95°C heat block for 15 minutes to neutralize the proteinase K.

10. Snap-cool the tubes in ice water and store them at −80°C until needed for the PCR.

REAGENTS

dNTPs, 25 mM (Promega, cat. no. U1240)

Taq DNA polymerase (Perkin-Elmer, cat. no. N801-0060)

Mineral oil (Perkin-Elmer, cat. no. 186-2302)
β-Globin primers, 20 μM
  Sense: GAA GAG CCA AGG ACA GGT AC
  Antisense: CAA CTT CAT CCA CGT TCA CC

Liquid hybridization probe:
  AAG TCA GGG CAG AGC CAT CTA TTG CTT ACA

10X PCR buffer (Perkin-Elmer, cat. no. N808-0006)

0.5-ml sterile PCR tubes (Perkin Reaction Tubes, cat. no. N801-0180)
β-Globin DNA dilution series: 5×10^4, 1×10^4, 5×10^3, 1×10^3, 5×10^2, 1×10^2 cell equivalents

PROTOCOL

PCR Assay for β-Globin DNA

1. In a pre-PCR area, assemble and label the PCR (0.5 ml) tubes and place them in a rack. Tubes to be labeled are: the assay blank (H_2O), clinical specimen DNAs, and β-globin DNA standard dilution series. Add 10 μl of sterile H_2O to the tube labeled as the assay blank.

2. Add 8 μl of H_2O and 2 μl of clinical specimen DNA to the tubes labeled as clinical specimens.

3. Add 10 μl of each dilution, working from the low concentration to the high concentration, to tubes appropriately labeled for β-globin DNA standard curve dilution series.

4. Determine the number of reaction tubes to be run and add two. Make a master mix by multiplying this number by the following volumes:

Component	Volume (μl/rxn)	Final concentration
Water	68.7	
10x Buffer	10.0	$[Mg^{++}]$ = 1.64 mM
β-Globin sense, 20 μM	5.0	1.0 μM
β-Globin antisense, 20 μM	5.0	1.0 μM
dNTPs, 25 mM each	0.8	200 μM each
Taq DNA polymerase, 5 units/μl	0.5 μl 90 μl/reaction + template = 100 μl	2.5 units/tube

5. Starting with the assay blank tube, continuing with the clinical specimen tubes, and finally with the β-globin DNA standard curve tubes, aliquot 90 μl of PCR master mix to each tube to bring the final volume of each reaction to 100 μl.

6. Add 50 μl of mineral oil to all tubes using a micropipettor and a disposable pipette tip. Change tips between each tube.

7. Vortex each tube and briefly centrifuge.

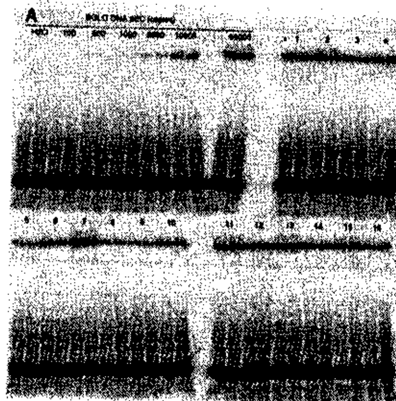
8. Place tubes into the preheated thermal cycler and begin the cycling according to the following schedule:

Denature	30 seconds at 95°C
Anneal	30 seconds at 50°C
Extend	3 minutes at 72°C
22 cycles	
Time delay	10 minutes at 72°C
Soak	10 minutes at 4°C (this can be left overnight)

9. Store samples at 4°C until used in the liquid hybridization assay, which should be performed no later than the next day, if possible.

ANALYSIS OF RESULTS

In this assay system, quantitation is achieved by direct comparison of unknowns with a standard curve. The β-globin control template consists of nucleotides −195 through +73 of the human β-globin gene listed in GenBank as HUMMBB5E. This sequence of approximately 278 bp is cloned into the *Sma* site of the vector pBluescript IIKS in the forward orientation (total length of the plasmid and insert is approxi-



B

DATA FROM PHOSPHOR-IMAGER

VOLUME = PHOSPHOR-UNITS

Background

ß-Globin RAW DATA TEMPLATE

B-Globin PEC	VOLUME	MEAN
H20 Background		0
Background		
100 copies	500	531
	562	
500 copies	2812	2884
	2955	
1,000 copies	4850	4850
	4850	
5,000 copies	12492	13174
	13855	
10,000 copies	45178	46038
	46897	
50,000 copies	155815	146632
	137449	

CODE #		VOLUME	MEAN
8/9/94	1	105346	109386
		113426	
	2	146588	144116
		141644	
	3	61711	62148
		62585	
	4	107010	116051
		125092	
	5	32798	38095
		43391	
	6	73812	74394
		74975	
	7	205660	206834
		208008	
	8	43339	43525
		43710	
8/10/94	1	42574	42918
		43262	
	2	84042	82149
		80255	
	3	148025	155284
		162543	
	4	102855	107775
		112694	
	5	149858	152772
		155685	
	6	94486	93726
		92966	
	7	108830	136474
		164117	
	8	69167	70705
		72243	

Figure 1 (*A*) Phosphorimage of a typical assay gel. (*B*) Raw data collected from the phosphorimager. The label pEC refers to the cloned assay standard. The numbered lanes are patient samples.

mately 3403 bp). The β-globin sense primer is a 20-nucleotide oligonucleotide located in the human genome at position −195 to −176. The antisense β-globin primer is a 20-nucleotide oligonucleotide located at position +53 to +73 and with the sense primer generates a 278-bp PCR product. The probe for the β-globin PCR product is complementary to the sequence positions −77 to −48 within the PCR product generated by the primers. The assay as described here ideally contains 20,000 cell equivalents, and thus the actual number of cell equivalents can be standardized for each sample.

The β-globin standard PCR products from this assay are analyzed using the liquid hybridization assay described in detail in the chapter by Vahey and Wong in Section 5. Briefly, the liquid hybridization is performed by mixing 30 μl of PCR product with 5×10^4 cpm of a ^{32}P-labeled oligonucleotide probe in hybridization buffer. The entire reaction mixture is heated and incubated for 5 minutes at 94°C and for 10 minutes at 55°C and then stored at 4°C.

Four microliters of loading buffer is added to 10 μl of sample and is applied to duplicate wells of an acrylamide minigel. Following electrophoresis to resolve the bound versus free probe, the gel is removed from the glass plates and exposed to a storage phosphor plate with subsequent analysis in a phosphorimager. See Figures 1 and 2.

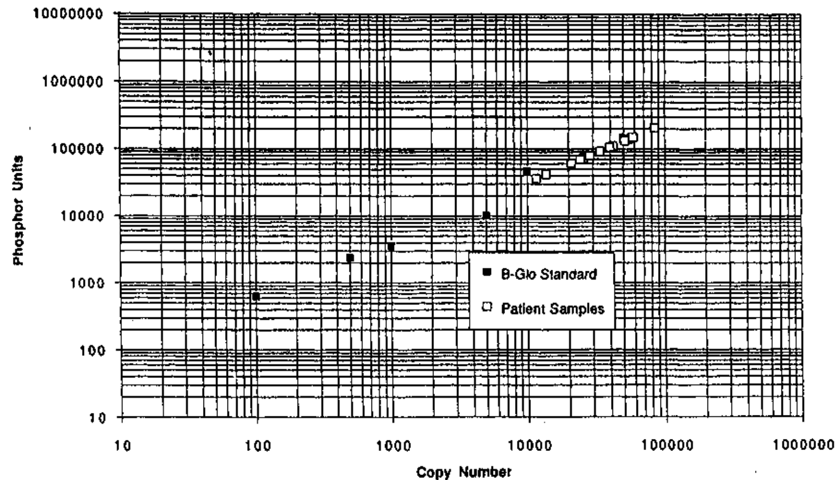


Figure 2 Plot of the standard curve and values of copy numbers (or cell equivalents) for the patient samples assayed.