Whitepaper

DNA-Free Reagents and Materials for Molecular Pathogen Analysis

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Summary

Generally, microbial DNA is present at very low loads in clinical specimens. Molecular analysis by amplification assays, including NGS, can be a challenge because of a potentially multiple input of contaminating DNA from exogenous sources. Besides air-borne, handling and cross contamination, materials and reagents used in the molecular laboratory can contain microbial DNA. Practically this is counteracted by limiting the number of amplification cycles which, however, leads to a loss of detection sensitivity of target sequences. In this contribution, the selection of certified microbial DNA-free components for sample collection, DNA extraction, PCR amplification and NGS analysis are discussed with respect to the aim of building up a reliable, standardised molecular system for the analysis of bacterial and fungal organisms at the limit of detection.

Introduction

Microorganisms appear to be present in clinical samples at low loads. For instance, Wain et al. [1] cultured *Salmonella typhi* from blood at a median load of 1 cfu/ml (range, <0.3 to 387 cfu/ml). In another, more broadly focused study Phillips and Bradley [2] plated blood samples from neonates on chocolate agar. Cell counts determined for Gram-positive bacteria, Gram-negative bacteria, yeasts of the genus *Candida* and *Malassezia* and mixed infections of *Candida* and Gram-positives ranged from 1 to >100 cfu/ml, 3 to 8 cfu/ml, 18 to 96 and 66 to >100 cfu/ml, respectively. It is interesting to note that van den Brand et al. [3] calculated from quantitative PCR a median equivalent of colony forming units of $1.35 \cdot 10^4$/ml (range: 55 to $1.3 \cdot 10^7$/ml). Although there appears to be no direct comparison of results from culturing and molecular assaying, this finding may suggest that microbial loads are underestimated by culturing at least in case of pediatric patients. Nonetheless, the available data indicates low microbial loads in some clinical specimens.

Low loads of microorganisms are challenging to be analysed by molecular methods. For PCR or Real-Time PCR diagnosis, a highly sensitive assay is therefore necessary [4]. This holds true also for the analysis of low microbial load communities by Next Generation Sequencing

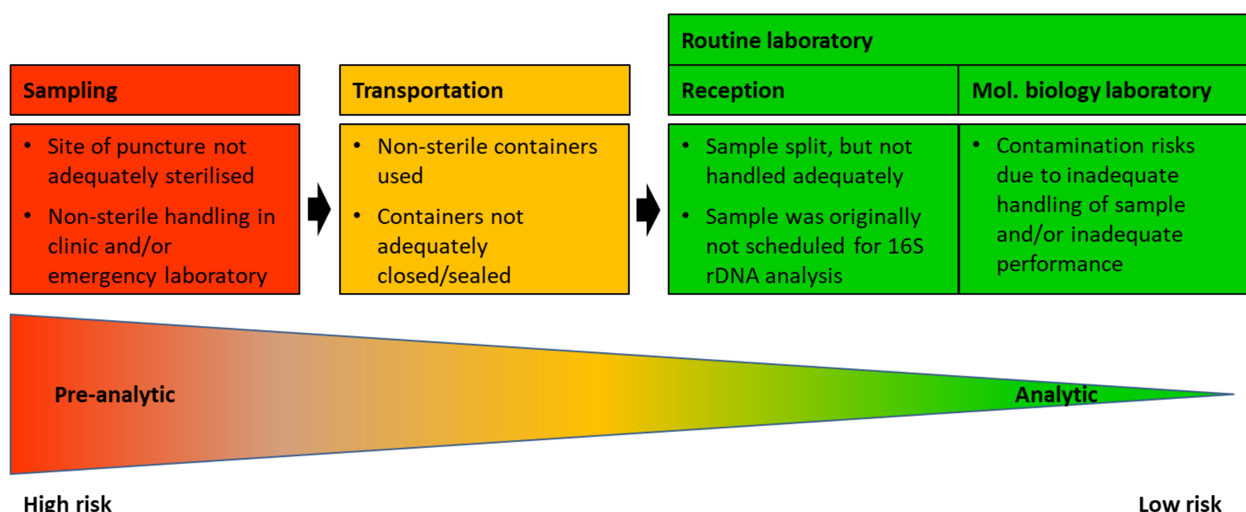

Fig. 1: Risk of contamination in the course of sampling, transport and analysis of clinical samples by 16S rRNA gene PCR. Modified after [6].

Table 1: *Microbial DNA contamination of materials and reagents employed in molecular analysis of pathogens in clinical samples* [a]

Process	% False positives (no. tests)	Origin	Reference
Sample collection			
Blood collection tubes	17 (185)	*Aspergillus* spp.	[8]
Blood serum tubes	10 (160)	*Aspergillus* spp.	[8]
	4 (50)	*Pneumocystis jirovecii*	[9]
Urine collection tubes	8 (25)	*Aspergillus* spp.	[8]
Forceps for tissue preparation	57 (23)	*Escherichia* spp., *Propionibacterium* spp., *Stenotrophomonas* spp. *Pseudomonas* spp.	[10]
Nucleic acid extraction and processing			
Zymolyase	n.d.	*Saccharomyces cerevisiae*	[11]
Lyticase	n.d.	unspecified fungus	[11]
DNA extraction	100 (20)	*Burkholderia* spp., *Pseudomonas saccharophilia*, *Ralstonia* spp., *Alcaligenes* spp.	[12]
	20 (20)	*Legionella* spp., *Aspergillus* spp.	[13, 14]
	n.d.	*Aspergillus* spp., *Candida* spp.	[15]
	n.d.	*Brucella* spp.	[16]
	<3 (36)	n.a.	[17]
Nucleic acid precipitation (glycogen)	22 (9)	*Acinetobacter lwoffii*	[18]
RNA stabilization reagent	5 (20)	*Aspergillus* spp.	[8]
PCR amplification			
Taq polymerase	100 (4)	unspecified bacterium	[19]
	100 (4)	*Pseudomonas* spp.	[20]
	8 (24)	*Sphingomonas* spp., *Moraxella* spp.	[20]
	2 (41)	*Acinetobacter junii*	[20]
	n.d.	*Pseudomonas* spp., *Serratia marcescens*, *Escherichia coli*, *Salmonella* spp., *Shigella* spp.	[21]
	10-30 (n.m.)	*Coxiella burnetii*	[22]
Primers	0-100 (18-66)	*Delftia tsuruhatensis*, *Klebsiella* spp., *Paenibacillus* sp.	[23]
PCR buffer	n.d.	*Acremonium* spp.	[11]
Pipetting			
Pipette tips	18 (32)	unspecified bacterium	[24]

[a] Samples of the same or different lots or samples from different manufacturers; signals were observed in negative PCR controls using DNA-free water; best match species were identified by sequencing of the amplicons and BLASTn search; n.d., not determined; n.a., not applicable; n.m., not mentioned.

(NGS) [5]. Clearly, analysis at the limit of detection can pose serious problems to accurate data collection as regards false positive results and wrong community structure by introduction of extraneous DNA. Nolte et al. [6] summarised the risks of contamination of 16S rRNA gene PCR during the workflow of sampling, transport to the routine laboratory and analysis of specimens (Fig. 1). According to this image, contamination risks are highest during sampling and transportation and out of control of the molecular laboratory. Contamination can happen through inadequate sterilisation at sampling, carry-over inoculation from laboratory surfaces and equipment, use of non-sterile sample containers and other factors.

Routine laboratories generally analyse samples for microbiological and clinical chemistry parameters. So, most samples are not scheduled for rRNA gene PCR analysis. When molecular analysis is demanded samples may be split at the reception under conditions that are not adequate for avoidance of contamination. Once arrived in the molecular biology laboratory, conditions for appropriate sample processing are under control. However, cross-contamination from one sample to another during extraction, aerosols from previous

amplifications and air-borne environmental sources, inappropriate handling, and contaminated reagents and consumables may lead to contamination [7]. Therefore, consciousness of potential sources of contamination is demanded when interpreting molecular results.

Sources of contamination

Reagents and consumables used in the molecular laboratory are generally not issued for the analysis of microbial sequences at very low concentration. Therefore, an important constraint of molecular analysis at the limit of detection is the potential presence of microbial DNA contaminating extraction chemicals, amplification reagents and consumables during the manufacturing process. Table 1 summarises results that indicate contamination of a variety of reagents and consumables by microbial DNA. The studies were conducted in the context of suitability of the material for sensitive molecular diagnosis of low microbial load samples. Contamination was found in the majority of materials studied.

DNA of fungal organisms including *Aspergillus* spp., *Pneumocystis* spp., *Saccharomyces cerevisiae*, *Candida* spp. and *Acremonium* spp. were detected in collection tubes for samples like blood, serum and urine, DNA and RNA extraction reagents as well as PCR buffer (Table 1). The origin of the contamination could also be assigned to eubacteria, among them environmental organisms like *Alcaligenes* spp. and *Pseudomonas* spp., skin colonizers and opportunistic pathogens like *Propionibacterium* spp., *Serratia marcescens* and *Sphingomonas* spp. as well as potential pathogens, including *Brucella* spp., *Coxiella burnetii*, *Escherichia coli* and *Legionella* spp. The rate of contamination was found to be high in some materials (>50% false positives), including forceps for tissue preparation, DNA extraction reagents and Taq DNA polymerases (Table 1). Evidence for low and tolerable loads of contaminating microbial DNA (2 and 8% false positive rate) was given for some Taq DNA polymerases (Table 1, ref. [20]).

The problem of false positive results in molecular analysis of pathogens through contaminated consumables, buffers and reagents has been addressed by a number of approaches. Millar et al. [25] propose a risk assessment model detailing the manipulations, contamination hazards and risks, and corrective action involved in the broad-range 16S rRNA gene PCR diagnosis of bacterial blood stream pathogens which may also serve as a guideline for other assays. The model divides the analytical process into three parts, sample collection, DNA extraction and amplification, all of which demand careful handling and the availability of molecular-grade, in particular DNA-free consumables and reagents. In the following sections, ways of decontamination of materials and reagents and employment of commercially available DNA-free materials are discussed as regards the setup of a reliable, highly sensitive system for the direct detection and identification of bacterial and fungal organisms in clinical and other sample materials with low pathogen loads.

Consumables for handling in molecular analysis

The selection of suitable plastic consumables employed for DNA extraction and molecular analysis, including pipette tips, sample tubes, centrifugation vials and PCR or Real-Time PCR tubes and plates is crucial for the avoidance of false positive results by contaminating DNA. Sterility and absence of nucleases as characters for molecular-grade articles are not a guarantee for the absence of contaminating DNA as evidenced by our own experience (Table 1). Therefore, as part of the setup of a system for low load pathogen DNA analysis, testing of products from different suppliers for the absence of bacterial and fungal DNA is recommended.

Radical gas treatment of plastics is common practise for the destruction of amplifiable sequences. There are some suppliers of consumables which declare their products bacterial DNA-free (Table 2). Only one among the three suppliers listed in Table 2, however, files testing for bacterial and fungal DNA. Nevertheless, it seems that absence of bacterial DNA may be an indicator of the absence of fungal DNA from our experience (see comment in Table 2). Nonetheless, to be sure as indicated above, consumables not explicitly labelled as tested for the absence of fungal DNA should be subjected to negative control run analysis in the laboratory.

Sample collection

Consumables for the collection and handling of samples are in line with analytical processes other than molecular diagnosis of microbial DNA present at low concentrations. Material for the collection and processing of blood to plasma and serum, stabilisation of blood cells and preparation of tissue biopsies has been shown to be a potential source of contaminating DNA of bacterial and fungal organisms (Table 1). So far, material routinely tested from lot to lot for the absence of microbial DNA does not seem to be available from commercial sources. Millar et al. [25] proposed to prepare lots of sterile DNA-free collection tubes, EDTA solution and water for blood drawing. Reduction of amplifiable bacterial sequences and cells over up to 4 orders of magnitude to below the limit of detection was

observed when surfaces of plastics were experimentally contaminated with DNA or micro-organisms and treated with methanol radicals or ethylene oxide [26, 27]. Radical-based treatment was regarded superior to UV or gamma irradiation which tends to have a negative influence on the plastic consumables.

DNA decontamination of water and buffers can be achieved by UV or gamma irradiation [28]. Water and buffers are also commercially available as molecular biology grade and certified human DNA-free products. Absence of microbial DNA, however, is mostly not indicated and should be tested by the user by PCR negative control runs. Other sources of certified bacterial and fungal DNA-free water exist, although available only as small volume products provided for PCR analysis (see Table 2, amplification reagents).

DNA extraction

As with other materials used for molecular biology, DNA extraction products are generally not designed for the purpose of ultra-sensitive detection of microorganisms at very low loads in clinical and other specimens. In fact, they generally contain contaminating DNA of bacterial and fungal origin (Table 1). Systematic studies have been performed to eliminate contaminating microbial DNA from extraction buffers by binding the DNA to silica-based membrane columns in a procedure employing filtration washing [12]. Ethylene oxide treatment of plastic consumables, including mini spin columns, was successfully employed for the destruction of DNA contaminants [27].

Industry has reacted to the increasing demand for microbial DNA-free reagents and consumables by the supply of ultra-clean products for DNA extraction from clinical samples. Table 2 lists certified bacterial and fungal DNA-free products dedicated to the extraction of microbial DNA for the analysis of low loads of microorganisms. The products address the manual, semi-automated and fully automated extraction of small and large sample volumes in the range 0.1 to 10 ml as well as tissue biopsies. Two products, MagNA Pure® (Roche) and easyMAG® (bioMérieux), extract total nucleic acids, while all others aim at the preparation of microbial DNA with reduced loads of human DNA. A variation of the standard easyMAG® protocol was described by Wiesinger-Mayr et al. [29] by which bacterial DNA preparations were greatly depleted of human DNA. Excess host DNA can be a factor of false positive results and loss of assay sensitivity as a consequence of unspecific primer binding and amplification of non-target sequences [30]. Recently, quantitative reduction of host DNA from samples was shown to dramatically increase microbial reads and thereby enhance the discriminative power of metagenomic whole genome sequencing analysis of prosthetic joint specimens [31].

Five of the six DNA extraction products in Table 2 are declared by the manufacturers to be routinely tested for the absence of bacterial and fungal DNA during the manufacturing process. As regards the product without information about testing for microbial DNA contamination, easyMAG®, there is evidence that also this system is suitable for sensitive analysis of pathogens, although demonstrated only for bacteria [28]. As discussed above, contaminating fungal DNA is likely to be absent, but should be proven before using the system for the development of a protocol for fungal DNA extraction.

PCR and NGS analysis

The record of references regarding contamination of amplification reagents mainly comprises of bacterial DNA (Table 1). As regards fungal DNA contamination, literature is scarce. Loeffler et al. [11] systematically studied contamination of reagents involved in the whole diagnostic process, including amplification. They found one component, the 10x PCR buffer of a certain lot of the product to be contaminated by fungal DNA which by sequence analysis of the amplicon could be assigned to *Acremonium* spp. This shows that care has to be taken in the selection of amplification reagents as was discussed before regarding sample collection and extraction.

Champlot et al. [28] conducted a systematic evaluation of various methods for the decontamination of PCR components. The authors provide protocols involving gamma irradiation of water and short UV irradiation by which PCR buffers and other liquids can be decontaminated efficiently and rather easily from exogenous DNA. They point out that sensitive components like Taq DNA polymerase, primers and dNTPs are inactivated by UV and therefore need a different treatment. Employment of a protocol using a heat-labile endonuclease resulted in 99.5% degradation of double-stranded DNA while the efficiency and sensitivity of the PCR assay was comparable to the control indicating that primers were not affected by the nucleolytic treatment. Although focused on the removal of bovine DNA contamination, the study provides valuable guidelines for the setup of a decontamination protocol for PCR components as regards fungal DNA. When screening the information provided by manufacturers, several PCR components, including Taq DNA polymerases, master mixes and molecular grade water are available which are certified bacterial and, in many cases, also fungal DNA-free (Table 2). The use of comer-

Table 2: *Materials and reagents for the analysis of low microbial loads*

Component	Brand	Manufacturer	Absence of DNA tested [a]		Comment
			Bacterial	Fungal	
Consumables					
Filter tips, tubes, centrifuge vials	MGrade®	Greiner Bio-One (Kremsmünster, Austria)	+	+	
	Biopur®	Eppendorf (Hamburg, Germany)	+	-	
	Biosphere® Plus	Sarstedt (Nümbrecht, Germany)	+	-	tested for absence of fungal DNA [b]
Extraction kits					
Manual protocols	MolYsis™	Molzym (Bremen, Germany)	+	+	up to 10 ml fluid clinical samples
Automated systems	Blood Pathogen Kit™	Seegene (Seoul, South Korea)	+	+	semi-automated; 1 ml blood; Seeprep12™ instrument (Seegene)
	SelectNA™ Blood Pathogen Kit	Molzym (Bremen, Germany)	+	+	semi-automated; up to 10 ml clinical samples; Liaison® IXT instrument (Diasorin, Saluggia, Italy)
	MagNA Pure® LC Microbiology kit	Roche Diagnostics (Penzberg, Germany)	+	+	automated DNA extraction; 0.1 ml samples; MagNA Pure LC 2.0 instrument (Roche)
	MolYsis SelectNA™*plus*	Molzym (Bremen, Germany)	+	+	automated DNA extraction; 1 ml samples, tissue biopsies; SelectNA™*plus* instrument (Molzym)
	EasyMAG®	bioMérieux (Marcy-l'Étoile, France)	-	-	modified automated protocol for 5 ml blood [29]; see text
Amplification reagents					
Taq DNA polymerases	Taq DNA Polymerase, DNA-free	Applichem (Darmstadt, Germany)	+	+	
	MolTaq 16S/18S	Molzym (Bremen, Germany)	+	+	

Table 2: *Materials and reagents for the analysis of low microbial loads (continued)*

Component	Brand	Manufacturer	Absence of DNA tested [a]		Comment
			Bacterial	Fungal	
	Hot MolTaq 16S/18S	Molzym (Bremen, Germany)	+	+	Hot start Taq DNA polymerase
	MTP™ Taq DNA Polymerase	Sigma-Aldrich (St. Louis, MO, USA)	+	-	
	DFS-Taq DNA Polymerase	Bioron (Ludwigshafen, Germany)	+	-	
	Taq DNA Polymerase	Amresco (Solon, OH, USA)	+	-	
	DNA free-Taq DNA Polymerase	XpressBio (Frederick, MD, USA)	+	-	
	DF Taq Polymerase E (DNA-free)	Genaxxon (Ulm, Germany)	+	-	
PCR master mixes, assays	Mastermix 16S/18S Basic	Molzym (Bremen, Germany)	+	+	master mix for assays with custom primers
	Mastermix 16S Complete	Molzym (Bremen, Germany)	+	+	broad-range 16S rRNA gene PCR assay for detection of bacterial DNA
	Mastermix 18S Complete	Molzym (Bremen, Germany)	+	+	broad-range 18S rRNA gene PCR assay for detection of fungal DNA
NGS assays	NGSeq 16S V3/V4	Molzym (Bremen, Germany)	+	+	master mix including primers/adapters for Illumina MiSeq® 16S metagenomic sequencing library preparation
Water	Microbial DNA-free Water	Qiagen (Hilden, Germany)	+	+	
	DNA-free Water	Molzym (Bremen, Germany)	+	+	
	PCR Water, DNA-free	Applichem (Darmstadt, Germany)	+	+	

[a] +, Information (homepage) on testing provided by manufacturer; -, no information available
[b] Own results, absence of fungal DNA shown at 40 cycles PCR employing Mastermix 18S Complete (Molzym)

cial products may be helpful in the reduction of microbial DNA contamination and the standardisation of sensitive bacterial and fungal target assays among laboratories. Importantly, when designing an analytical assay for the detection of minute amounts of fungal DNA, DNA-free products should also guarantee a high amplification activity [21].

Even though buffers and reagents of the amplification reaction are available DNA-free, either as single components or as master mixes (Table 2), primers designed for specific targets are generally produced under conditions which introduce considerable levels of microbial DNA into the primer preparation (Table 1). This is a frequently experienced problem which demands special care regarding decontamination (see above). To this end, Molzym provides complete systems (SepsiTest™-UMD, Micro-Dx™) that include contamination-free buffers, reagents and consumables for manual or automated DNA extraction and for Real-Time PCR amplification as well as primers for sequencing analysis of bacterial and fungal pathogens.

DNA contamination in extraction and amplification reagents is also problematic in microbiome analysis by next generation sequencing methods. Recently, Thoendel et al. [32] noticed a tremendous impact of contaminating DNA in whole genome amplification kits on pathogen analysis of sonicate fluids from prosthetic joint biopsies by metagenomic shotgun sequencing. Edelmann et al. [33] confirmed this view by a comparative study employing DNA extraction and amplification master mixes from different suppliers for targeted sequencing of the 16S rRNA gene. In this study, the authors assessed the removal of human DNA before library preparation and the reduction of bacterial contaminants in 16S-PCR reagents by a selection of commercial kits. The kits showed different rates of depletion of human DNA and contamination by bacterial DNA. One combination, Molzym's automated SelectNA™*plus* DNA extraction system and NGSeq 16S V3/V4 for library preparation proved to reduce the human DNA content of the DNA preparation at the highest rate (11-fold) and contain the least contaminant sequencing reads (<5%), respectively. These results prompted the authors to conclude that the system aids an efficient NGS-based workflow towards a standardised method in infection diagnosis.

Conclusions

In the last years, manufacturers of molecular grade products have become aware of the problem of DNA contamination and now supply materials and reagents with very low loads of contaminating microbial DNA. In terms of standardisation, products manufactured contamination-free under high quality standards are inevitable for the exact PCR diagnosis of pathogens and NGS analysis of metagenomic structures in clinical routine.

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