

## Setting Up a Polymerase Chain Reaction Laboratory

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### Summary

One of the most important attributes of the polymerase chain reaction (PCR) is its exquisite sensitivity. However, the high sensitivity of PCR also renders it prone to false-positive results because of, for example, exogenous contamination. Good laboratory practice and specific anti-contamination strategies are essential to minimize the chance of contamination. Some of these strategies, for example, physical separation of the areas for the handling samples and PCR products, may need to be taken into consideration during the establishment of a laboratory. In this chapter, different strategies for the detection, avoidance, and elimination of PCR contamination will be discussed.

**Key Words:** False-positive PCR; anti-contamination strategies.

### 1. Introduction

One of the most important attributes of the polymerase chain reaction (PCR) is its exquisite sensitivity. However, this high sensitivity has also given PCR its main weakness, namely, its tendency to produce false-positive results owing to exogenous contamination (1,2). Contamination avoidance is therefore the single most important consideration when setting up a PCR laboratory (3), especially one designed to generate diagnostic information (4–7). In many situations, precautions that are normally taken in the handling of microbiological materials are equally applicable to PCR-related procedures (7).

### 2. Sources of Contamination

There are four main sources of PCR contamination. The most important one is PCR products from previous amplifications, the so-called carryover contamination (3). Because of the enormous amplification power of PCR and its ability to generate up to  $10^{12}$  product molecules in a single reaction, this is the

most serious source of contamination. When such large amounts of PCR products are generated repeatedly over a period of time, the potential for contamination becomes increasingly high. This is further compounded by the fact that many diagnostic applications require PCR to perform at its highest sensitivity, namely, at the single-molecule level. Under these circumstances, even one of the billions of molecules generated from a single reaction is enough to generate a false-positive result. The second source of contamination is cloned DNA previously handled in the laboratory. The third type is sample-to-sample contamination. This source of contamination is most detrimental to samples that require extensive processing prior to amplification. The fourth source is the ubiquitously present template DNA in the environment from the laboratory personnel and reagents used for DNA extraction and PCR (8–10).

### 3. Principles of Contamination Avoidance

Like many problems, avoidance is better than cure, and PCR contamination is no exception. The main principles of contamination avoidance in PCR are:

1. Strict physical separation of individual PCR-related maneuvers: we recommend the use of three distinct areas for the sample preparation stage, the PCR setup stage, and the post-PCR stage. This applies as much to the performance of laboratory procedure as to equipment. Thus, every piece of equipment, no matter how small, should be restricted to each area. This applies to laboratory notebooks, which should not be carried between different areas. If transfer of items is essential, then the direction should be from the pre-PCR area to the post-PCR area and never the reverse.
  - a. Sample preparation area: this area is for the processing of sample materials, such as the extraction of DNA and RNA. No PCR products should ever be allowed into the area. Dedicated equipment and reagents should be reserved solely for sample preparation purposes, including pipetting equipment and laboratory coats. Gloves should be worn at all times and changed frequently. In general, the simpler the sample processing is, the less chance there is of introducing contamination. Dedicated storage facilities, e.g., freezers, should be available for sample preparation alone.
  - b. PCR setup area: it is recommended that the setting up of PCR reactions be performed in a laminar flow hood. The defined area of the hood facilitates the maintenance of cleanliness of the area. Dedicated equipment and storage facilities should be available near the PCR setup area. A separate area should be available for the addition of samples to the PCR reagents. DNA or RNA samples should never be allowed inside the PCR setup hood.
  - c. PCR machine: the location of the PCR machine depends on the exact amplification requirements. For PCR applications involving a single round of PCR and in which it is not required that individual PCR tubes be opened for the addition or sampling of reagents prior to analysis, the PCR machine may be

located in the post-PCR area (*see item d*). However, for applications in which the PCR tubes must be opened, e.g., for nested PCR, the PCR machine should be located at a fourth isolated area separated from sample preparation, PCR setup, and the post-PCR areas. In nested PCR, a dedicated set of pipets should be allocated for this purpose.

- d. Post-PCR area: this is the area reserved for the analysis of PCR products, including electrophoresis, restriction analysis, and mass spectrometry. No items from the post-PCR area should be allowed back into the aforementioned areas. It is important to note that this includes items such as notebooks and pens.
2. Laboratory practice designed to minimize the risk of contamination:
    - a. All PCR reagents should be aliquoted, and reagents that can be autoclaved should be so treated.
    - b. Use and change gloves frequently. Kitchin et al. have advocated the use of face and head masks, as certain individuals appear more prone to the shedding of contaminants (*11*).
    - c. Positive displacement pipets or aerosol-resistant pipets should be used.
    - d. When multiple reactions are needed, it is helpful to set up a master mix to reduce the number of maneuvers, and thus reduce the chance of possible contamination.
    - e. The number of PCR cycles should be kept to a minimum, as excessively sensitive assays are more prone to contamination (*12*).
    - f. When given a choice, disposable items are preferable to items that must be washed prior to being reused.
    - g. If possible, different personnel should be allocated to the pre-PCR and post-PCR parts of the project. If this is not practical, then it is preferable to schedule the project or work week such that the pre-PCR and post-PCR procedures are performed on different days.
    - h. The use of closed PCR systems, e.g., the TaqMan® system (*13*), which use fluorescence signals for detecting PCR products, can eliminate the opening of the amplification vessels and post-PCR sample handling. Therefore, carryover contamination using these systems is much less of a problem than conventional systems. This is especially important for clinical diagnostic applications (*5*).
  3. Use of specific anti-contamination measures:
    - a. Ultraviolet (UV) irradiation: Sarkar and Sommer describe the use of UV irradiation to damage any contaminating DNA prior to the addition of DNA template (*14,15*). As this method relies on the crosslinking of adjacent thymidine residues, the sequence of the PCR target influences the decontaminating efficiency of the method (*16*). Certain primers appear to be more sensitive to the damaging effect of UV light, and may need to be added after the irradiation step. Furthermore, the hydration status of DNA appears to have a great influence on its susceptibility to UV irradiation in that dry DNA seems much more resistant to the damaging effect of UV (*16,17*). This latter fact means that

there are limitations to the use of UV for sterilizing dry laboratory surfaces. Ultimately, clean laboratory practices and physical separation remain the most important anti-contamination measures, with UV irradiation providing an additional margin of protection.

- b. Restriction enzyme treatment: restriction enzymes that cleave within the target sequence for PCR may be used to restrict any contaminating sequence prior to the addition of the target (18,19). Following decontamination, the enzyme is destroyed by thermal denaturation (i.e., 94°C for 10 min; thus, thermostable restriction enzymes such as *TaqI* should not be used for this purpose) before addition of the template DNA. In a model system, Furrer et al. showed that restriction with *MspI* (10 U for 1 h) reduced contamination by a factor of 5 to 10 without impairing the efficiency of PCR (19).
- c. DNase I treatment: this approach is similar to that in **item b** except that DNase I is used. Furrer et al. showed that prior treatment with 0.5 U of DNase I for 30 min reduced contamination by a factor of 1000 without impairing the efficiency of PCR (19).
- d. Incorporation of dUTP and treatment with uracil-*N*-glycosylase (UNG): as the carryover of PCR products from previous amplification experiments constitutes a predominant source of PCR contamination, the ability to selectively destroy PCR products, but not template DNA, presents one way to reduce contamination. Such an approach is described by Longo et al., who substituted dUTP for dTTP during PCR (20). Carryover PCR products containing dUs can then be destroyed prior to subsequent amplification experiments by incubation with UNG. It should be noted that when dUTP is used instead of dTTP, the MgCl<sub>2</sub> concentration often must be readjusted: typically, dUTP is used at 600 mM with 3 mM MgCl<sub>2</sub>. Following the initial thermal denaturation, UNG activity is destroyed; thus, the newly synthesized PCR products are not degraded. However, UNG may regain some of its activity when the temperature is below 50°C, and thus an annealing temperature of over 50°C should be used (21) and all completed PCR containing UNG should be kept at 72°C until analysis. UNG treatment has been reported to result in a 10<sup>7</sup>- (22) to 10<sup>9</sup>-fold (23) reduction in amplicon concentration. In our experience, there is a very slight reduction in sensitivity in PCR systems incorporating dUTP and UNG treatment, although up to a 10-fold reduction in sensitivity has been described (22). This method can also be applied to reverse-transcription (RT)-PCR because PCR products which contain deoxyribose uracil are digested by UNG preferentially to ribose uracil-containing RNA with the optimization of the concentration of UNG and the time and temperature of enzyme digestion (24,25). However, it should be remembered that this method is only effective against dU-containing PCR products. Thus, carryover contamination owing to conventional PCR product lacking dUs cannot be eradicated using this method.
- e. Incorporation of isoprosalen compound: Cimino et al. describe adding a photochemical reagent before PCR and activation after the amplification is completed (26). The reagent will then crosslink the two strands of the PCR product

and render them unamplifiable. The crosslinking is most effective at 5°C and under UV intensity of more than 27 mW/cm<sup>2</sup> (e.g., in an HRI-300 chamber) (27). This method has been shown to be similar in decontaminating efficiency to the UNG method, and results in the elimination of at least 10<sup>9</sup> copies of contaminating PCR products (23).

- f. Exonuclease digestion: it was demonstrated that certain exonucleases, e.g., exonuclease III and T7 exonuclease, when added to fully assembled PCR reactions, were able to render carryover PCR product molecules non-amplifiable but would spare identical target sequences in genomic DNA (28,29). In a model system, a 30-min incubation with exonuclease III was able to degrade  $5 \times 10^5$  copies of carryover amplicons (28). Several mechanisms for the selectivity against PCR products have been postulated: Zhu et al. attributed it to the relatively long chain length of genomic DNA, which might resist degradation by exonucleases better than the comparatively short PCR products (28), and Muralidhar and Steinman, in an ingenious series of experiments, demonstrated that part of this selectivity has a geometric explanation (29). Thus, for any stretch of DNA to be amplifiable by a specific pair of primers following T7 exonuclease treatment, the primer binding sites should be situated on the same side with respect to the geometric center of the molecule. As it is extremely unlikely that a particular genomic target would straddle the center of any stretch of genomic DNA (essentially produced by random shearing during DNA extraction), this form of exonuclease treatment would spare the genomic target. The situation with carryover PCR products, however, is completely different, as the primer binding sites are located at opposite ends of the molecules and thus would span the geometric center of the molecule. Exonuclease treatment for the prevention of PCR carryover, therefore, possesses the chief advantage of uridine incorporation and glycosylation in that the completed reaction tubes do not have to be reopened for the addition of the target and/or *Taq* polymerase. Furthermore, exonuclease treatment has the added advantage of being able to destroy even nonuridine-containing PCR products, and would be very useful in an already contaminated environment.

#### 4. Detection of Contamination

Monitoring for contamination is probably as important as measures to prevent it. It is a reality that contamination will be experienced by most, if not all, workers using PCR. To facilitate the monitoring of contamination, the following measures should be undertaken:

1. Negative controls should be included in every PCR experiment. To detect sporadic contamination, multiple controls are usually required. Different negative controls, testing the different stages in the PCR process at which contamination may occur, should be included. PCR reagent controls will only test for contamination of the reagents, but not the sample preparation stage.

2. In certain applications, PCR products from different samples are expected to have different sequences, e.g., sequence variations in bacteria occurring at different times. In these situations, sequencing of PCR products (30) or methods that reflect the sequence variation, e.g., heteroduplex analysis (31) and single-strand conformation polymorphism (SSCP) analysis (32), are helpful in verifying the genuineness of a positive result.

## 5. Remedial Measures

Once contamination has been detected, all diagnostic work should be stopped until the source of contamination has been eliminated. In many situations, discarding all suspected reagents is all that is required to cure the problem. In cases in which the equipment is contaminated, thorough cleansing or even replacing the culprit equipment may be necessary. In serious situations, changing to a new primer set that amplifies a different target segment of DNA may be the only method of solving the problem.

## 6. Automation

Automated nucleic acid extraction (e.g., MagNA Pure®) and liquid handling systems (e.g., Biomek® FX) are now available for the high-throughput nucleic acid extraction and preparing of PCR mixtures. The yield and contamination rates of the automated nucleic acid extraction methods have been shown to be comparable with the manual methods (33–35). These automated platforms are particularly useful in diagnostic laboratories handling a large amount of samples or samples with high infectious risks.

## 7. Conclusion

Contamination is the single most important obstacle to using PCR reliably for diagnostic purposes. Contamination can only be avoided by meticulous attention to good laboratory-operating details and the exercise of common sense. When coupled with monitoring systems aimed at detecting contamination, reliable PCR, even at high sensitivity, should be a realizable goal.

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