Single-Use Based BioProduction Process for Manufacturing of High Quality Recombinant Enzymes for mRNA and DNA Workflows

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ABSTRACT

Molecular research and therapy relies heavily on tests utilizing polymerase chain reaction (PCR) and in vitro transcription (IVT) technologies. PCR enables sensitive detection, specific identification, and accurate quantification of nucleic acid sequences and IVT is used to manufacture mRNA. To avoid false positives and uncertainty in the assay results, it is crucial that the reagents used in PCR and IVT applications be free of foreign contaminants, such as DNA, RNA, HCP, endotoxins originating from the manufacturing environment, human operator, cross-contamination from shared equipment, or host cells expressing recombinant proteins. To remove the risk of these types of contamination, Thermo Fisher Scientific has developed and implemented a new process for manufacturing such enzymes. We use a closed system based on single-use technology to drastically minimize the risk of contamination inherent to the conventional manufacturing process. To help ensure conformance to strict purity requirements, we subject our DNA-free PCR reagents and IVT enzymes to stringent quality tests. 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 and IVT reagents that meet all quality and regulatory requirements for therapeutic use.

INTRODUCTION

To date, most molecular tests are built on nucleic acid amplification methods, predominantly 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]. A signal from contaminating DNA can interfere with detection of allow-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. 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.
IVT technology is currently used to manufacture mRNA for potential therapeutic use. For such purpose GMP-grade T7 RNA Polymerase, RNase inhibitor RNAseOUT and Inorganic Pyrophosphatase are necessary for efficient mRNA synthesis. Afterwards DNase is used to remove template DNA leading to mRNA purification and functionalization for therapeutic applications where IVT enzymes must meet strict quality and regulatory requirements (Figure 2 and Table 3).

MATERIALS AND METHODS

At Thermo Fisher Scientific, we have adapted singe 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 4. 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.

RESULTS

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

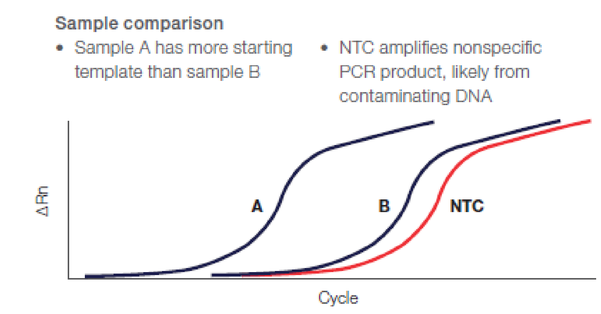


Figure 2. IVT enzymes and workflow showing the synthesis of mRNA used for passive immunotherapy. mRNA enzymes meet all quality attributes necessary for therapeutic applications.

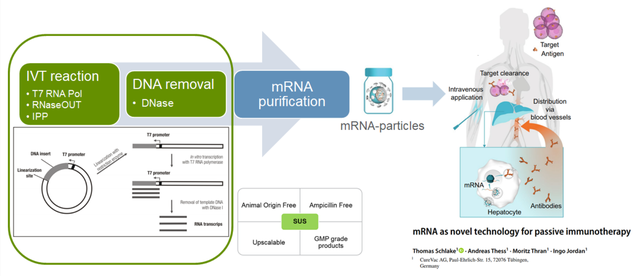


Table 1. Quality control standards for “DNA-free” enzymes.

Enzyme of interest	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	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

Table 2. Quality control standards for “DNA-free” enzymes.

Purity test	Requirement
Functional 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

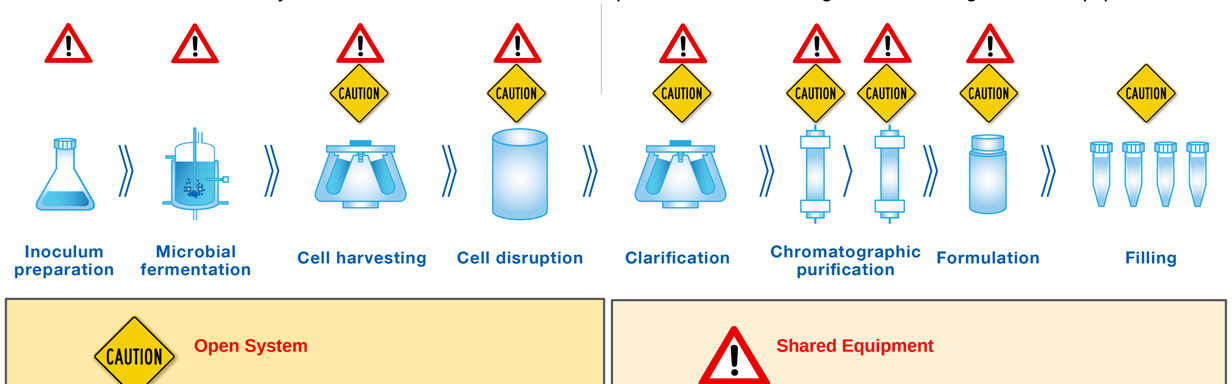
Table 3. Additional Quality control standards for “mRNA” enzymes.

HCP, Endotoxin and Heavy metal contamination

- Functional activity - U/mg
- Endotoxin level: <10 EU/mg
- HCP: <100 ng/mg
- Heavy metals: ICHQ3D
- Microbial contamination: 1 < CFU/mL
- Mycoplasma not detected
- Host RNA < 100 pg/mg

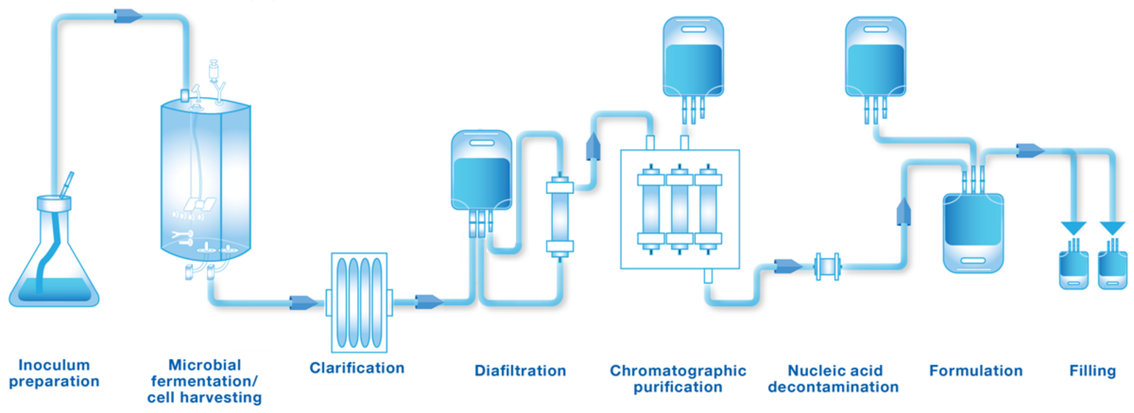
Enzymes for mRNA and DNA workflows manufactured utilizing our SUS technology are subjected to rigorous quality control testing. First, functional assays evaluate the protein activity and confirm that these enzymes retain the same functional characteristics as enzymes produced by conventional methods. Second, the enzymes are tested for their purity, to verify that nucleases and contaminating DNA/RNA 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 verify that SUS-manufactured enzymes are free of DNA from human operators and plasmids used for recombinant protein expression.

Figure 3. Conventional manufacturing process for recombinant enzymes, with risk of DNA/RNA contamination. The process of enzyme preparation is repeatedly exposed to potential DNA/RNA contamination from open environments and human operators. In addition, there is a risk of carryover DNA/RNA 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. 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.

Figure 4. 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 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. The single-use technology (also commonly known as “disposable”) utilizes plastics intended for one-time use and disposed of after use. 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 4. 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 DNA contamination from the host cells used for expression of recombinant enzymes may still be present. In the manufacturing of PCR and IVT enzymes in an SUS, additional proprietary steps were added to remove the majority of host-cell DNA/RNA/HCP in the early stage of production, and the last traces of host-cell DNA/RNA are trapped using the nucleic acid decontamination step after the chromatographic purification.

CONCLUSIONS

Conventional PCR and IVT reagents, which have been shown to contain contaminating DNA/RNA, fall short of being able to provide the reliability required to detect low abundance DNA/RNA targets. To support developers of DNA based assay and IVT kits, Thermo Fisher Scientific is the first to offer DNA/RNA-free PCR and IVT reagents manufactured using a closed single-use system technology. Unlike other PCR and mRNA reagents tested by less sensitive analytical methods, we have developed proprietary qPCR assays which enable the detection of minute quantities of contaminating DNA and RNA.

REFERENCES

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