1 Setting Up a PCR Laboratory

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Development of the polymerase chain reaction (PCR) as a basic component of the molecular biology laboratory has occurred very rapidly from its inception in 1985. Since then, more than 15,500 articles have been published in which this technique was used. (See Table 1 for additional information sources for PCR.) As PCR became more widely used, scientists rapidly learned more about it and, as a result, learned that the PCR had its strong points and its deficiencies. Very quickly, PCR demonstrated its power to amplify very small amounts (e.g., a single copy) of template nucleic acid and to amplify different nucleic acids (e.g., DNA and RNA). At the same time, laboratory personnel learned that this biochemical reaction had a unique deficiency; namely, a strong susceptibility to contamination from its own product. Early experience with the PCR soon showed that additional precautions were needed (Lo et al. 1988; Kwok and Higuchi 1989). This chapter is devoted to establishing a PCR laboratory whose operations will give reliable and contamination-free results.

CONTAMINATION ISSUES

PCR contamination remains an issue for laboratories performing forensic procedures and detection of infectious agents (Pellett et al. 1999; Scherczinger et al. 1999). There are a number of approaches to control of PCR contamination, and the degree of stringency that is required in a laboratory is often determined by the assay being performed.

TABLE 1. Listing of Internet Web sites and URLs for PCR information

#	Web site (URL)	Comment
1.	http://highveld.com/pcr.html	PCR Jump station (Web portal)
2.	http://www.dnalc.org/resources/BiologyAnimationLibrary.htm	Downloadable animated video of PCR (PC or MAC)
3.	http://ncbi.nlm.nih.gov	GenBank site for checking amplimer (primer) sequences
4.	http://www.mbpinc.com (then select "Tech Reports" for monograph)	PCR contamination monograph
5.	http://info.med.yale.edu/genetics/ward/tavi/PCR.html	Guide to multiplex PCR

Amplicon Aerosol

The single most important source of PCR product contamination is the generation of aerosols of PCR amplicons that is associated with the post-PCR analysis. Methods for eliminating this aerosol range from physical design of laboratories and use of specific pipettes to chemical and enzymatic approaches. The choice of method is often dependent on the frequency of amplification of a target amplicon and the relative amounts and concentrations of the amplicons created by the PCR.

Target Template Contaminants

In addition to post-PCR contamination, the target template itself can be a source of contamination. For example, DNA templates are typically more troublesome as contaminants because they are more stable than RNA targets. Detection of infectious agents typically demands the most stringent contamination efforts, whereas detection of other targets, such as those from inherited disease, may require less contamination control. Regardless of the template to be detected, good laboratory practices should be followed (Kwok and Higuchi 1989) (see details below).

Real-time PCR Systems

PCR systems exist that provide direct measurement of amplicon accumulation during the reaction. These real-time PCR systems offer an alternative approach to the traditional post-PCR analysis methods. From a contamination control perspective, the collection of data during the amplification reaction by using a fluorescence-based detection system eliminates the need to handle the sample. Thus, when these PCRs are completed, the detection and analysis are complete, the reaction tubes remain sealed, and there is no amplicon escape. Arranging the PCR laboratory to perform these homogeneous (or real-time) PCRs requires a different approach, which is addressed later in this chapter. Because the major use of PCR as a laboratory tool still depends on a separate post-amplification manipulation, this approach is the one primarily addressed.

CONTAMINATION PREVENTION APPROACHES IN THE PCR LABORATORY

The PCR laboratory typically is involved with activities that include sample preparation, PCR reaction assembly, PCR execution, and post-PCR analysis. These activities are summarized in Figure 1. When arranged in this linear fashion, these activities can be collected into two major groups, the pre-PCR activities (sample preparation and PCR preparation) and the post-PCR activities (PCR execution and analysis).

Use of the PCR for research and diagnostic purposes requires that some additional procedural limitations be observed so that the reaction yields valid results. As awareness of the PCR's susceptibility to contamination became known, Kwok and Higuchi (1989) presented some additional guidelines for researchers using the PCR. Consistently observing these guidelines is essential for successfully operating a PCR laboratory on a long-term basis. They form part of a network of protocols focused on maintaining a PCR laboratory in a contamination-free condition.

Contamination can arise from several different sources, such as previous amplification and purification of plasmid clones, repeated isolation of template (genomic) nucleic acids, and previously amplified molecules ("amplicons"). Although most attention in a PCR lab-

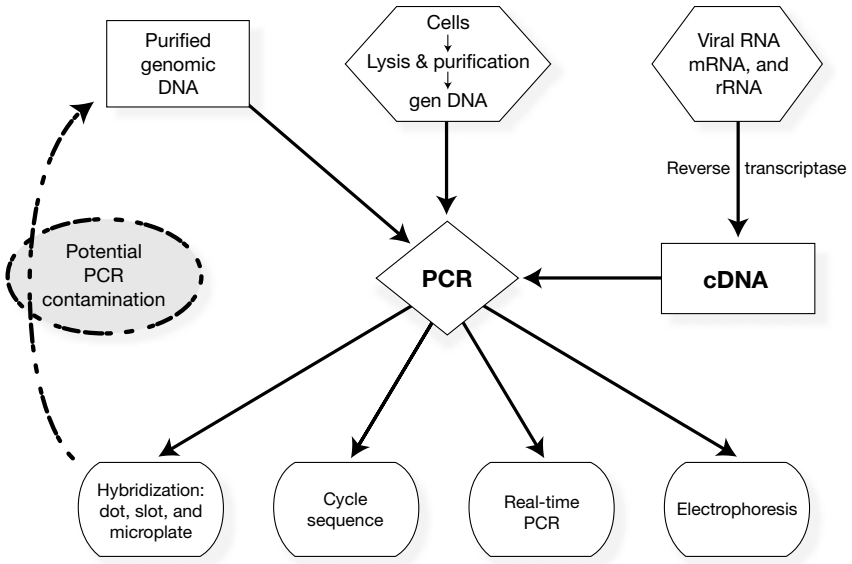

FIGURE 1. Outline of sample processing and analysis in a PCR laboratory.

oratory is focused on the last category of contamination, the other two sources should not be ignored. A prudent approach to controlling their contamination is to segregate the more standard recombinant DNA activities into separate areas of the laboratory and, in particular, to limit the performance of PCR activities to its own area.

For PCR amplicon contamination, it is the control and removal of the PCR amplicons that form the basis for the contamination control program. When PCR is used in research laboratories, either a greater variety of templates or amplifications of a specific template will be studied or manipulated and, thus, controlling amplicon contamination may be less challenging. In a diagnostic laboratory, there can be more opportunities for PCR contamination due to the repeated analysis of selected templates and the fact that PCR assays may be performed at or near the detection limit of PCR. The last possibility is especially demanding and thus requires a much more rigorous approach to controlling PCR contamination.

The essential parts of this contamination control program include space and time separation of pre- and post-PCR activities, use of physical aids, use of ultraviolet (UV) light, use of aliquoted PCR reagents, incorporation of numerous positive and negative or blank PCRs (H_2O substituted for template), and use of one or more various contamination control methods that use chemical and biochemical reactions. The underlying theme in these actions is the recognition that amplicon contamination cannot be seen, felt, or a priori detected before it happens. Use of consistent, careful technique coupled with liberal incorporation and monitoring of PCR blanks will ensure a vigilant, proactive approach to PCR contamination.

Space and Time Separation

As illustrated in Figure 1, the main source of the feedback contamination is the amplicons generated by the previous PCR. By separating the source of the amplicons' (e.g., post-PCR) activities from the pre-PCR activities, the potential for contamination is significantly reduced. This separation is best illustrated by separating the facilities in space, so that there are two rooms where these activities occur (Fig. 2). If this is not achievable, different areas designated for sample preparation and PCR setup can be located away from the area for

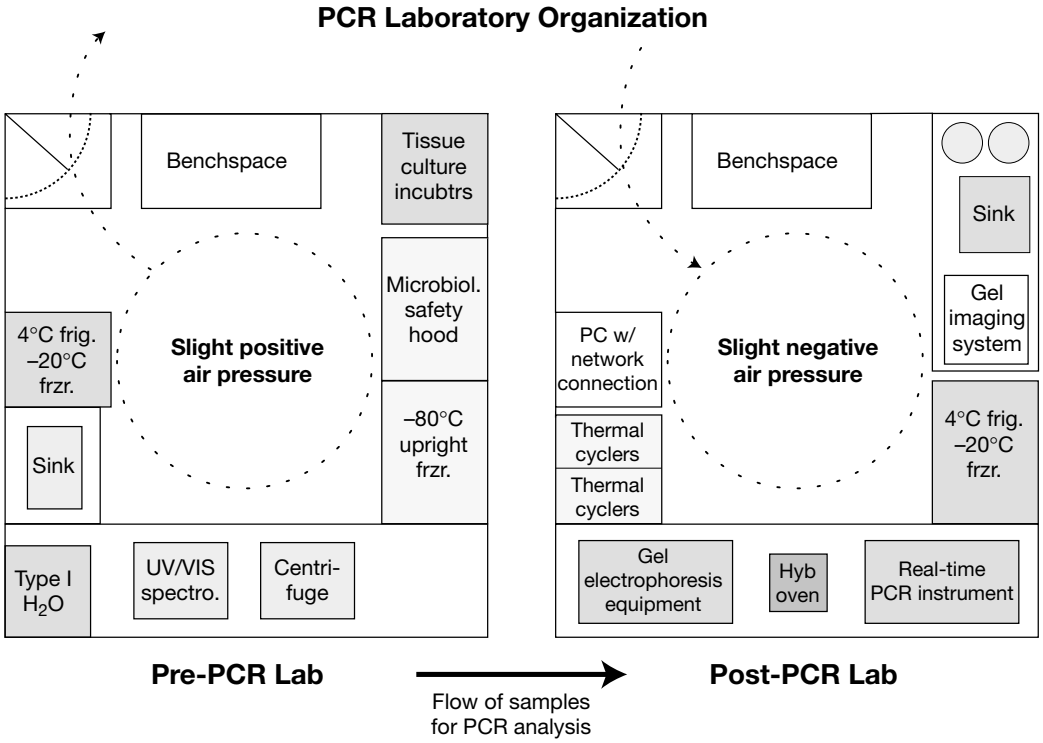

FIGURE 2. Organization of a PCR laboratory with separate pre- and post-PCR rooms.

post-PCR analysis. If all activities are to be performed in a single room, sample preparation should occur inside a laminar flow hood, preferably equipped with a UV light. The walls of the hood should be wiped with a fresh (or freshly made) 10% bleach solution (1 part regular bleach: 9 parts water) before processing samples or preparing PCR samples. Waste materials that contain PCR amplicons should not be allowed to accumulate in an area that is also frequented by other personnel who may be eventually involved with template isolation and purification. Additionally, the laboratory should consider establishing a daily schedule for performing PCR. Sample preparation and pre-PCR should be morning activities. Once completed, the pre-PCR supplies and equipment can be stored, and the afternoon can be devoted to the post-PCR analysis.

Laboratory Space Arrangement

As mentioned above, the ideal arrangement of the PCR facility is to have the pre- and post-PCR areas located in separate rooms (see Fig. 2), each with dedicated resources. A source of deionized water needs to be present in both rooms, as well as dedicated centrifuges, storage freezers/refrigerators, and storage of supplies. Even telephones, computers, and other electronic communications should also be dedicated.

Rarely is laboratory space allocated strictly for PCR; it often must be shared with other procedures. In a research laboratory, cell culture and other biochemical protocols such as analysis for enzymes and other biochemical species, protein and nucleic acid isolation, cloning, transfection, and purification may also occur. Thus, PCR protocols may likely be integrated into the laboratory's operation with consequent need to share facilities and bench space.

In the case of the diagnostic laboratory, a much more limited menu of activities occurs that is focused primarily on sample isolation and processing, although other possible activities such as cloning or sequencing can happen as well. There is usually a greater demand to detect and quantify low amounts of template in biological samples in the diagnostic laboratory, and this places a greater need on preventing amplicon contamination proactively.

A research institution may be unable to have separate facilities unless an arrangement can be developed for a "core" PCR setup facility, with each laboratory performing its own PCR and subsequent amplification. Where there is a requirement for detection of very low amounts of template nucleic acid (DNA or RNA), separate facilities offer the greatest likelihood that contamination-free results can be obtained over a long time frame.

An alternative to two-facility arrangements is found in real-time PCR methods. Because the results of each PCR are provided throughout the reaction, there is no need to open the PCR containers, and these can be discarded while still sealed. At least one instrument manufacturer is developing a completely self-contained automated system (the AmpliPrep: Taqman from Roche Molecular Systems, Alameda, CA) that would eliminate the need for two separate rooms. However, there may be situations where the contents of individual tubes and capillaries created during real-time PCR analysis need to be analyzed independently, and so these containers would need to be opened. This should happen in a different room from where the real-time PCRs were set up and performed.

Equipment in PCR Laboratories

To ensure that pre-PCR and post-PCR events remain separated, each room must have its own separate set of equipment, including pipettors, reagents, pipettor tips, racks, and so forth. Moreover, these items should not leave the area to which they are assigned. Each should be labeled as to location and used in that location only. Lab coats should be dedicated for both areas as well. Because pipetting forms the basis for most PCR analysis, each area needs its own dedicated pipettors that are never exchanged between work areas. To assist with this, color-coded pipettors (e.g., green for pre-PCR work, red for post-PCR work) can be used. When pre-PCR pipettors and tips are not in use, they should be stored in airtight bags to keep them clean. Reactions should be constructed using master mixes, and the template should always be added last using positive displacement tips to prevent pipettors from becoming cross-contaminated while pipetting samples that contain template. These types of pipettors and tips are available from several sources and can be purchased in sterilized packs. It is important to remember that barrier tips cannot be autoclaved.

Pre-PCR Activities

The definition of pre-PCR is the protocols and equipment required for the isolation of nucleic acid and the assembly of the reaction to amplify the samples. During the last 10 years, there has been much progress in developing devices that perform these activities in an automated fashion. Most PCR laboratories still perform these tasks using manual procedures.

What is the minimum needed to equip a PCR laboratory for sample preparation, PCR reagent preparation, and PCR assay setup? Because most of the activities revolve around pipetting of liquids, these activities should be examined most closely; in particular, the manual pipettors and pipette tips. As discussed previously, positive displacement tips or barrier methods should be used to pipette the template into the reactions as the last step.

There is a risk of creating aerosols in the preparation for RNA and DNA templates. If a large number of specimens of one type are processed on a routine basis, the laboratory may

wish to treat this method with care and perform it in a hood or biosafety cabinet (Fig. 2). Because of the effectiveness of ultraviolet light (UV) for amplicon control, use of UV inside the cabinet prior to sample preparation or PCR reagent preparation is advisable. Alternatively, any one of a number of small, benchtop-size cabinets that use UV irradiation can also be utilized. These are dedicated to PCR use and are large enough to contain several pipettors, racks, and some reagents.

Environmental Considerations

- *Air handling.* For extremely high-performance PCR laboratories that will be involved with detecting very-low-prevalence DNA or RNA molecules (e.g., infectious disease agents in clinical samples), additional measures may be necessary to prevent contamination from the air being recirculated between the pre- and post-PCR laboratories. In this case, the air handlers need to be separate and the air pressure individually adjusted in each laboratory. In the pre-PCR laboratory, there should be a slight positive pressure compared to the air in the connecting hallway. The post-PCR laboratory, in contrast, should be at slightly reduced pressure to pull air in from the outside and thereby prevent escape of amplicons from the completed PCR samples being analyzed inside the lab (Fig. 2). Finally, the air handlers for the pre- and post-PCR laboratories need to be connected to separate air ducts, and each must lead to a separate location for exhaust.
- *UV irradiation.* It is possible to exploit further the sensitivity of nucleic acid to UV by using UV to sterilize the entire pre-PCR laboratory. This can be done by having UV lights placed in the ceiling fixtures and connecting their activation to a lock-out mechanism on the exit door so they only illuminate when the last person in the lab closes and locks the external lab door. If this type of hardware is installed, it must be accompanied by a ventilation system to eliminate the UV-generated ozone and a rigidly enforced schedule of monitoring the performance of the UV bulbs. These light fixtures accumulate a residue arising from the precipitation of oxidation products on the glass of the bulb. If this is not removed monthly, the UV system is not effective.
- *Protective clothing.* To further prevent PCR amplicons from leaving the post-PCR lab, each investigator should have a dedicated post-PCR lab coat. Additionally, each investigator should have a general molecular biology lab coat and a separate coat for pre-PCR. In extreme cases, a disposable gown and booties should be worn.
- *Adhesive paper at lab entrances.* This approach effectively prevents trace amounts of dust and debris from entering the laboratory. It is a rather expensive approach to controlling contamination, but may be worth the expense for selected applications.

Sterilization of Reagents

Because PCR laboratories perform some molecular biology methods that require sterile reagents, some may need to be autoclaved. The single most critical reagent is water. Sterile USP water can be quickly converted to PCR water by filtering it through two 0.45-micron nitrocellulose filters. These filters have a very high binding capacity for nucleic acid and proteins. If the laboratory is involved in amplification of very small quantities of bacterial DNA, the USP water should be autoclaved separately from all other reagents before filtration. In general, reagents and solid items destined for the pre-PCR lab should be autoclaved separately from other supplies. It is important to note that spent tissue culture fluids, bac-

terial culture supernatants, bacterial media plates with recombinant cultures or plasmids, and samples from the post-PCR lab represent a large potential reservoir of contaminating DNA and should also be autoclaved separately from any material that will enter the pre-PCR lab.

Contamination Control

As mentioned earlier, a variety of approaches can be used to control PCR amplicon contamination. They can be grouped into two broad methods: (1) methods that use physical means to prevent dispersion of PCR amplicons and (2) methods that exploit some type of chemical reaction to render the amplicons incapable of serving as templates in a new round of PCR. Each of these has a place in the PCR laboratory, and most successful PCR laboratories use a spectrum of these methods to effectively control contamination.

Physical Methods

This category includes the physical barrier approaches. The most popular is the use of either positive displacement or barrier pipette tips to prevent aerosols. These barrier methods prevent the reintroduction of small amounts of a contaminating aerosolized sample into the next sample that is pipetted (Fig. 3). Use of these tips is generally recommended in the pre-PCR areas of the laboratory where samples are being processed and template nucleic acids (DNA and RNA) are being isolated and purified. Use of these tips is necessary and cost-effective in the post-PCR laboratory because there is already a large amount of amplicon present.

An adjunct to these tips is the use of a laminar flow hood or biological safety cabinet to facilitate preparation of PCR samples and reagents. When they are prepared in such an enclosure, there is much less chance of an external source of PCR amplicon contaminating the samples and reagents being manipulated for the subsequent PCRs.

Chemical Methods

A number of chemical approaches have been developed during the last 20 years. However, only a few have seen any real success in becoming routine in their use and application for controlling PCR contamination.

- *UV photolinking.* This approach can be used in both a pre-PCR and post-PCR setting. The basis for this reaction is that adjacent pyrimidines on a DNA strand can be cross-linked when exposed to UV light of 254 nm (Gordon and Haseltine 1982). The reaction is very fast and can be effective for bigger amplicons; i.e., those greater than 700 bp. Smaller amplicons are harder to inactivate because there are fewer adjacent pyrimidines. There is also a role for interstrand cross-linking in UV inactivation. Once cross-linked, the pyrimidine dimers cannot be excised and so the DNA polymerase is sterically blocked, or the DNA cannot completely denature, and the synthesis reaction is effectively halted.

  UV photolinking is most often used in a pre-PCR setting in which the equipment is installed in a small tabletop cabinet that is used for sample preparation. All of the items to create a PCR are placed inside the cabinet and then illuminated before the PCRs are assembled. There are some caveats with this approach, however, that are worth mentioning. First, there is a safety concern about exposure to UV light, and this must be addressed. Second, the photoreaction favors thymidine over cytidine by about 10:1.

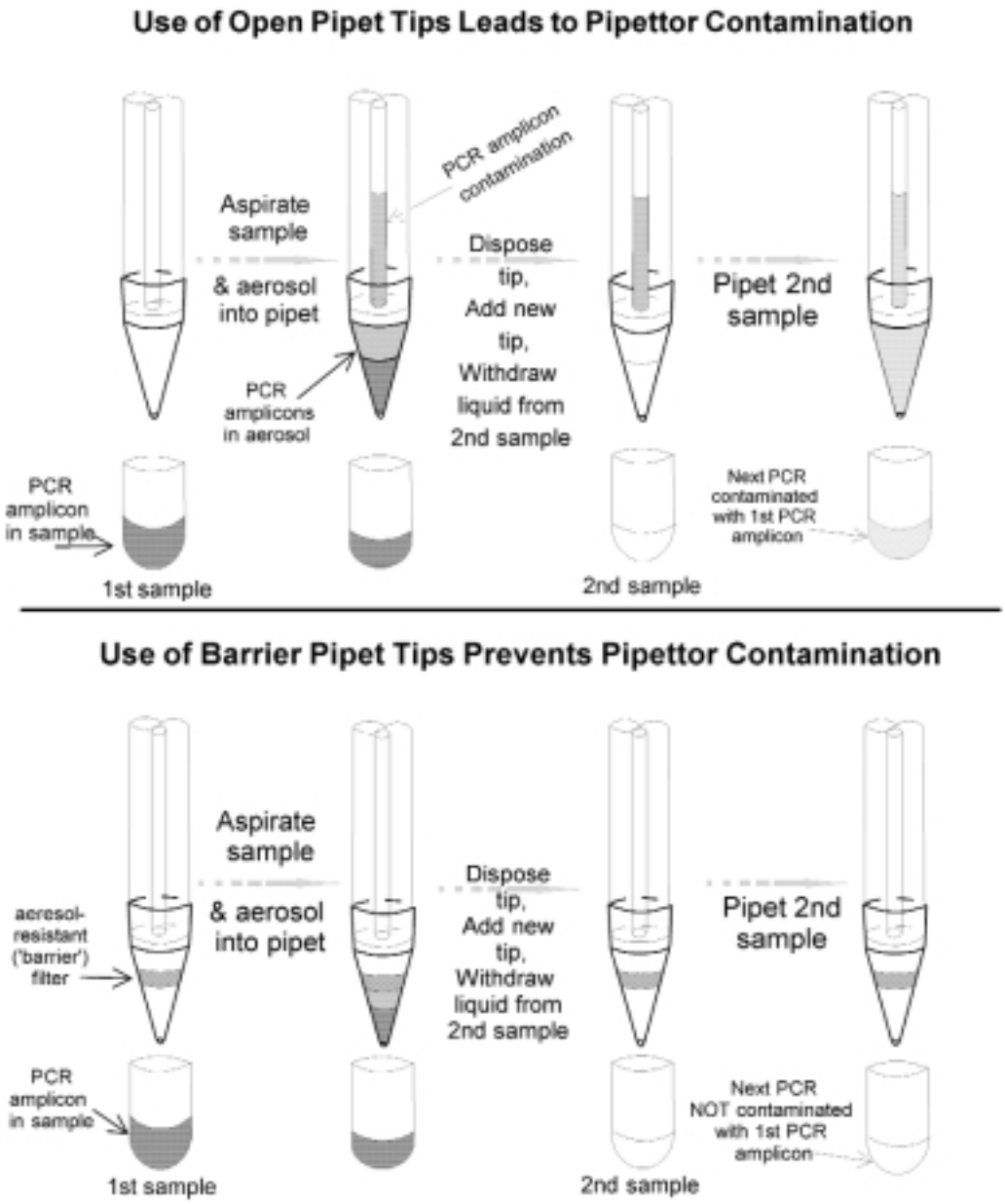

FIGURE 3. Use of barrier tips to prevent amplicon contamination in the PCR laboratory.

Therefore, amplicons that are AT-rich are more efficiently disabled than AT-poor sequences. Third, decreasing length of the amplicon usually gives a lower rate of protection; therefore, short amplicons are not well controlled. The approach is nevertheless effective and should be used when possible.

- *Uracil-DNA-glycosylase.* This enzyme (also known as UDG) is very effective at destroying PCR amplicons when vigorously used for sample preparation (Longo et al. 1990; Thornton et al. 1992). The method is so important in contamination control that it is covered separately in Chapter 2. Briefly, at the pre-PCR step, dTTP is substituted with

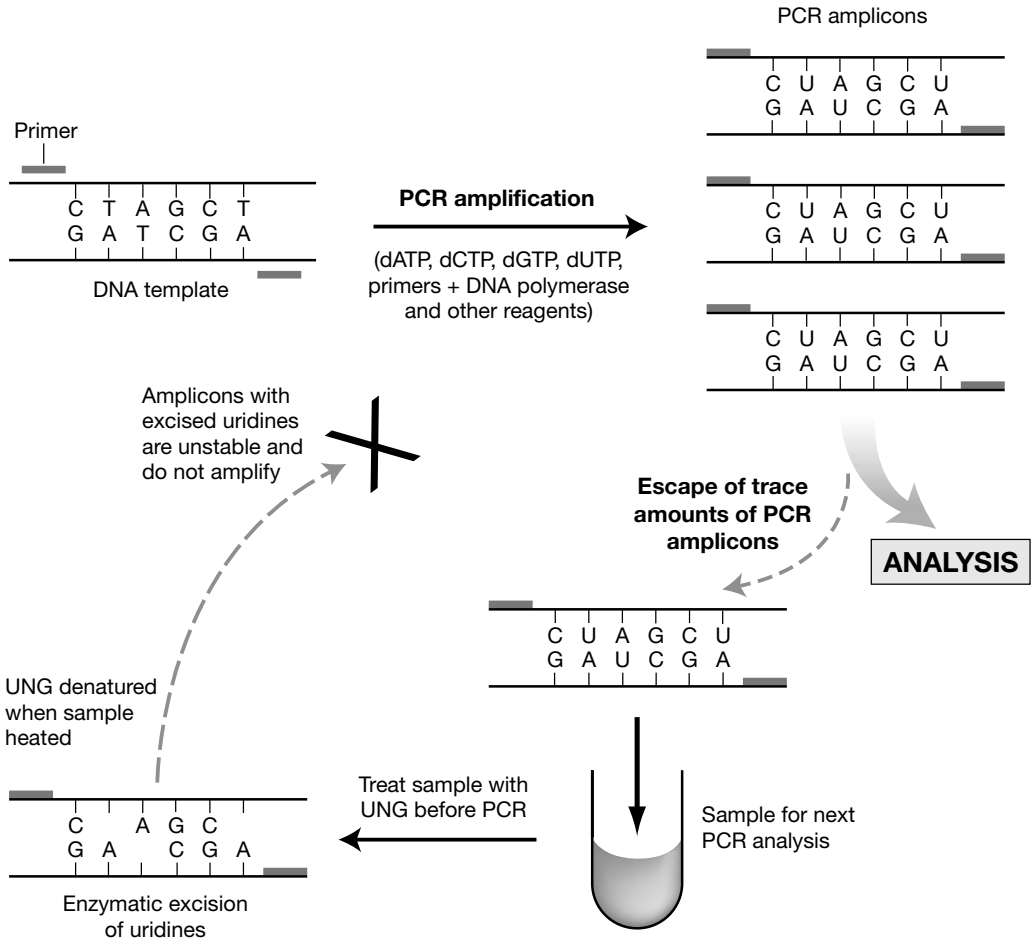

FIGURE 4. Use of the UDG reaction to prevent PCR contamination.

dUTP, and UDG is included in the reaction mix. All other reaction components remain the same. During the PCR, DNA polymerase substitutes dU for dT in the growing DNA strand. In the final product, there is now dU instead of dT in the DNA sequence. Before any new sample is processed, it first is exposed to UDG enzyme. If UDG comes across any U-containing DNA strands, the U's are cleaved, leaving the strand with gaps. Following heating in the next PCR, the abasic strands fall apart and cannot be amplified. The use of UDG provides the added advantage of a hot start by degrading all PCR products made prior to the first full cycle. This is discussed in Chapters 2, 3, 4, and 16.

Most PCR laboratories use one or more of these reactions in conjunction with laboratory operation and design to control PCR amplicon contamination effectively (Newton 1995). Selection of the best combination of these techniques is driven by factors such as types of templates, their prevalence, and others, as discussed earlier in this chapter.

PATENTS AND LICENSES

The use of PCR is covered by U.S. patents that were issued in the mid-1980s (Mullis 1987; Mullis et al. 1987). If results from PCR are to be used for diagnostic purposes and/or a fee

for service will be arranged, then an additional license(s) will need to be negotiated for the use of the PCR with Roche Molecular Systems (Alameda, CA). This arrangement may also be necessary for other in vitro amplification reactions such as nucleic acid sequence-based amplification (NASBA; BioMerieux, Durham, NC), transcription-mediated amplification (TMA; GenProbe, San Diego, CA), and strand displacement amplification (SDA; Becton Dickinson, Franklin Lakes, NJ) and their corresponding licensing organizations.

Not only are licenses required for the use of the amplification reaction(s), but there are also some PCR-based diagnostic tests for specific genes that are patented and therefore require an additional use license to be negotiated. In each case, laboratory personnel should clarify the license issue(s) prior to performing and offering test results.

SUMMARY

This chapter has provided a brief overview of essential items that need to be considered when creating a PCR laboratory from the "bare walls." Many times, some of the needed resources are already present, making the conversion process easier. However, it should still be recognized that a PCR laboratory has its own unique requirements, and those should be carefully considered when evaluating what is needed to start and operate this kind of laboratory successfully.

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