Bacterial contamination of blood components, particularly of platelet concentrates (PCs), represents the greatest infectious risk in blood transfusion. Although the incidence of platelet bacterial contamination is approximately 1 per 2000 U, the urgent need for a method for the routine screening of PCs to improve safety for patients had not been considered for a long time. Besides the culturing systems, which will remain the criterion standard, rapid methods for sterility screening will play a more important role in transfusion medicine in the future. In particular, nucleic acid amplification techniques (NATs) are powerful potential tools for bacterial screening assays. The combination of excellent sensitivity and specificity, reduced contamination risk, ease of performance, and speed has made real-time polymerase chain reaction (PCR) technology an appealing alternative to conventional culture-based testing methods. When using real-time PCR for the detection of bacterial contamination, several points have to be considered. The main focus is the choice of the target gene; the assay format; the nucleic acid extraction method, depending on the sample type; and the evaluation of an ideal sampling strategy. However, several factors such as the availability of bacterial-derived nucleic acid amplification reagents, the impracticability, and the cost have limited the use of NATs until now. Attempts to reduce the presence of contaminating nucleic acids from reagents in real-time PCR have been described, but none of these approaches have proven to be very effective or to lower the sensitivity of the assay. Recently, a number of broad-range NAT assays targeting the 16S ribosomal DNA or 23S ribosomal RNA for the detection of bacteria based on real-time technology have been reported. This review will give a short survey of current approaches to and the limitations of the application of real-time PCR for bacterial detection in blood components, with emphasis on the bacterial contamination of PCs.

Despite considerable advances in the safety of blood components, transfusion-associated bacterial infection remains an unresolved problem, with significant transfusion-related morbidity and mortality rates. Platelet concentrate (PC) transfusion-associated sepsis is now recognized as the most frequent infectious complication in transfusion therapy, surpassing by up to 2 orders of magnitude the incidence of transfusion-associated viral transmission.\(^1,2\) The bacterial contamination of PCs is a major problem because of the current requirement to store PCs at room temperature with agitation (aerobic conditions) to preserve platelet function. At 22°C to 24°C, bacteria grow more easily than under lower temperature conditions, so that small bacterial inocula can grow to very high numbers within a short period. Consequently, older units are most likely to have high bacterial inocula and are therefore more likely to cause sepsis in recipients.

The bacterial contamination rate of whole blood held at room temperature for 2 to 20 hours is reported to be 0.34%.\(^3\) Recent reports suggest that platelet-related bacteremia occurs at a frequency approximately 50 times greater than that for red blood cells.\(^3\) Approximately 80% of red cell–associated sepsis involved psychrophile bacteria or microbes capable of growth at refrigerated temperatures, including *Yersinia enterocolitica* (46%), *Pseudomonas* sp (25%), and *Serratia* sp (11%).\(^5\) Cell-free products such as plasma and cryoprecipitates are stored frozen and are rarely associated with bacterial contamination.\(^1\) Therefore, in the scope of bacterial safety, highest attention is given to PCs because they represent a good growth medium and the storage conditions support bacterial growth.\(^6\) The reported prevalence of bacterially contaminated PCs varies from 0.08% to 0.7% in countries that perform prospective testing, depending on their technology, testing protocols, and additional intervention methods.\(^7,8\)

Different screening methods have been developed for bacterial detection and can be divided into culture and rapid-detection methods\(^9-18\); but to date, none of these methods is sufficient as a perfect
preventative screening for the detection of contaminated units. General diagnostic difficulties for the detection of bacterial contamination in blood products are the limited diagnostic window, the heterogeneity of the target organisms, and the advanced demand on sensitivity to detect low bacterial cell numbers present at the beginning of the storage of blood products (less than 100 colony forming units [CFU] in 200 mL). These problems especially occur in sterility controls of PCs because only 5 days of storage is allowed, and the low initial contamination level of the product is below the detection limit of all methods. Even the most sensitive assay based on the cultivation of bacteria (eg, automatic blood culturing systems) particularly detects 1 CFU per assay, but slow-growing bacteria such as *Propionibacterium* sp may be missed or are detected too late (5-7 days after PC preparation), when blood products have already been transfused. In conclusion, the detection of bacterial contamination of blood components generally requires time for the organisms to proliferate.2,9,19 This is in contrast to viral contamination of blood products, in which the virus or the immune response to the virus usually can be detected from a sample obtained at the time of donation.

In line with this diagnostic dilemma, several rapid methods have been developed for sterility testing of blood products.5,17 Moreover, molecular genetic techniques are theoretically appealing for the detection of bacteria in blood components because of their high sensitivity and specificity and the rapidity of obtaining results. At present, the high potential of nucleic acid amplification techniques (NATs) for application in sterility testing has not been exhausted. Such assays, adaptable to transfusion medicine, are not commercially available so far, although several working groups have developed and validated home-brewed NAT assays. This review will give a short survey of the present situation with real-time molecular diagnostics regarding the sterility testing of blood products.

**MOLECULAR TECHNOLOGY**

**Real-Time PCR**

Real-time PCR has revolutionized the way clinical microbiology laboratories diagnose human pathogens. The technique is extremely sensitive, rapid, capable of high throughput, and relatively easy to perform.20-23 With its ability to measure PCR products as they accumulate in “real time,” it has become possible to determine the amount of PCR product accumulated during the exponential phase of bacterial growth, which can in turn be used for the quantitation of nucleic acids. This testing method combines PCR chemistry with fluorescent probe detection of amplified products in the same reaction vessel. In general, both PCR and amplified product detection can be completed in 1 hour or less, which is considerably faster than conventional PCR and detection methods.24 Real-time PCR has several advantages over other PCR-based quantification approaches, including the elimination of postamplification handling, no cross-contamination with PCR products, easier automation, and processing of large numbers of samples. Sensitive and specific detection is possible by using novel fluorescent probe technology. Three types of nucleic acid detection methods have been used most frequently with real-time PCR testing platforms in clinical microbiology: 5’ nuclease (TaqMan probes), molecular beacons, and hybridization probes. These detection methods all rely on the transfer of light energy between two adjacent dye molecules, a process referred to as *fluorescence resonance energy transfer*.20,24,25 In real-time PCR assays to detect bacterial contamination in blood products, TaqMan probes were preferred10,14 because of the limited target regions that can be used for primer and probe hybridization. Especially the use of the locked nucleic acid and minor groove binder probe technology in combination with short oligonucleotides permits a better efficiency of PCR because of lower interference during the amplification process. The shorter length gives the probes better sequence specificity and lower fluorescent background in comparison with conventional probes.26-28 Furthermore, the chemical modification of the probes can improve the hybridization affinity for complementary sequences, increase the melting temperature (Tm) by several degrees, and therefore improve the specificity of the assay. Using real-time PCR for the bacterial detection, several points have to be considered. The main focus refers to the choice of the target gene and the assay format. At present, there are two real-time NAT assays published that detect bacterial contamination in PCs.14,18 These two NAT assays will be compared in the following sections, and several other different approaches for the detection of bacterial contamination will be presented.
Choice of Target for Broad-Range Bacterial NAT

A general requirement for both the primer and the probe sequences is their unique ability to identify a specific organism or an organism group (eg, transfusion-relevant bacteria species). Moreover, the PCR primer has to be able to identify the target sequences in the specimen of interest with high efficiency and specificity (eg, blood, plasma, PC). The target nucleic acid sequence should be conserved in bacteria. If sequence data of the intended target area show a significant frequency of polymorphisms, a more conserved site should be chosen.20

For screening methods, all potentially occurring bacteria species have to be detected with nearly the same efficiency. Consequently, broad-range amplification techniques are preferred for this purpose, rather than the specific detection of a limited group of bacterial genera (eg, Enterobacteriaceae29) or species (eg, Y enterocolitica30,31). In blood products, a relatively limited spectrum of bacterial species has been isolated, depending on the product itself.1 In PCs, skin-associated gram-positive bacteria (mainly Staphylococcus sp, Bacillus cereus, and Propionibacterium acnes) have been implicated in most contamination cases (up to 71%), but gram-negative organisms (mainly members of the Enterobacteriaceae) also account for most (82%) of the transfusion fatalities.1,4,5 A number of broad-range PCR methods for the identification and detection of bacteria have been reported, targeting the 16S ribosomal RNA (rRNA),14,29,31-35 23S rRNA,10,18 tuf genes (encoding elongation factor EF-Tu),36 rpoB (encoding RNA polymerase β-subunit),37 and groEL genes (encoding heat shock proteins).38 These targets are present in all bacteria and accumulate mutations at a slow, constant rate. Hence, these genes were used as molecular clocks in a phylogenetic analysis of bacteria.39 However, these housekeeping genes exhibit different degrees of conservation (Fig 1). Highly variable regions of genes contain unique signatures for each bacterium, as well as information about the relationships between different bacteria. These DNA sequences are used for phylogenetic analysis, identification, and specific detection of bacteria. Conserved

<table>
<thead>
<tr>
<th>Multi-copy Genes</th>
<th>Single-copy Genes</th>
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</thead>
<tbody>
<tr>
<td><strong>mr (ribosomal RNA operons)</strong></td>
<td>tuf A (other rpoB, groEL)</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>high (rDNA genes), variable (ITS)</td>
<td>Degree of conservation: variable</td>
</tr>
</tbody>
</table>

Fig 1. Comparison of bacterial marker molecules used as targets in broad-range NAT. The molecule size and copy numbers are attributed to *E coli*. The number of variable and therefore informative positions for the bacteria domain is depicted as described by Ludwig et al.39 The information bits are expressed as a logarithm (base 2) of the number of possible character states (4 n = 20 aa) times the number of *E coli* positions. Abbreviations: groEL, gene that encodes the 60-kd heat shock protein GroEL; rpoB, gene that encodes the RNA polymerase β-subunit; rrn, ribosomal RNA operon; tDNA, transfer DNA; tRNA, transfer RNA; tufA, gene that encodes the translational elongation factor EF-Tu.
regions of sequence are found in all known bacteria; hence, these regions are used for broad-range PCR to amplify intervening, variable, or diagnostic regions. In screening assays, these conserved regions are targets for broad-range primers and probes that permit the detection of bacteria independently of bacterial culture or knowledge of phylogenetic origin. The 16S ribosomal DNA (rDNA)–based techniques have historically been most commonly used; hence, in comparison to other genes, for example, 23S rRNA, a high number of complete sequences for the 16S rRNA are currently available. Although, the overall phylogenetic information content of the 23S rRNA molecule is greater than that of the 16S rRNA molecule (Fig 1), the 16S rRNA broad-range PCR remains the criterion standard. Recently, the development of broad-range PCR targeting the 16S-23S rRNA intergenic transcribed spacer (ITS) or 23S rRNA gene has become popular. The limited DNA sequence data and the lower degree of conservation of the other bacterial housekeeping genes such as tuf, groEL, or rpoB have been neglected, but could be used for other diagnostic questions, for example, viability assays. Although the degree of conservation of the target gene is the major criterion in broad-range bacteria PCR, there are several additional points to consider when designing a molecular genetic bacterial screening assay. For bacterial screening tests, the sensitivity of the assay is crucial. Thus, the copy number of the target molecule is a main factor. Looking at the DNA level, multicopy genes such as rRNA genes exhibit up to 12 copies per bacterial cell, compared with single-copy genes such as groEL or tufA that reveal only one copy per bacterial cell. This ratio has evidently shifted when looking at the RNA level of these genes. Depending on the growth rate, microorganisms can synthesize between 1000 and 50,000 copies of rRNA molecules per cell, stabilized in the ribosomes in nucleoprotein complexes. The use of RNA molecules as a target in NAT achieves enhanced sensitivity. This was demonstrated in bacterial reverse transcriptase PCR (RT-PCR). The use of ITS regions for molecular genetic detection at the RNA level is disadvantageous because these targets are lost during processing of the primary transcript. When using RNA as a target in NAT, the copy number controlled by the gene expression and half-life of RNA is crucial. Thus, the regulation of gene expression and stability of the synthesized RNA is of importance. For example, the groEL gene, encoding the heat shock protein Hsp60, is induced by stress; components of the translation machinery such as EF-Tu or rRNA are extensively expressed in growth phases. The elongation factor EF-Tu is one of the most abundant proteins in bacteria, present in about 100,000 molecules per cell, which is as much as 5% of the total cell protein. It is encoded by 2 different genes called tufA and tufB. The amounts of EF-Tu relative to ribosomes vary from 3-fold to 14-fold depending on the growth conditions (corresponding to the transcription of tuf genes).

Molecular Viability Markers

Ribonucleic acid stability is an important factor in gene regulation. In contrast to DNA, messenger RNA (mRNA) is turned over rapidly in viable cells, whereas rRNA has a stability comparable to DNA. Most mRNA species have half-lives measured in seconds to minutes. For example, tufA mRNA has a half-life of 3.0 minutes. A wide range of stabilities was observed for individual mRNAs of Escherichia coli, although approximately 80% of all mRNAs have half-lives between 3 and 8 minutes, which is much shorter than that described for rRNA molecules. The rRNA molecules 16S and 23S rRNA do not indicate the viability status of cells killed under in vitro conditions. Hence, detection of mRNA by RT-PCR as opposed to DNA-based methods is considered a better indicator of cell viability. The viability question is of importance when positive results are obtained with DNA-based NAT. Hence, the detection of bacterial DNA always indicates sterility problems, especially in blood products. However, PCR-positive results can lead to misinterpretation. This problem is of major importance with clinical specimens from patients who undergo antibiotic therapy. When using NAT for bacterial detection, for example, for diagnosis of sepsis, endocarditis, or meningitis, it has to be considered whether the detected bacterial species are viable. At present, molecular genetic methods cannot determine bacterial viability without doubt; and therefore, cultural methods are still the criterion standard. However, in some instances, the latter strategy has failings, namely,
when slow-growing or uncultivable bacteria are involved. First approaches give hope for the future that molecular genetic viability testing will supplement culture methods.\textsuperscript{10,49} It has been demonstrated that the detection of bacterial \textit{groEL} mRNA with RT-PCR is a good marker for viability, in contrast to DNA or rRNA, which can still be detected days after the death of the bacteria.

**QUALITY CONTROL**

Nucleic acid amplification technique assays have to be validated according to the general and legal requirements referring to accuracy, precision, specificity, sensitivity (detection limit, quantitation limit), linearity, range, and robustness.\textsuperscript{57-61} In the International Conference on Harmonisation guidelines for the validation of analytical processes, various aspects of the validation of qualitative tests are indicated, which also apply partly to the NAT methods. These requirements are not all currently fulfilled for bacterial NAT. Both qualitative molecular genetic bacterial detection (sterility testing) and quantitative determination of bacterial load are taking place.\textsuperscript{14,44} The main focus in sterility testing of PCs refers to qualitative NAT assays. An important criterion for the evaluation of an analytical method (including qualitatively) is the determination of the lower detection limit, requiring defined standards. However, standards are not yet available for microbiological diagnostics and are thus produced by each laboratory individually. Ascertaining the target values of these standards is extremely problematic because no metrologically correct measuring system for these parameters exists. In addition, reference methods for bacterial quantification are not available. Target values for the so-called standards are therefore attained using either the routine methods biologically characterizing the standard or the same method used to screen the sample material. The routine methods used in microbiology are less reliable (incorrect and imprecise). The current practice is quantification via either (1) bacterial titer expressed as CFU or (2) the nucleic acid molecule for detection expressed in copies or genome equivalents. These problems are discussed in the following section.

\textit{Determination of the Lower Detection Limit}

A comparison between the detection limits of the different bacterial NAT assays is difficult because standardized methods and reference materials are not yet available. Real-time NAT assays permit the determination of the initial template concentration and, therefore, an accurate estimation of the molecule number or the lower detection limit of the assay. For viral approaches, there is a direct correlation between viral load and copy number determined by real-time NAT.\textsuperscript{24,28} This is restricted for quantitation of bacteria and can be applied only when the result is expressed in copy numbers and not in CFUs, CFU equivalents, or cell number.

For viral NAT assays, standardization was achieved by using well-characterized reference material, that is, calibrated material using a common standard unit (international units).\textsuperscript{52,63} For bacteria, there are several problems and questions regarding the establishment of a standard preparation. Bacteria are living organisms and in contrast to viruses are changeable regarding their metabolism. Thus, conservation of a representative state of bacterial growth is difficult or impossible. Nevertheless, first steps were taken to generate bacterial standards that can be used for spiking experiments of blood components.\textsuperscript{64} The bacterial standards that can be purchased from the Paul Ehrlich Institute (Langen, Germany) can be characterized by their growth kinetics using an impedance registering system and are frozen in human albumin during their logarithmic growth period. The CFU before and after freezing of the bacteria can thus be described as constant.\textsuperscript{16,65} To determine the analytical sensitivity testing of bacterial NAT, CFU standards can thus be used.\textsuperscript{18} This represents a compromise because bacteria stored frozen have downregulated metabolisms. In this lag phase of growth, fewer RNA copies per cell can be detected than in growing cultures. Hence, in RNA-based NAT, the analytical sensitivity of the method is underestimated because of the fact that this standard does not reflect the real cell number as occurring in growing cultures, for example, bacterial propagation in PCs. However, the introduction of CFU standards will help compare different DNA-based methods regarding their sensitivity. Besides the molecular genetic methods, these bacterial standards are very useful for spiking experiments, where defined inocula are needed.\textsuperscript{16,18,65,66} However, the extrapolation of results from an assay in spiked studies using fresh cultures of “healthy” inoculates or frozen bacterial standards to routine analysis can justifiably be questioned.\textsuperscript{57} When determining CFU, results
depend on various parameters, including culture conditions, differences in species and strain, overall cell count, living cell count, metabolic state, growth behavior, etc. Huge amounts of noncultivable dead cells or free nucleic acids that can be amplified by DNA-based NAT complicate the equalization of bacterial titer and CFU. Moreover, bacterial quantitation by real-time PCR assays is influenced by the variation in the number of gene copies in a given bacterial species. The copy numbers in a single chromosome of the main target in broad-range PCR, the rRNA operon, have varieties between 1 \((Mycoplasma \text{ sp.}^{[57]})\), 4 \((Pseudomonas \text{ aeruginosa})\), 7 \((E \text{ coli}^{[42]})\), and 9 \((Staphylococcus \text{ aureus}^{[43]})\). In addition, chromosomal replication can further increase the numbers of a given rRNA operon. The number of replication forks is directly related to the generation time, which in turn depends on the metabolic state of the bacteria at the time of sampling.\(^{44}\) Ignorance of the exact number of copies of rRNA operons in any given species at the time of sampling represents the main limitation to an absolute determination of bacterial numbers by real-time PCR based on rDNA.\(^{44}\) One real-time PCR assay developed based upon 16S rDNA amplification describes an analytical sensitivity of 1 CFU equivalent per PCR for the gram-negative bacterium \(E \text{ coli}(\text{corresponding to 50 CFU/mL})\) in PCs,\(^{14,68}\) but without any given definition of the growing conditions of the cultures. However, gram-positive bacteria occur more frequently in PCs,\(^{1,5}\) which make especially high demands on the nucleic acid extraction procedure. As a consequence of these major differences in efficiency of nucleic acid extraction and the variation of copy number interspecies and intraspecies, standard curves for the quantification of bacterial load are barely applicable for this purpose. The absolute quantification of CFU equivalents or cell numbers with standard curves is thus problematic. For instance, 1 CFU of \(S \text{ aureus}\) contains more cells than 1 CFU of \(E \text{ coli}\). This refers to the well-known dilemma regarding NAT vs culture-based methods, where we are actually comparing “apples” with “oranges.” Among other things, the measured CFU level depends on bacterial viability; thus, stationarily grown cultures, such as the standards used in this study, are not comparable with logarithmically grown cultures.

Most commonly, quantification or determination of analytical sensitivity is performed with DNA standards (amplicons, plasmids) for PCR or RNA standards for RT-PCR. These molecules represent parts of the target organism’s genome and are used to determine the bacterial load expressed in genome equivalents or copy numbers. This control forms the basis of an external standard curve created from the data produced by the individual amplification of a dilution series of exogenous control. The concentration of an unknown sample, which is amplified in the same reaction but in a separate vessel, can then be found from the standard curve. However, this quantitation strategy only images the amplification steps of nucleic acids but ignores the nucleic acid extraction step, the most critical within the NAT assay.

As discussed, the overall procedure for molecular genetic detection of bacteria is thus difficult to image. It would therefore be better to separate screening of the NAT assay (referring to the molecule for detection) and sample preparation (nucleic acid extraction). The NAT assay can then be characterized by the nucleic acid molecule for detection, whereas the lower detection limit, precision, and correctness of the method can all be determined. Sample preparation, that is, nucleic acid extraction, should be evaluated using defined amounts of molecule spiked in the matrix for screening (here PCs). To measure the efficiency and yield of the nucleic acid released during lysis of the bacteria, defined bacterial suspensions should be used. They should be produced under standardized culture conditions and be characterized with regard to overall cell count (counted using flow cytometry) and bacterial titer (expressed as CFU). The performance of the detection method can then be evaluated more successfully using the correlation between the bacterial count and the molecule count. Overall, improved standardization, or at least an established validation program, is required for bacterial NAT in the future. A further problem when evaluating these approaches is the comparability of the methods.

**Quality Control and Quality Assurance**

Importance has to be attached to the internal quality assurance scheme and, finally, participation in external proficiency trials, also called external quality assurance programs.\(^{57,58}\) Such trials for qualitative PCR assays are available for most clinically relevant viral and bacterial pathogens. Such external quality assurance programs for
bacterial screening NAT assays are not available today, and comparisons of different methods or laboratory findings are difficult. All PCR assays should include an internal amplification control (as discussed below), a reagent control, and the processing of both negative and positive controls (run control).20,57 Hence, screening of PCs for bacterial contamination with NAT assays should include controls that can be used to check the test suitability to ensure the reliability of the analytical procedure whenever used. This run control should be at a concentration near the lower detection limit of the assay to challenge the detection system, yet at a high enough level to provide consistent positive results. For viral NAT assays, this is realized by including a working reagent, for example, plasma.

Fig 2. Amplification plots of routine platelet screening using real-time RT-PCR. Nucleic acids of culture-negative tested PCs were extracted from 2.4 mL PC using magnetic separation technology and analyzed on the Rotorgene platform.18 For positive control (solid), PC was spiked with S. epidermidis (10⁷ CFU/mL). For the 2 low positive run controls (A, B: dashed), PCs were spiked with the PEI standards S. epidermidis (A, 50 CFU/mL) or K. pneumoniae (B, 50 CFU/mL). Abbreviations: FAM, 6-carboxy-fluorescein; JOE, 2',7'-dimethoxy-4',5'-dichloro-6-carboxyfluorescein; Norm. Fluoro. FAM 23S rRNA, normalized fluorescence of the amplification plots detecting the bacterial 23S ribosomal RNA in the FAM channel; Norm. Fluoro. JOE β2 microglobulin, normalized fluorescence of the amplification plots detecting the IC β2 microglobulin messenger RNA in the JOE channel; negative control, H₂O (dotted).
spiked with a hepatitis C virus sample calibrated against the WHO Hepatitis C Virus International Standard. For bacterial NAT assays, the choice of control material is more difficult because of the lack of standardization, as discussed below. Recently, PCs spiked with bacterial standards were applied for processing positive controls, whereby the bacterial titer was twice the 95% detection limit of the assay. To take account of the bacterial differences, controls for gram-positive (Staphylococcus epidermidis) and gram-negative bacteria (Klebsiella pneumoniae) were used, as shown in Figure 2. In addition, a sterile PC aliquot should be used as a nonreactive processing control to demonstrate that nonspecific PCR amplification and detection of amplified product are not occurring. In addition, a negative control is optionally used to demonstrate that the reagents are not contaminated with target nucleic acid and can be used to compensate for background signal generated by the reagents. This is of extreme importance for broad-range bacterial NAT assays, where contamination is the omnipresent problem, as discussed in “Limitations of Bacterial NAT Assays by Nucleic Acid Contamination.”

Internal Control

In addition, consideration should be given to the incorporation of an internal control (IC) in NAT assays. An inherent problem in diagnostic PCR is the presence of amplification inhibitors that may cause false-negative results. Therefore, the addition of an amplifiable nucleic acid in the PCR assay serves as an IC, an important quality control, and has already been described for early PCR experiments. The use of an IC is mandatory for blood screening NAT tests, as decided by the German federal licensing agency Paul Ehrlich Institute. An IC for diagnostic NAT assays should be easy to produce and to standardize, without affecting the efficiency of bacterial target amplification.

An exogenous IC is added before nucleic acid isolation (extraction control) or amplification (amplification control), where coamplification is performed within the same reaction. Ideally, these ICs hybridize to the same primers, have identical amplification efficiencies, and contain discriminating features, such as length or sequence variations, targeted by hybridization probes. However, these competitive ICs can lower the amplification efficiency, which results in a lower detection limit. Therefore, noncompetitive IC templates are used, where the target and IC are amplified with different primer sets. The detection of model viruses spiked to clinical specimens is a well-established system for exogenous ICs to monitor the efficiency of extraction and amplification. Especially bacteriophages such as lambda or MS2 are often used and provide process control in many NAT assays. The advantage of such model viruses is the stability of RNA and the control of decapsulation of the viral DNA and RNA during the extraction procedure. One disadvantage is that amplification of the IC may not accurately reflect amplification of the target.

An endogenous IC is a template that occurs naturally within the specimen being analyzed. In gene expression analysis and virus screenings, housekeeping genes are often used as ICs and references for transcript quantification. The same approach was chosen for NAT to detect bacterial contamination in blood components. Mohammadi and coworkers assessed the efficacy of DNA extraction by coamplification of the human HLA-DQA gene that was coextracted from PCs. In addition, the potential inhibition of the bacterial amplification system was controlled by spiking PCRs with bacterial control DNA from Bordetella avium.

Another approach was performed by Störmer et al. The coamplification of the human glyceraldehyde-3-phosphate dehydrogenase mRNA served as an IC. Because of better performance in duplex RT-PCR, another transcript from the human housekeeping gene β2 microglobulin was chosen.

REAL-TIME PCR FOR BACTERIA SCREENING OF BLOOD COMPONENTS

The high sensitivity, specificity, and rapidity of results of molecular biologic methods have made them appealing for the detection of contaminated blood products. Until today, culture methods, which require a long time to indicate the presence of bacteria, have remained the criterion standard, although there have been various approaches to detecting bacteria in PCs. Polymerase chain reaction has opened up new detection possibilities for slow-growing pathogens, intracellular bacteria, and viable but nonculturable pathogens. The detection of bacteria in blood by real-time PCR
<table>
<thead>
<tr>
<th>Sample</th>
<th>PCR Mix</th>
<th>Detection limit (CFU/mL)</th>
<th>Authors</th>
</tr>
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<tbody>
<tr>
<td>Whole blood</td>
<td>100</td>
<td>5000</td>
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<td>Harris and Hartley, 2003</td>
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<td>260</td>
<td>12-16</td>
<td>Sen, 2000</td>
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<td>Rider and Newton, 2002</td>
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<td>NS</td>
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<tr>
<td>Blood culture</td>
<td>1000</td>
<td>Real-time PCR</td>
<td>Not specified</td>
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</table>

Abbreviations: all, a chromosomal gene of *Y enterocolitica* that encodes a 17-kd outer membrane protein; *groEL*, gene that encodes the 60-kd heat shock protein GroEL; NS, not specified; *virF*, virulence gene of *Yersinia* species.
has been described in only a few studies, as shown in Table 1. It is stated that the ideal test should be simple to perform; rapid; not prohibitively expensive; capable of detecting all organisms at low levels; and applicable for erythrocytes, platelets, and plasma products. Depending on the sample type, the extraction of nucleic acids requires a different treatment. Problems associated with the PCR detection of bacteria in clinical samples include the extraction difficulty of nucleic acids from the sample and their subsequent purification to remove PCR inhibiting substances. Samples of whole blood or blood components are notoriously bad PCR targets because they contain inhibitory factors such as hemoglobin. Therefore, displayed studies are often optimized for the use of a specific sample matrix, especially for PCs. The use of a nonamplified chemiluminescence-linked universal bacterial rRNA probe was one of the first approaches that was able to detect $2.1 \times 10^5 \text{ CFU/mL}$ PC in 60 to 90 minutes. This NAT assay can detect a wide variety of bacteria and has been recently improved by semiautomation.\textsuperscript{74,75} Subsequently, the amplification of bacterial rRNA sequences has been evaluated by several investigators. Feng et al\textsuperscript{11} investigated the use of PCR in the detection of \textit{Y. enterocolitica} in whole blood that detected $5 \times 10^3 \text{ CFU/mL}$. This assay requires 6 hours but is capable of detecting only 1 bacterial pathogen. Recently, Sen developed a 5′ nuclease TaqMan probe PCR assay based on the nucleotide sequence of the 16S rRNA gene from \textit{Y. enterocolitica} and detected as little as 30 CFU/mL in 2 hours.\textsuperscript{29}

Broad-range primers were used by Harris and Hartley\textsuperscript{33} in conventional PCR that detects 1 to 10\textsuperscript{3} CFU/mL. Notably, a number of broad-range PCR methods targeting the 16S rDNA for identification and detection of bacteria based on real-time PCR have been reported.\textsuperscript{14,29,30,34,35,77}

### Table 2. Comparison of Two Platelet Bacteria Screening Strategies Using Real-Time NAT

<table>
<thead>
<tr>
<th>Sampling</th>
<th>23S rRNA RT-PCR\textsuperscript{18}</th>
<th>16S rDNA PCR\textsuperscript{14}</th>
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<tr>
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<tr>
<td>Nucleic acid extraction</td>
<td>2.4 mL</td>
<td>200 µL</td>
</tr>
<tr>
<td>Kit</td>
<td>Chemagic viral DNA/RNA kit</td>
<td>MagNA Pure total nucleic acid isolation kit</td>
</tr>
<tr>
<td>Instrument</td>
<td>Magnetic Separation Module I (Chemagen)</td>
<td>MagNA Pure LC Instrument (Roche)</td>
</tr>
<tr>
<td>Technology</td>
<td>Magnetic bead technology</td>
<td>Magnetic bead technology</td>
</tr>
<tr>
<td>Time</td>
<td>75 min</td>
<td>25-40 min</td>
</tr>
<tr>
<td>Nucleic acid</td>
<td>RNA and DNA</td>
<td>DNA</td>
</tr>
<tr>
<td>NAT</td>
<td>Real-time RT-PCR</td>
<td>Real-time PCR</td>
</tr>
<tr>
<td>Decontamination method</td>
<td>None</td>
<td>Ultrafiltration of reagents using Plasmid Maxiprep binding columns, Sau3AI digestion of the PCR mix, UNG DNA</td>
</tr>
<tr>
<td>Target</td>
<td>23S rRNA</td>
<td>16S rDNA</td>
</tr>
<tr>
<td>Volume equivalent of nucleic acid input*</td>
<td>0.24 mL</td>
<td>0.02 mL</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>22-29 CFU/mL</td>
<td>50 CFU/mL</td>
</tr>
<tr>
<td>Real-time instrument</td>
<td>Rotorgene 3000 (Corbett Research)</td>
<td>ABI 7700 (Applied Biosystems)</td>
</tr>
<tr>
<td>Rime for RT-PCR</td>
<td>65 min</td>
<td>62 min</td>
</tr>
<tr>
<td>Time for whole process</td>
<td>4 h</td>
<td>4 h</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCR inhibition control</td>
<td>Coamplification of β2 microglobulin mRNA</td>
<td>Coamplification of human HLA-DQA DNA</td>
</tr>
<tr>
<td>Run control</td>
<td>Coamplification of spiked \textit{B. avium} DNA (50 CFU/mL)</td>
<td>Coamplification of spiked \textit{B. avium} DNA (50 CFU/mL)</td>
</tr>
</tbody>
</table>

\*Volume (V) equivalent of PC set in NAT ([V_{template} / V_{elute}] \times V_{sample} per NAT reaction).

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Nadkarni et al described the use of universal primers and probes targeting a conserved region of the 16S rDNA to estimate the total bacterial load in clinical samples. Several investigators used this primer-probe system and adapted it for sterility testing of blood components. Mohammadi et al developed a broad-range PCR assay based on real-time PCR technology to monitor bacterial contamination in PCs. This assay enables the detection of 50 CFU equivalents per milliliter PC. To remove contaminating DNA from the reaction mix, different approaches using enzyme digestion, UV irradiation, and ultrafiltration were discussed.

A different approach was the development of RNA targeting real-time RT-PCR assays for platelet bacteria screening. Two real-time RT-PCR methods targeting conserved regions of the eubacterial 23S rRNA gene or the groEL gene (encoding the 60-kd heat shock protein Hsp60) were developed and optimized for use in the routine laboratory with a detection limit of 22 to 29 CFU/mL PC. Only these two investigators have presented their real-time approach for platelet bacteria screening to date. As shown in Table 2, both appeal for their sampling 24 hours after donation (apheresis-derived PC) or preparation (pool PC) at the earliest. The 16S rDNA targeting assay combines an automated DNA extraction from a 200-μL sample and real-time PCR in 4 hours. Decontamination of the reagents in the extraction kit is achieved by filtration through plasmid-binding columns, whereas the PCR mix is decontaminated using enzyme digestion. Amplification of the coextracted human HLA-DQA DNA provides an extraction control, and that of spiked B avium DNA an amplification control.

In contrast, the 23S rRNA targeting assay combines an automated high-volume DNA and RNA extraction from a 2.4-mL sample and real-time RT-PCR in 4 hours without decontamination treatment. Coamplification of β2 microglobulin mRNA serves as extraction as well as an amplification control. Furthermore, two run controls are included in each run, which contain low bacterial titers to control the sensitivity of the whole process, as shown in Figure 2.

Sampling Strategy for Bacteria Screening

The efficacy of bacterial screening methods and their usefulness as routine bacterial detection techniques are influenced by many factors, such as bacterial growth kinetics, the sample volume, and the time of sampling. In contrast to viruses, bacteria are not static and proliferate during storage; thus, the choice of the sampling day for the bacterial screening is crucial. If sampling is too late, it will limit the availability of components; but if it is too early, it will trigger the release of components that would be detected as bacterially contaminated if tested later. The initial levels of bacteria in the PC units are usually exceedingly low, below the detection limits of current screening systems and below the level considered clinically significant (10^5 CFU/mL). Therefore, sampling on the day of collection invariably misses bacterial contamination. With longer times between sampling and testing, there is a higher probability that all contaminated units will be identified.

Bacterial growth is variable and depends on the initial bacterial load. Three bacterial groups can be distinguished: bacteria that have no capacity to proliferate, bacteria that survive and are able to grow, and bacteria that remain in the lag phase of growth for up to 6 days. Culture studies performed both on packed red cell units and platelets have shown that culture on the day of collection invariably misses bacterially contaminated PCs. It has been shown that sampling for cultural detection on day 2 would detect most of the bacteria capable of causing bacterial sepsis despite a very low initial inoculum.

Studies to determine the optimal time for sampling PCs for real-time PCR screening showed that the sampling time is obviously not that critical when PCs are contaminated with fast-growing organisms, resulting in a sufficient bacterial load in the blood product being tested. The detection of slow-growing organisms remains more problematic because the most common organisms concerning platelet contamination, such as S epidermidis, exhibited a characteristically extended lag phase before rapid proliferation after 48 hours. Therefore, it was concluded that sampling should not be carried out earlier than 24 hours after preparation (apheresis-derived PC) or 48 hours after blood donation (pooled PC) because this preenrichment should enable most of the contaminated PCs to be detected, as shown in Figure 3. Aseptic sampling could be achieved by the connection of a sterile transfer
pack container to the PC unit using a sterile tubing welder.  

Another critical factor is the sample volume that could have a significant impact on the amount of product left for transfusion, particularly for random donor units. Therefore, a larger sample volume improves the sensitivity but depletes the product. The required sample volume should be such that the given sample will contain at least 1 organism. As shown in the nucleic acid extraction section, commercially available extraction methods are able to process only a sample volume of up to 1 mL. The risk of sampling errors is therefore enormously high and cannot comply with requirements mentioned above when sampling is processed too early. For the first time, the use of a high-volume extraction method was adapted for use in platelet bacteria screening to minimize sampling errors and to improve the sensitivity of the method used.

A different approach is incubation of the sample at a higher temperature,  that could shorten the detection times for slow-growing organisms. With regard to sampling errors, taking the sample at an early stage for incubation at a different temperature means that contaminated units could be missed. Moreover, further incubation in a synthetic growth medium at 35°C after sampling and after 24-hour storage of the PC is another sampling strategy approach. Nevertheless, 24-hour storage of the PC unit seems to be indispensable. This screening procedure is a justifiable compromise between the sensitivity of NAT assays, the diagnostic window period, and the delay of the PC supply.

**Nucleic Acid Extraction From Blood Products**

The prerequisite benefit from the advantages of PCR is efficient protocols for the extraction of nucleic acids. The overall sensitivity of the assay is determined by the nucleic acid yield, its purity, and the amount of sample equivalents that can be transferred to the amplification reaction. Until today, an array of commercially available nucleic acid isolation systems has been developed, offering standardized, quality-controlled reagents with optimized compositions for all steps of the process. However, some fail to detect a minor amount of

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**Fig 3.** Determination of the optimal sampling time for bacterial screening using real-time RT-PCR on a slow-growing organism. One single apheresis-derived PC unit was spiked with approximately 1 CFU/mL of *S. epidermidis* and stored at 22°C. Samples were taken in duplicate before inoculation (negative control at 0 hour) and at different times (4, 14, 17, 24, 38, 44, 60, 72, and 96 hours after inoculation) and enumerated by plating culture (line including points indicates the bacterial growth representing the bacterial load at the time of sampling). Nucleic acids were extracted using magnetic separation technology and analyzed on the Rotorgene\textsuperscript{18} (threshold cycle \([C_T]\) values of the real-time RT-PCR detection are displayed in bars). *Staphylococcus epidermidis* showed an initially long lag phase and rapid growth after 44 hours of storage. Samples taken before and at 4, 14, and 17 hours after inoculation were tested negative \([C_T > 45]\) in real-time RT-PCR. Bacteria were detected for the first time when sampling was performed 24 hours after inoculation. Here the amplification of a PCR product is first detected. This is determined by identifying the cycle number at which the reporter dye emission intensity rises above a background noise. That cycle number is referred to as the *threshold cycle*. 

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\[C_T\]
bacterial DNA among an abundance of human DNA. Therefore, an optimal sample preparation procedure should efficiently break very resistant bacteria cell walls, without being too harsh for the nucleic acids released from the cells that are easily lysed, and should remove substances that may inhibit amplification, such as heme. Moreover, the released nucleic acids should be maintained into a small volume of aqueous solution to protect them from degradation. With regard to the sensitivity of the assay, a maximum amount of the sample has to be set in the nucleic acid extraction procedure. Extraction of clinical specimens can be accomplished either by manual or by automated methods. Conventional manual sample preparation methods are labor-intensive and susceptible to contamination, handling variations, or errors. Automation aspires to avoid human errors, to improve exactness, to reproduce results, and to permit analysis of samples in large numbers. For PCs, commercially available extraction methods have been successfully used to detect bacterial contaminations. Manual Nucleic Acid Extraction

Classic phenol extraction was successfully used to prepare DNA for amplification in the early diagnostic applications of PCR. However, the use of corrosive and toxic agents represents a safety hazard in the clinical laboratory. Several manual extraction kits using noncorrosive agents have been developed. Researchers are therefore able to choose the technique most suited to their target, source, or starting material. Classic methods of DNA and RNA isolation are based on column or precipitation methods. These techniques require centrifugation or vacuum steps and often have long processing times, as well as volume limitations. Moreover, the use of ethanol to precipitate the nucleic acids can inhibit the PCR when it is not properly removed. Manual extraction of bacterial nucleic acids from PCs was performed with glass-fiber fleece immobilized in a special plastic filter tube and subjected to centrifugation. For efficient lysis of bacteria, mechanical and enzymatic degradation of cell walls was carried out. This approach leaves room for the option of isolating total nucleic acid or, after DNase treatment, pure RNA.

Automated Nucleic Acid Extraction

Magnetic separation techniques have several advantages over standard separation procedures. This process is usually very simple, with only a few handling steps. Automated systems are typically closed systems to minimize the contamination risk and walkaway systems that do not require constant attention. Furthermore, the recovery of nucleic acids from automated instruments is consistent and reproducible. However, besides these benefits, potential drawbacks such as the cost of equipment and space in the laboratory also have to be considered. Most recent developments use magnetic beads that bind the nucleic acids to their silica surface and transfer them through the various steps of the extraction process. This technology was successfully evaluated for the extraction of viral nucleic acids for routine blood donation screening and adapted for the extraction for bacterial nucleic acids from PCs for sterility testing by Störmer and colleagues. Using the Chemagic viral DNA/RNA kit in conjunction with the Magnetic Separation Module I (Chemagen, Baesweiler, Germany), high-volume simultaneous extraction of total DNA and RNA is performed. This method uses magnetic beads, consisting of an iron oxide core surrounded by a nucleic acid–binding matrix, for direct capture of total nucleic acids. After cell lysis, nucleic acids bind to these magnetic beads and can be separated using the Chemagic Magnetic Separation Module I. The advantages of extraction with this technology are the absence of a precipitation step, which often causes problems with yield and purity; the simultaneous extraction of both species of nucleic acids; and the possibility of using a high sample volume of 2.4 mL with regard to minimizing the sampling error. Nucleic acid isolation of up to 12 high-volume samples in one batch on the Chemagic Separation Module I is accomplished in approximately 75 minutes. A different method is the coverage of magnetic beads with nucleic acid–binding matrices. The MagNA Pure system (Roche Diagnostics, Mannheim, Germany) consists of a fully automated bench-top instrument and ready-to-use nucleic acid isolation kits with prefilled cartridges, permitting automated isolation of nucleic acids from 1 to 8 samples within 25 to 40 minutes.

Limitations of Bacterial NAT Assays by Nucleic Acid Contamination

Several factors have limited the direct amplification of bacterial nucleotide amplification in blood products. The major practical problems associated
with the use of broad-range rDNA PCR are the contamination of the assay by exogenous bacterial DNA of the nucleotide amplification reagents, particularly of the bacterially derived enzymes, and the presence of bacterial DNA sequences commonly found in human blood. This complication has led to a number of diagnostic laboratories abandoning the idea of adopting broad-range rDNA PCR techniques as part of their diagnostic service. To exclude carryover contamination from previously amplified DNA, the enzyme uracil-N-glycosylase (UNG), which cleaves the uracil base from the phosphodiester backbone of uracil-containing DNA, could be used. The enzyme has no effect on thymine-containing DNA. Because of the use of recombinant enzymes that were expressed and purified from *E. coli*, additional contamination of the reagents by any kind of eubacterial DNA and RNA cannot be excluded by using UNG; and this limitation still represents a serious problem.

*Taq* DNA polymerase has often been found to be contaminated with bacterial DNA or rRNA because of its high affinity for DNA, which is copurified during enzyme production. Several investigators have reported this problem in broad-range bacterial PCR and have attempted to overcome it using a number of different procedures. Recently, Corless et al have described numerous problems associated with use of the TaqMan system and 16S rDNA PCR. Attempts to decontaminate PCR materials have involved methods to destroy DNA, including UV irradiation, 8-methoxypsoralen treatment, DNAse treatment, restriction enzymes, and combinations of these methods. Meier et al investigated a method to eliminate contamination of DNA with psoralen. Psoralens are known to intercalate into double-stranded nucleic acids and form a covalent interstrand cross-link after photoactivation with UV light. Therefore, use of 8-methoxypsoralen to extinguish the template activity of contaminating DNA has been suggested. Some investigators solved the problem of contaminated reagents by passing PCR reagents through centrifugal filter devices because these filters permit decontamination of all PCR reagents, including UNG, *Taq* polymerase, primers, and probes, which was not possible using other methods such as DNase treatment. Furthermore, an additional approach to remove contaminating DNA from the isolation kit reagents was filtration using a nucleic acid binding column. However, it was found that most decontamination methods decreased PCR sensitivity; and thus, none of these methods has been shown to be entirely effective or reproducible. As a result, some conclude that reducing the number of PCR cycles is the most effective way of avoiding the amplification of contaminant DNA that gives false-positive results; but this would lower the sensitivity of the assay as well. Nucleic acid free enzymes are needed, but they are still not available. The expression of DNA polymerases in eukaryotic systems would solve the purification problem, but patent claims or the in vitro diagnostic medical devices directive represents insurmountable obstacles.

**CONCLUSIONS AND FUTURE PERSPECTIVES**

Real-time NAT is a powerful tool in clinical diagnostics and will possibly acquire enhanced significance for sterility testing in transfusion medicine. Initial skepticism about molecular genetic tests has been observed, as occurred when NAT assays were introduced for the virus screening of blood products. The application of broad-range bacterial NAT is demonstrated in several studies. These methods are highly specific, very sensitive, and rapid. The relatively short turnaround time of NAT assays provides the potential for them to be used before the release of PCs for transfusion.

Nucleic acid amplification technique screening does not prevent all transfusion-associated bacterial septic reactions, as is the case for other diagnostic methods. Not every bacterial contamination plays an important role because not all species or even isolates of certain species are able to grow within human plasma or PCs. For instance, *Propionibacterium* species were detected more frequently in PCs with culture-based methods than with NAT. Inoculating blood culture systems and anaerobic cultivation detects these bacteria after approximately 3 to 7 days under optimized growth conditions; here the relevance for transfusion medicine has to be discussed. Anaerobic *P. acnes* show only slow or no growth under PC storage conditions. In addition to slow growth, the levels of bacteria in blood components are thought to be too low to result in sepsis upon immediate transfusion. Hence, their clinical significance may be low.
Hitherto, systematic studies of the outcome of patients transfused with *P. acnes*-contaminated PCs to demonstrate the transfusion relevance are missing. *Propionibacterium acnes* is not usually virulent, and only a few cases have been described in transfusion-related sepsis. In all cases, *P. acnes* was not isolated from the patients; and a cause-and-effect relation was not confirmed. Depending on the species level, differences of growth in PCs are observed. Therefore, detection of bacteria in PCs with culture-based methods must mean that these bacteria neither will propagate in this milieu nor cause transfusion-related sepsis.

Because of the low bacteria titers of about 10 to 100 bacteria per donation initially, the sensitivity of the detection method plays a crucial role. Microbiological detection requires a long incubation time, whereas NAT assays should be sensitive and fast enough for the routine contamination screening of PCs. To overcome sampling errors, a minimum preincubation of PCs has been proposed. This preenrichment should enable most of the contaminated PCs to be detected using current NAT methods; but it is to be expected that rare, slow-growing bacteria might escape such a detection scheme. An essential requirement is the standardization of NAT assays for the broad-range detection of bacteria. The dilemma is the lack of comparability to NAT methods and to other screening methods used for sterility testing.

In the future, molecular genetic sterility testing may also be requested for other blood components. Besides cellular therapeutic agents, for example, human stem cells, dendritic cells, or T cells, molecular genetic tests are of interest because methods prescribed in international pharmacopedia (European Pharmacopedia, Japanese Pharmacopedia, United States Pharmacopedia) are not applicable in all cases. Because of methodological limitations or time-consuming diagnostics, rapid methods will have to be employed, particularly if the blood product has a restricted durability or is prematurely needed for patient therapy. For such tissue cultures, the sterility assay has to also detect *Mycoplasma* species and fungal pathogens. Therefore, NAT assays developed for PC screening will have to be adapted and validated for this latter purpose.

In conclusion, the high potential of bacterial NAT screening of blood components has been demonstrated; but further studies are needed to prove its applicability for the routine contamination screening by transfusion services.

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