Single-use technology in production of DNA-free PCR reagents

Abstract

Molecular research relies heavily on tests utilizing polymerase chain reaction (PCR) technology. PCR enables sensitive detection, specific identification, and accurate quantification of nucleic acid sequences. To avoid false positives and uncertainty in the assay results, it is crucial that the reagents used in any PCR test be free of foreign DNA, such as DNA originating from the manufacturing environment, human operator, cross-contamination from shared equipment, or host DNA of cells expressing recombinant proteins. To remove the risk of these types of DNA contamination and enable better assays, Thermo Fisher Scientific has developed and implemented a new process for manufacturing DNA-free enzymes. We use a closed system based on single-use technology to drastically minimize the risk of DNA contamination inherent to the conventional manufacturing process. To help ensure conformance to strict purity requirements, we subject our DNA-free PCR reagents to stringent quality tests to verify that products are free of contaminating bacterial, human, and plasmid DNA. This combination of manufacturing systems and quality testing delivers PCR reagents that are orders of magnitude cleaner than other "DNA-free" reagents on the market.

Background

For over 20 years, molecular research has been a driving force behind advances in health care. Any condition that has a confirmed nucleic acid target (e.g., DNA or RNA) can likely be detected or identified with molecular testing. To date, most molecular tests are built on nucleic acid amplification methods, predominantly PCR.

PCR relies on DNA polymerases to amplify a few initial target DNA molecules up to 10^6- to 10^7-fold, enabling fast and sensitive detection of microbial pathogens or important genomic markers. PCR-based assays allow early, species-specific identification, uncovering of antibiotic resistance, and accurate quantification of pathogens from trace amounts of their DNA.

Due to their high sensitivity, PCR-based tests are vulnerable to amplifying minute quantities of contaminating nucleic acids, potentially leading to ambiguous false-positive results. The presence of contaminating DNA has a greater impact if highly conserved amplification targets, like the bacterial 16S rRNA gene, are used for broad-range detection [1]. Since the 16S rRNA gene is present in multiple copies in the genomes of all known bacteria, the absence of any traces of contaminating bacterial DNA is crucial for an assay intended to detect a bacterial target.

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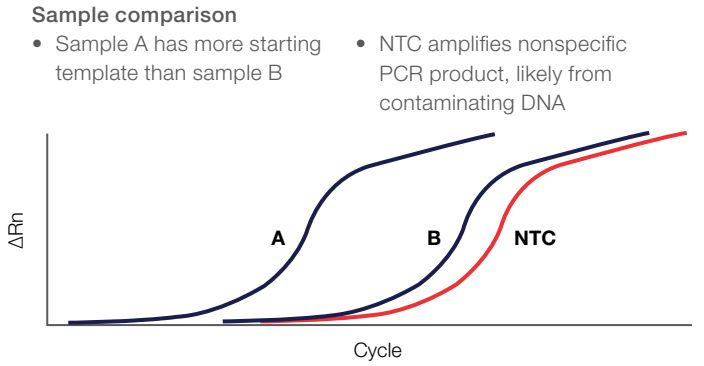
This problem of DNA contamination is exacerbated when the target DNA is in low abundance. Therefore, a signal from contaminating DNA can interfere with detection of a low-copy DNA target, as seen in Figure 1, compromising the sensitivity and reliability of the assay.

DNA contamination in PCR reagents

The potential risks and implications associated with contaminated PCR reagents have been well reported [2]. Commercially available lots of *Taq* DNA polymerase have been shown to contain 10–1,000 genome equivalents of bacterial DNA per unit of enzyme [3]. Reported DNA removal methods vary in efficiency and are not universal. In addition, decontamination is often achieved at the cost of decreased detection sensitivity, which may lead to false-negative results and jeopardize the value of the assay [4].



- Most samples have low target or template concentrations, which make them more like sample B.
- The no-template control (NTC) response can be caused by many things, including traces of exogenous DNA in PCR reagents.
- The closer the C_t of sample B is to that of the NTC, the less certain the result will be; good results (low false-positive rates) require good separation between the NTC and sample curves.
- If the target concentration in the sample cannot be increased (e.g., most samples), the only way to improve the assay is to move the NTC curve further to the right (larger C_t values).
- Using DNA-free PCR reagents will generally move the NTC curve to the right to allow better results:
  - Better certainty from the same samples (i.e., better separation of sample and NTC)
  - Lower limits of detection with the same certainty (i.e., detection of lower levels of target while still having separation between sample and NTC)

Figure 1. The importance of DNA-free PCR reagents for assays with low-copy targets.

Commercial providers of *Taq* DNA polymerase acknowledge the concern over DNA contamination and offer "DNA-free" products for PCR assays. These alternatives differ from conventional PCR reagents in the stringency of their quality control (Table 1). The alternatives are specifically tested to measure levels of residual DNA in the reagents. While not consistent between manufacturers, these tests often examine the presence of *E. coli* DNA, and sometimes also human or fungal genomic DNA. The methods used to measure the contamination levels are endpoint or quantitative PCR. The requirement usually is that no amplification be detected in the absence of DNA template, after a certain number of PCR cycles. However, the result is still ambiguous because a negative answer in PCR may simply mean that the DNA polymerase is not sensitive enough to detect the low amount of contaminating DNA in the reaction, or the primers used are not adequate to detect DNA of different targets or organisms.

While is it unclear how these "DNA-free" products for PCR assays are manufactured, it is generally thought that the major source of contamination in commercial *Taq* DNA polymerase originates from steps in the enzyme manufacturing process or addition of other reagents, contaminated with DNA, to the final product.

Table 1. Quality control standards for "DNA-free" enzymes.

Taq DNA polymerase	Quality control
Supplier 1	Bacterial: <10 copies of bacterial gDNA/enzyme unit (based on 16S rRNA gene amplification)
	Human: <1 genome equivalent of mammalian gDNA/enzyme unit (based on mitochondrial DNA amplification)
	Fungal: <1 genome equivalent of fungal gDNA/enzyme unit (based on 18S rRNA gene amplification)
Supplier 2	Bacterial: no detectable PCR product in NTC with primers specific to the *E. coli* 16S rRNA gene (gel analysis)
Supplier 3	Bacterial: <3% false positives (amplification of 16S rRNA gene; gel analysis)
Supplier 4	Bacterial: <37 fg of *E. coli* genomic DNA (amplification of 16S rRNA gene; gel analysis)
	Human: <100 fg of human genomic DNA (gel analysis)
Supplier 5	Bacterial: <1 fg of *E. coli* genomic DNA/enzyme unit
Supplier 6	Bacterial: no detectable PCR product with *E. coli* genomic DNA (gel analysis)
Supplier 7	Bacterial: <0.2 copies of *E. coli* genomic DNA/enzyme unit (based on 16S rRNA gene)

Figure 2. Conventional manufacturing process for recombinant enzymes, with risk of DNA contamination. The process of enzyme preparation is repeatedly exposed to potential DNA contamination from open environments and human operators. In addition, there is a risk of carryover DNA contamination from previous manufacturing material through shared equipment.

Conventional manufacturing process

Recombinant DNA polymerases are commonly expressed in *E. coli* cells. However, this conventional manufacturing process fails to adequately remove residual nucleic acids from the bacteria used in production. Host-cell nucleic acids are not the only contaminating DNA commonly found in PCR enzyme preparations. Figure 2 illustrates the conventional manufacturing process for recombinant enzymes. The process consists of multiple steps where the enzyme preparation is repeatedly exposed to the open environment and the human operator. Many of these manufacturing procedures are conducted in common-use equipment (e.g., fermentation tank or chromatographic column) that is shared for the manufacturing of other proteins. If cleaning and decontamination of shared equipment is not sufficiently rigorous, the risk of cross-contamination (including nucleic acids) from previous fermentations or manufactured material can be significant.

Lessons from the biopharmaceutical industry

Eliminating the risk of contamination is not a challenge unique to applications requiring sensitive detection. Similar concerns are well understood by the biopharmaceutical industry, where quality and safety of the manufactured drugs must be ensured. To meet the purity standards required for pharmaceuticals, while needing to be flexible and responsive in making a variety of drugs of various amounts within the same production facility, the pharmaceutical industry has adopted continuous bioprocessing systems utilizing closed systems and processes. These systems and processes, often based upon single-use technology, have successfully decreased dependence on environmental controls and improved agility, flexibility, and production robustness while delivering the purity levels required [5]. The single-use technology (also commonly known as “disposable”) utilizes plastics intended for one-time use and disposed of after use.

Manufacturing line based on a closed single-use system

At Thermo Fisher Scientific, we have adapted single-use technology for the novel production of enzymes, similar to that of the biopharmaceutical industry. The main steps of a closed and single-use system (SUS) are illustrated in Figure 3. All stages of enzyme manufacturing utilize disposable single-use bioprocessing systems in which single-use components—fermentors, containers and bags, filters, and chromatography columns—are connected by sterile single-use tubes. Buffers and washing solutions are prepared in single-use bags and filtered for sterilization. A 100% closed system helps ensure that the entire manufacturing process is never exposed to the surrounding environment and human operators. Since an SUS does not depend on common-use equipment, the enzyme preparation is protected from potential cross-contamination.

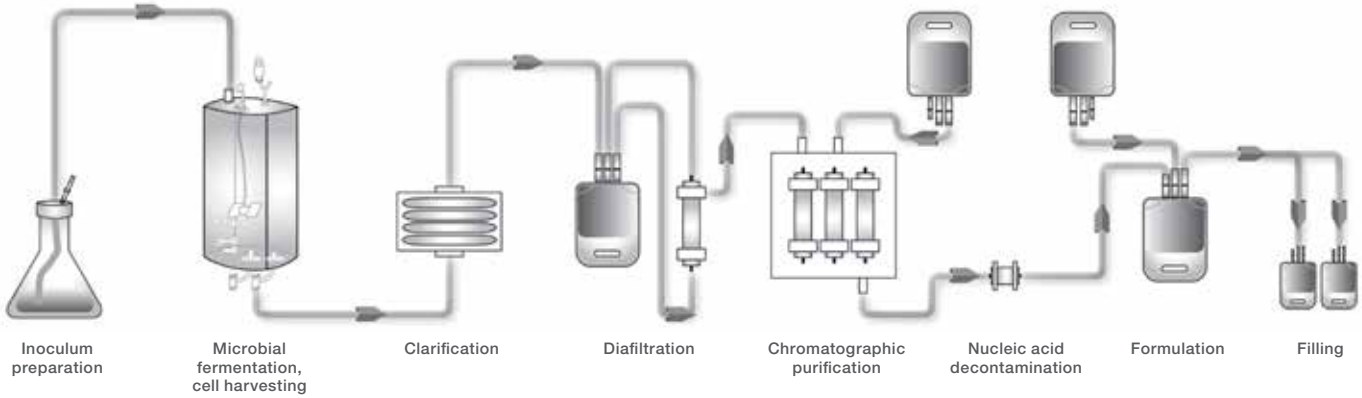


Figure 3. Closed SUS–based manufacturing process for recombinant enzymes. A completely closed system using disposable single-use bags, tubes, and connectors, reduces the potential DNA contamination from the environment, human operator, and cross-contamination to a negligible level.

The SUS manufacturing line is housed in a dedicated, purpose-built space under a controlled environment corresponding to clean room specifications Class D, C, and B (Class D for upstream processes such as inoculum preparation, fermentation, clarification, and diafiltration; Class C for downstream processes including purification, nucleic acid decontamination, and formulation; and Class B for filling).

With closed SUS–based manufacturing, the probability of contamination with exogenous DNA has been reduced. However, the potential for DNA contamination from the host cells used for expression of recombinant enzymes may still be present. In the manufacturing of PCR enzymes in an SUS, additional proprietary steps were added to remove the majority of host-cell DNA in the early stage of production, and the last traces of host-cell DNA are trapped using the nucleic acid decontamination step after the chromatographic purification.

Quality control measures

Enzymes manufactured utilizing our SUS technology are subjected to rigorous quality control testing. First, functional assays evaluate the protein activity and confirm that these enzymes retain the same functional characteristics as enzymes produced by conventional methods.

Second, the enzymes are tested for their purity, to verify that nucleases and contaminating DNA are not present. Proprietary quality control tests, relying on highly sensitive qPCR assays, are used to confirm that nucleic acid contaminants are absent. These new tests, combined with our SUS technology, demand a redefinition of what it means for PCR reagents to be DNA-free. For example, one unit of *Taq* DNA polymerase manufactured using our SUS technology contains less than 0.01 genome equivalents of bacterial DNA (see Table 2 for all DNA purity tests). Since the test detects conserved coding sequences of bacterial 16S rRNA genes, we are able to verify that any DNA from *E. coli* or any other bacteria that has been transferred into the final product is undetectable (within the limits of detection). We also verifiy that SUS-manufactured enzymes are free of DNA from human operators and plasmids used for recombinant protein expression.

Table 2. Purity requirements for our DNA-free *Taq* DNA polymerase variants.

Purity test	Requirement
***Taq* DNA polymerase purity**	Exonucleases and endonucleases: undetected RNases: undetected
DNA contamination detection	Bacterial gDNA (16S rRNA gene detection): ≤0.01 copy/enzyme unit Human gDNA (*Alu* sequence detection): ≤0.001 copy/enzyme unit Plasmid DNA (*ori1* sequence detection): ≤0.01 copy/enzyme unit

Conclusion

Increasingly, as kit developers continue to push for lower sample volume for their assays, higher sensitivity is required to detect target DNA in these assays. When only a few target molecules are available, even minute quantities of contaminating DNA may lead to false positives. Conventional PCR reagents, which have been shown to contain contaminating DNA, fall short of being able to provide the reliability required to detect low-abundance DNA targets. To support developers of DNA-based assay kits, Thermo Fisher Scientific is the first to offer DNA-free PCR reagents manufactured using a closed single-use system technology. These reagents are verified to levels that are orders of magnitude cleaner than other commercially available products, and are an ideal choice in applications requiring high sensitivity and reproducibility.

References

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