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Regulatory Concern of Polymerase Chain Reaction (PCR) Carryover Contamination

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Abstract

Currently, DNA amplification techniques have become important detection tools. However, the extreme sensitivity of such techniques can easily result in contamination. This is a major problem in using these techniques routinely in a regulatory agency such as the Food and Drug Administration (FDA) because false-positive polymerase chain reaction (PCR) results will fail our mission. Preventing PCR carryover contamination and a capacity to rapidly determine false PCR positives are crucial. In the past, several methods have been used to prevent amplicon carryover contamination. In this chapter, we provide practical suggestions for PCR carryover contamination detection and prevention that work well with most PCR applications in our laboratory.

Keywords: polymerase chain reaction (PCR), carryover contamination, nested PCR, real-time PCR, single-tube nested real-time PCR

1. Introduction

Polymerase chain reaction (PCR) amplification techniques have provided means for the rapid and sensitive detection of pathogens [1]. The number of applications of PCR is still growing, and more and more amplification-based techniques are now used in FDA field laboratories to detect pathogens, such as *Salmonella*, *Escherichia coli* 0157:H7, *Shigella*, *Vibrio*, hepatitis A virus (HAV) and noroviruses (NoVs) [2]. A significant challenge facing us is that the sensitivity of PCR can easily result in contamination and consequently in false-positive PCR. A small amount of previously amplified PCR product or potential target sequences that infiltrate laboratory supplies and equipment or that are present in an aerosol can easily contaminate the sample and PCR reagents in the tests. Therefore, prevention of carryover

contamination from previous PCR amplifications has become a high priority. As a first line of defense to prevent contamination of PCR with a previously generated amplicon, mechanical separation of the PCR laboratory into different rooms or laboratory benches is needed. Secondly, chemical, UV, and enzymatic methods can be applied to inactivate any prior amplicon generated in the laboratory. Additionally, rapid identification of contaminants and their sources is needed to prevent false-positive PCR results. For this purpose, we developed a rapid method to detect PCR carryover contamination by DNA sequencing. The combination of the above methods plus good laboratory technique should be able to totally eliminate PCR carryover contamination and allow us to perform accurate and sensitive PCR routinely in regulatory setting.

1.1. Polymerase chain reaction

In 1983, Dr. Kary Mullis at Cetus Corporation conceived of polymerase chain reaction. There is not any technique that has had a greater impact on the practice of molecular biology than PCR. PCR-based methods are powerful techniques [3]. This technique is centered around the ability of sense and anti-sense DNA primers to hybridize to a DNA of interest. When put into use, agents of infectious diseases can be detected at extremely low levels. After extension from the primers on the DNA template by DNA polymerase, the reaction is heat-denatured and allowed once again, to anneal with the primers. After another round of extension, a multiplicative increase in DNA products is observed. When critical controls are set, this technique becomes a quantitative process. Therefore, a minute amount of DNA can be efficiently amplified in an exponential fashion to result in an easily manipulable amount of DNA. The current sensitivity and detection limit is at a level as low as 10–50 copies per ml. Although the PCR is extremely easy and fast, PCR product carryover contamination impedes the routine use of these techniques routinely in regulatory laboratories.

1.2. PCR-based technology

1.2.1. Reverse transcription PCR (RT-PCR)

PCR uses DNA as a starting material. When RT-PCR is carried out, the starting material is RNA. In this method, RNA is first transcribed into complementary DNA (cDNA) by reverse transcriptase from total RNA or messenger RNA (mRNA). The cDNA is then used as a template for the quantitative PCR (qPCR). Real-time quantitative PCR (RT-qPCR) is used in a variety of applications such as food-borne RNA virus and avian flu virus detection. With this technique, we can detect the target RNA at an extremely low level in samples. RT-PCR is an increasingly popular method for RNA virus detection, but DNA contamination in RNA preparations is also a concern. In order to minimize the possibility of carryover contamination in RT-PCR, it is critical to minimize the number of handling and pipetting steps.

1.2.2. Real-time quantitative PCR (qPCR)

Traditional detection of amplified PCR product relies upon gel electrophoresis. qPCR is an advanced form of the traditional PCR. It is a major development in PCR technology that enables

the reliable detection and measurement of products generated during every cycle of the PCR process. This technique became possible after the introduction of an oligonucleotide probe that was designed to hybridize within the target sequence. Due to the 5' nuclease activity of Taq polymerase, amplification of the target-specific product can be detected through cleavage of the probe during PCR. These assays are very sensitive and can detect as few as 10–100 viral copies per reaction. qPCR techniques have evolved into a variety of other branches including real-time PCR by Taqman (Roche), LightCycler by (Roche), SmartCycler by (Cepheid), etc. Some of them are now widely in use for virus and bacteria detection in regulatory laboratories. Unlike other PCR methods, qPCR does not require post-PCR product handling, preventing potential PCR product carryover contamination.

1.3. PCR contamination

All the PCR methods are powerful techniques. Unfortunately, the exquisite sensitivity of these techniques makes them vulnerable to contamination [4, 5]. One of the most important rules when performing PCR is to avoid contamination. This chapter will outline necessary precautions to prevent contamination as well as procedures for detecting and cleaning suspected contamination.

2. Potential sources of contamination

2.1. Cross contamination between samples

A large number of target organisms in sample handling may lead to pre-amplification sample cross contamination [6, 7]. The sources of contaminants between samples are diverse and can all contribute to the contamination of the finished PCR product. These sources may include reagents, disposable supplies, sample carryover, improper handling procedures, etc.

2.2. Cross contamination between nucleic acids

Cross contamination between nucleic acids is a major problem in all PCR laboratories. Nucleic acids from organisms or plasmid clones derived from organisms that have been previously analyzed and that may be present in large numbers in the laboratory environment could be a source of contamination. Contaminants can also be introduced by unrelated activities in neighboring laboratories. These sources of contamination are problematic as they may lead to pre-amplification cross contamination [8–10].

2.3. PCR product carryover contamination

The most important source of contamination is from the repeated amplification of the same target sequence, which leads to accumulation of amplification products in the laboratory environment. Even minute amounts of carryover can lead to false-positive results. A typical PCR generates theoretically as many as 10^8 copies of target sequence [11]. If uncontrolled, amplification products will contaminate laboratory reagents, equipment, and ventilation

systems. Carryover of previously accumulated amplified DNA is considered the major source of contamination.

3. Methods to control contamination

Contamination between samples and from previous PCR amplicon generation is a significant potential source of invalid PCR results [12]. The first two forms of contamination described above can be easily avoided by using careful technique and good quality control practices. Generally, most PCR-based assays consist of three steps: DNA sample processing, PCR amplification, and amplification product detection (excluding real-time PCR). It is in the latter step that carryover contamination often occurs through methods that include gel electrophoresis, solid phase hybridization, solution hybridization, and capillary electrophoresis [7]. Methods to prevent amplification product carryover contamination have been developed in the past ten years [13–17]. Basically, there are mechanical, chemical, UV light irradiation, and enzymatic methods and closed-tube PCR detection formats, all of which can help to prevent amplification product carryover contamination [7]. The following section will focus on more recent practices and methods that have been used in our laboratory to eliminate carryover contamination.

3.1. Mechanical method

Our laboratory was designed and operated in a way that prevents contamination of reactions with PCR products from previous assays and cross contamination between samples. It includes the separation of areas of the laboratory where samples and reagents are prepared from the areas where amplification is performed and amplification products are analyzed. This unidirectional workflow can reduce the opportunity for contamination to occur. A typical PCR laboratory should be divided into at least three to four different areas—(1) sample preparation, (2) PCR mix preparation, (3) PCR product detection, and (4) RNase free area—if the PCR method involves RNA sample.

3.2. Chemical method

General cleaning practices are important for controlling PCR carryover contamination. All surfaces in the PCR area should be routinely decontaminated to prevent cross contamination. The PCR work bench is required to be cleaned with 10–15 % sodium hypochlorite solution (bleach), followed by removal of the bleach with 70 % ethanol.

3.3. UV irradiation method

UV irradiation is an easy method to inactivate amplification product involved in carryover contamination. The method is based on the ability of UV light to induce thymidine dimer formation in the DNA that makes the contaminating nucleic acid inactive as a template for further amplification (**Figure 1**). A good practice is to expose all of the PCR supplies to UV light for 5–20 min as the nucleic acid will be damaged by absorbing the UV light energy at

254 nm wavelength [19]. UV irradiation is an integral feature of our PCR laboratory, and the Spectrolinker XL-1500 (Spectronics Corporation, Westbury, NY) is used to eliminate contamination that may occur during PCR tests. All of our PCR tools are stored in a UV light box (C.B.S Scientific, Co. Del Mar, Ca). PCR master mix preparation and specimen setup are also carried out in this UV light box.

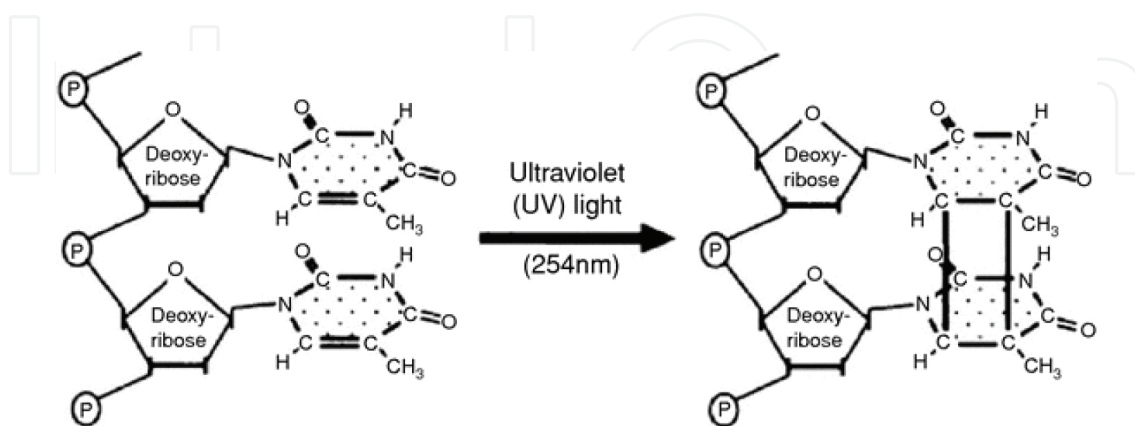


Figure 1. Action of UV light on the nucleic acids [18].

3.4. Enzymatic method

Uracil-DNA glycosylase (UNG) is a DNA repair enzyme [20] that can recognize and remove uracil residues from DNA (**Figure 2**). In 1990, the use of UNG to inactivate PCR products was first reported [21]. This method employs uracil (dUTP) instead of thymine (dTTP) during PCR to generate amplification products with distinguishing characteristics relative to the native DNA template. Because the newly synthesized amplicons contain dUTP, they are susceptible to hydrolysis by UNG. This method is the most widely used contamination control technique in our laboratory.

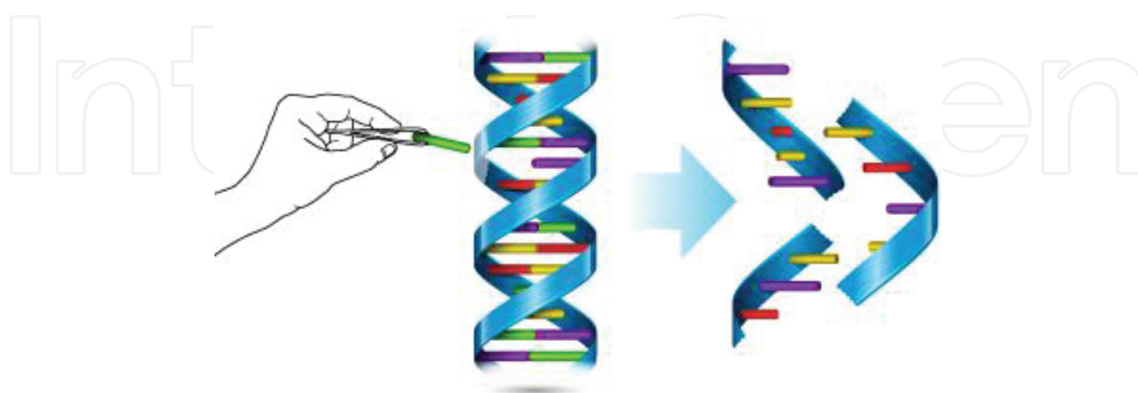


Figure 2. Replace dTTP with dUTP during PCR amplification and the PCR product will contain uracil. Prior to PCR, the PCR mixture is treated with uracil-DNA glycosylase (UNG). During the denaturation step, temperature is elevated to 95°C, resulting in cleavage of apyrimidinic sites and fragmentation of carryover DNA. As the template contains thymine, it will not be affected by the UNG treatment (source: Sopachem Life Sciences).

Briefly, this carryover prevention technique consists of three steps:

1. The dUTP is incorporated into all PCR products, substituting dTTP or incorporating dUTP during synthesis of the primers [15, 22].
2. Before PCR, mixtures are treated with UNG (Applied Biosystems, Foster City, CA) at room temperature for 10 min to hydrolyze and remove any contaminating amplification products that may be present in the PCR mixtures. This technique also has a hot start function [15].
3. UNG is thermally inactivated at 95°C for 5 min prior to the actual PCR.

3.5. Another amplification format without the risk of carryover contamination

3.5.1. Real-time PCR-based technology to avoid contamination

In traditional PCR, amplification and detection of the target DNA sequence occur separately. To determine if a sample contains the target sequence, post-amplification handling of the amplicon is required. A more recent technological development, real-time PCR [23], allows for the simultaneous amplification and detection of a target sequence through the use of fluorescent-labeled probes (**Figure 3**). In comparison to conventional PCR, real-time PCR can reduce the chance of carryover contamination. The new generation of amplification technology simultaneously amplifies and detects target DNA without exposing the amplification products to the laboratory environment. Currently, we developed several real-time PCR methods, such as single-tube real-time PCR and single-tube nested real-time PCR (**Figure 4**) to simultaneously detect multiple pathogens in a closed system which has substantially reduced the possibility of false-positive results due to amplification product carryover contamination [24, 25].

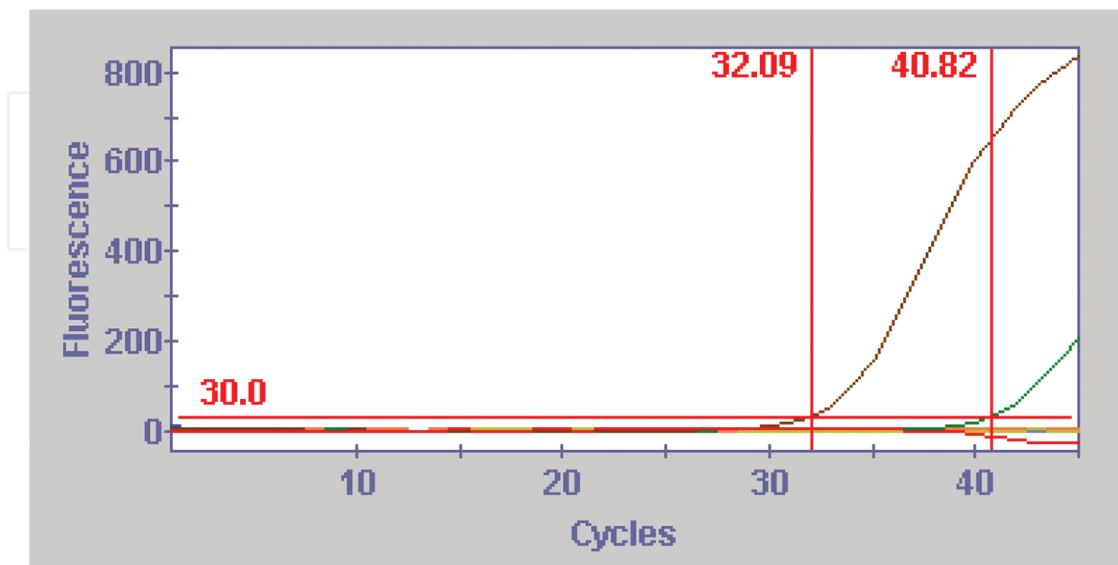


Figure 3. A typical result of graphical view from our laboratory.

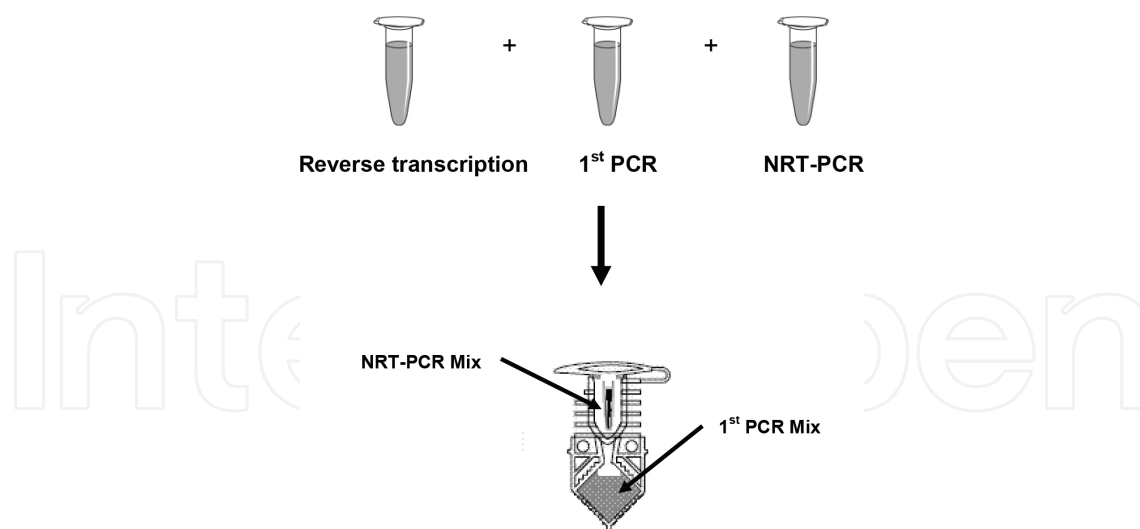


Figure 4. Single closed-tube nested real-time-PCR system: in order to reduce the chance of carryover contamination, all reactions including reverse transcription, conventional PCR, first PCR, nested PCR, and real-time TaqMan detection are performed in a single closed tube [24].

4. Method to detect contamination

In the context of this discussion, contamination is defined as the unwanted presence of a PCR amplicon. At times, PCR contamination is present but difficult to ascertain. If a significant contamination problem appears in a PCR laboratory, we need to walk through the procedure of testing for contamination and, if necessary, replace all reagents. The PCR parameters considered for potential sources of contamination include amplification setup, amplification product handling, and DNA aerosol and storage. Carryover contamination is determined by the following methods in our laboratory.

4.1. Internal controls

Appropriate control reactions are helpful in determining whether DNA contamination has occurred. It is important to use a special PCR-positive control which is different from the sample DNA, such as a DNA fragment with a deletion or base alteration in the region of amplification [26]. PCR products can be assessed on a gel to distinguish the control from the native PCR products. Negative controls are also very important and must be included with each run because the first sign of contamination trouble is usually the appearance of an amplification product in the negative or blank controls [27].

4.2. DNA sequencing

Techniques to sequence PCR products were developed in our laboratory in the past few years [15, 28]. This confirmatory sequencing ensures that the PCR product has the expected sequence. The direct comparison of PCR product sequences from a sample and a control is the

best way to determine whether two PCR products are similar or different. After comparison of the DNA sequence variation between the PCR products and the control, the cross contamination of samples can be detected. In some suspected cases, we directly sequenced PCR products by using the ABI BigDye Terminator Cycle Sequencing kit with a 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Briefly, each cycle consists of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and extension at 60°C for 4 min. After 25 cycles, the fluorescent extension products are purified by a simple isopropanol precipitation step. Software is available on websites [29] to perform a wide range of different types of sequence alignment. DNA sequence data were analyzed by the Geneious, the GenBank sequence database, and the BLAST program from the National Center for Biotechnology Information (NCBI). Accurate identification of any contamination is required for the proper function of FDA field laboratory. The DNA sequencing method should be an ideal technology for this purpose.

5. Discussion

PCR is a very powerful and extremely sensitive amplification technique, but there is always the peril that a tiny amount of contamination of the DNA target may lead to false-positive results. It has become necessary to systematically address the issue of PCR contamination, especially in the FDA, a regulatory agency. To overcome this issue, effective methods have been successfully developed and used to avoid carryover contamination in our regulatory laboratories in the past few years [15, 24, 25].

- We have effectively established and maintained a unidirectional workflow from a PCR clean to a PCR dirty area, thereby reducing the opportunity of contamination to occur.
- Samples were set up on a bench that was isolated from PCR product testing areas.
- All PCR master mixes were prepared in a separate room or at least on a separate bench. Also, we always used a separate laboratory coat, gloves, tubes, and filter pipette tips in the different PCR working areas.
- A separate aliquot of water stock for each round of PCR was addressed.
- All PCR work benches were decontaminated with 10–15 % bleach and 70 % alcohol. All the pipettes, pipette tips, tubes, racks, and gloves were UV-irradiated.
- A different pipette tip was used when pipetting each of the PCR reagents, even the same master mix to each tube.
- The PCR tubes were kept closed during the procedure. The tubes were opened only when necessary because of potential aerosols that are dangerous with respect to contamination. Minimizing the number of pipetting and mixing steps in PCR master mix preparation is also very important from the perspective of aerosol contamination.
- It is very important to schedule PCR when not handling plasmids to prevent cross contamination.

- dUTP was incorporated into all PCR products which can subsequently be selectively destroyed by UNG.
- Optimization of PCRs is also important. G + C-rich products may be more difficult to inactivate by UNG because of the lower concentration of uridine triphosphate(UTP).
- Positive controls consisted of a low copy number of the desired nucleic acid target and should never be prepared or stored with the samples.
- Other amplification methods, such as real-time PCR [23] or closed-tube PCR [25] which can reduce the chances of carryover contamination, are now being used more routinely in our laboratory.
- A rapid DNA sequencing method to precisely detect contamination was established.

6. Conclusion

Standard precautions should always be employed during all PCR-based testing, whether it is real-time PCR or conventional PCR. All the regulatory laboratories should have their own appropriate controls and systematic measures to prevent and detect contamination. When contamination does occur, we need to accurately determine which reagent is contaminated. All of us should also understand that our individual working habits directly affect our work quality. We believe all the above methods can reduce the risk of contamination and ensure the efficacy of all PCR results.

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