MagNA Pure System Application Note No. 5 March 2015

High-throughput detection of bacterial, fungal and viral nucleic acids in routine microbiological sample types using one generic Pathogen Universal Protocol on the MagNA Pure 96 System

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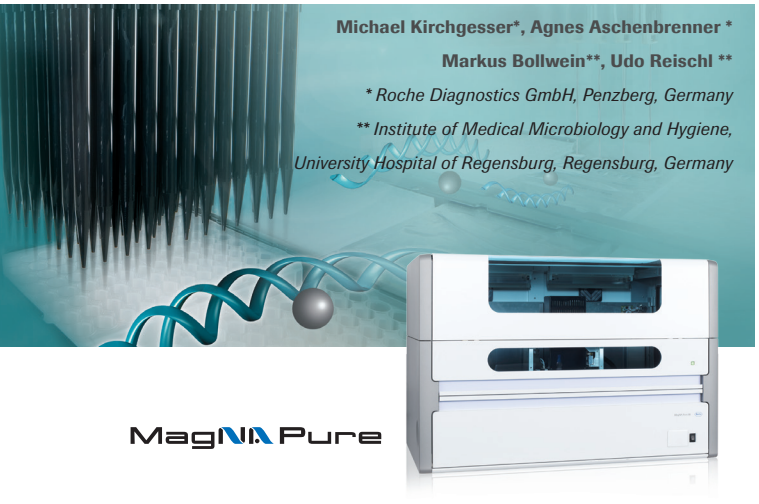


Abstract

The objective of this study was to compare the MagNA Pure 96 Pathogen Universal protocol to the MagNA Pure LC DNA Isolation Kit III that is well established in our microbiological laboratory. For this we isolated nucleic acids from ten different sample materials: body fl uids (CSF, urine, sputum), feces, swabs, as well as whole blood, EDTA-, Citrate-plasma and serum. This spectrum is typical for a microbiology laboratory and includes easy and diffi cult-to-process samples, as sputum and stool are. Into these sample types we spiked classifi ed pathogen stocks from in-house cultures. Subsequently real-time PCR analysis of fungal, bacterial and viral targets was done using the LightCycler® 480 Instrument.

The 200 µl protocol performed effi cient and sensitive nucleic acid extraction from up to 96 samples requiring less than 60 minutes runtime. It yielded superior or similar results compared to the respective MagNA Pure LC protocol. Higher input volumes, 500 or 1000 µl, available only for the MagNA Pure 96 System, generally increased sensitivity of detection. Direct use of some routine microbiological samples without dilution by pretreatment reagents, resulted in improved Crossing Point (CP) values. For normal sample types including whole blood this made pretreatment dispensable. A pretreatment step did however improve pathogen detection in the more diffi cult-to-process samples, such as sputum, swabs and stool.

Our results demonstrate that both methods allow efficient and convenient isolation of high quality nucleic acid.Compared to the Viral NA Universal protocol, the Pathogen Universal protocol is further optimized for diverse sample materials, including diffi cult samples and for the detection of various pathogen types. The MagNA Pure 96 Instrument thus enables high throughput sample preparation applications in microbiology laboratories.

Introduction

In microbiology laboratory routine, a variety of different sample types are analyzed. Typical sample types include difficult to-process samples of respiratory and fecal origin, such as sputum, swabs and stool. Individual samples can exhibit great diversity with regard to viscosity, cell content and the presence of potential inhibitory substances. There is also a broad range of fungal, bacterial and viral pathogen nucleic acid targets, which vary in composition and concentration depending on the actual status or site of infection.

For automated nucleic acid isolation, Roche offers the MagNA Pure Systems, optimized for different throughput situations of laboratories:

- MagNA Pure Compact Instrument for 1-8 samples per run
- MagNA Pure LC 2.0 Instrument for 8-32 samples per run
- MagNA Pure 96 Instrument for 8-96 samples per run

All three systems use the same basic principle and chemistry to produce comparable yields and nucleic acid purity (1, 2, 3).

We have used the proven MagNA Pure LC System for automated nucleic acid isolation for several years to detect diverse microbiological pathogens (see Table 1A). In the present study, we investigated the isolation efficiencies and quality of bacterial, fungal and viral nucleic acids from different typical specimen in microbiology, as well as from blood or blood component samples. To compare the performance of the MagNA Pure 96 System with the MagNA Pure LC System, we selected representative examples of pathogens and sample matrices (see Table 1B). One of our objectives was to evaluate a universal generic protocol that permits diverse sample materials to be analyzed in the same run. Furthermore we compared different methods for the inactivation of pathogens by lysis of bacterial and fungal organisms.

For the MagNA Pure 96 System the DNA and Viral NA Small Volume Kit (06 543 588 001) and the DNA and Viral NA Large Volume Kit (06 374 891 001) were used in combination with the Bacteria Lysis Buffer (04 659 180 001). On the MagNA Pure LC System the DNA Isolation Kit III (Bacteria, Fungi, 03 264 785 001) was used.

	Sample types	Gram-positive Species	Gram-negative Species	Fungi	Virus
A.	Sputum Swabs Fecal samples Bronchoalveolar lavage (BAL) Urine Cerebrospinal fluid (CSF)	Actinomyces israelii, Bacillus cereus Corynebacterium xerosis Enterococcus faecalis Listeria monocytogenes Propionibacterium acnes Staphylococcus aureus Staphylococcus epidermidis Streptococcus agalactiae (B-Strep.) Streptococcus pneumoniae	Bacteroides fragilis Citrobacter freundii Enterobacter cloacae Escherichia coli Haemophilus influenzae Helicobacter pylori Klebsiella pneumoniae Legionella pneumophila Moraxella catarrhalis Neisseria gonorrhoeae Proteus vulgaris Pseudomonas aeruginosa Salmonella enteritidis Serratia marcesceus Shigella sonnei Yersinia enterocolitica	Aspergillus fumigatus Candida albicans	
B.	Sputum Swabs Fecal samples Bronchoalveolar lavage (BAL) Urine Cerebrospinal fluid (CSF) Whole blood EDTA-Plasma Citrate-Plasma Serum	Staphylococcus aureus Enterococcus spec.	Escherichia coli	Candida albicans	Cytomegalovirus Epstein-Barr virus Hepatitis A virus Influenza A virus (H1N1)

Table 1: A. Overview on sample types and species that are detected in our routine, using the MagNA Pure LC System and the DNA III Isolation Kit (Bacteria).
B. Overview on selected representative sample types and species that were tested in comparison on MagNA Pure LC and MagNA Pure 96 Systems.

Materials and Methods

Pathogen stocks and sample material

Specific pathogens that are representative for the pathogens tested in our routine were spiked into nine different sample types (see Table 1B). Sample types included sputum, BAL, swabs, urine, stool, CSF, serum, EDTA-plasma, Citrate-plasma and whole blood. The pathogen material was taken from characterized in-house stock cultures. The sample material had previously tested been negative for these pathogens. Phosphate Buffered Saline (PBS) served as an inhibitor-free control sample material to calculate extraction efficiencies.

Virus

Cytomegalovirus (CMV, single stranded DNA)
Epstein-Barr virus (EBV, double stranded DNA)
Hepatitis A virus (HAV, RNA)
Influenza A virus (H1N1, RNA)

Bacteria

Staphylococcus aureus (S. aureus)
Enterococcus spec.
Escherichia coli (E. coli)

Fungi

Candida albicans (C. albicans)

Sample pretreatment and nucleic acid isolation

Nucleic acid isolation was done using the MagNA Pure 96 System and the DNA and Viral NA Small Volume (200 µl sample volume), respectively Large Volume Kit (500, 1000 µl sample volumes). The samples were pipetted into the MagNA Pure 96 Processing Cartridge, and had been pretreatedwhen necessary. Sample pretreatment prior to any automatedprocessing is commonly recommended when working with potential infectious pathogens. Some of the protocols for the MagNA Pure Systems that use a guanidinium-containing lysis buffer, therefore have an external lysis step that can be done under a safety hood. Many bacteria however need an additional heat or enzyme activated step to be effectively inactivated or lysed, additionally the bacterial lysis buffer does not contain guanidinium. An incubation step at +65°C enhances lysis and liquefaction. An incubation step at +95°C was used to inactivate highly infectious pathogens in a sample. Very viscous samples must also be pretreated, in order to get them liquefied and thus optimal processable. A liquefaction step using DTT is therefore optional for very viscous samples, such as sputum or mucous stool. In the present study, different pretreatment protocols were used as indicated in Figure 1. A generic standard protocol for routine and difficult samples was established and tested for samples that are typical for bacteria and fungi. A modified protocol without heat step at 95°C was used for blood samples and compared to a protocol with the heat step. Blood samples are typical for virus targets, which might suffer from a highly elevated temperature.

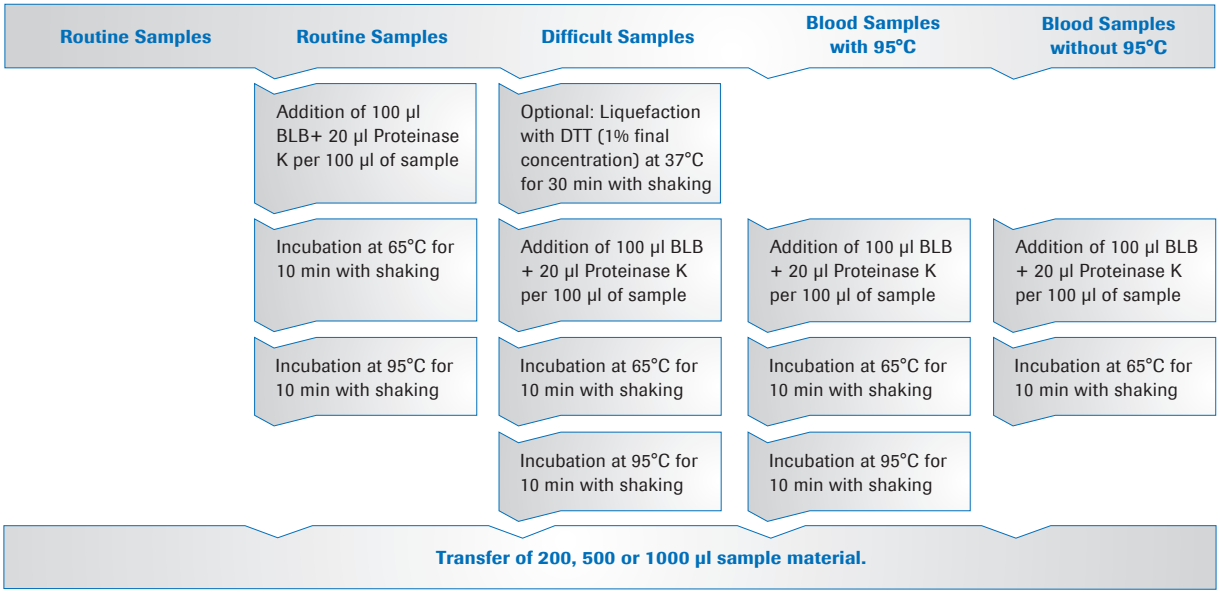


Figure 1: Pretreatment protocols for different sample types

When a pretreatment step using reagents is done, target input amounts for NA isolation are reduced by half, compared to samples processed without pretreatment. This theoretically accounts for the difference of one CP found in real-time PCR (see below).

Material and Methods

Real-time PCR Detection

Real-time PCR Detection
For all pathogens mentioned in table 1 we have an established in-house assay using the LightCycler® 480 System (Roche Diagnostics). LightCycler® Fast Start DNA Master Hybridisation Probes (12239272001) was used for all bacterial and fungal targets (see e.g. 3, 6), as well as for HAV, CMV, EBV, and E. coli. HAV was detected by a one-step RT-PCR method. Real Time ready Influenza A/H1N1 Detection Set (05640393001) and the Real Time ready RNA Virus Master (05 619 416 001) were used for H1N1 detection (see 4). All PCRs were done using 5 µl of eluate and 15 µl Mastermix.

For 200 µl and 500 µl sample volumes, the MagNA Pure 96 Instrument processed up to 96 samples per run in less than one hour. For 1000 µl sample volumes, up to 48 samples were processed per run, because sample lysates were divided into two portions by the MagNA Pure 96 robot for subsequent processing. The setup of the MagNA Pure Instrument with reagents and disposables, as well as software programming, required together 5 minutes.

Reference MagNA Pure LC protocol

The MagNA Pure LC DNA Isolation Kit III (Bacteria, Fungi) was used as a reference for comparison with the Pathogen Universal Protocol. When using the DNA Isolation Kit III, the same pretreatment was performed for all samples, according to the pack insert. Up to 32 samples consisting of 200 µl pretreated sample material (corresponding to 100 µl original sample input volume) were processed in a single run. 15 minutes were required for the setup of the instrument with reagents and disposables, and the programming the software. A purification run with a full batch took 95 minutes. Processing less samples requires less time, applicable for batch sizes in multiples of 8, and ranges from 2 (8) – 72 (24) minutes.

Results

Performance of the MagNA Pure 96 Pathogen Universal Protocol

Results of the three different sample volumes with and without pretreatment for S. aureus and CMV are shown inFigures 2A and 2B, respectively. Similar results were obtained for EBV and HAV (data not shown). LightCycler® System analysis yielded positive results for all samples under study, indicating effective NA isolation, absence of inhibitory substances, and successful detection. of the respective pathogenic target organisms. For the 200 µl sample volume, compared to the analysis with nucleic acid purification with the MagNA Pure LC System, the data obtained using the MagNA Pure 96 System were slightly better for S. aureus or similar for CMV. In addition, we observed that a larger sample input volume of 500 or 1000 µl and its related higher amounts of target nucleic acid usually indeed produced the expected lower Cp values.

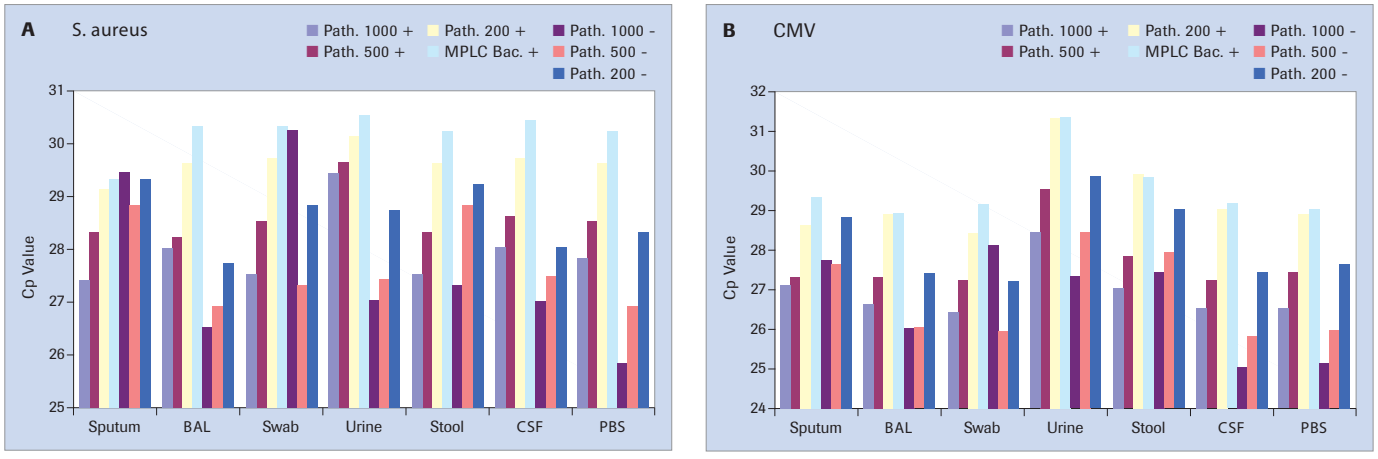


Figure 2: Comparison of different sample input volumes of the MagNA Pure 96 Pathogen Universal Protocol for NA isolation from different sample materials with or without pretreatment; Panel 2A: Detection of S. aureus; Panel 2B: Detection of CMV.

Path. 1000 (500, 200) +: MagNA Pure 96 Pathogen Universal Protocol using 1000 (500, 200) µl with pretreatment
Path. 1000 (500, 200) –: MagNA Pure 96 Pathogen Universal Protocol using 1000 (500, 200) µl without pretreatment
MPLC Bac. +: Reference representing the MagNA Pure LC instrument using the DNA Isolation Kit III with pretreatment
Pretreatment reduced target input amounts for NA isolation by half, resulting in a higher CP value by 1.

Results

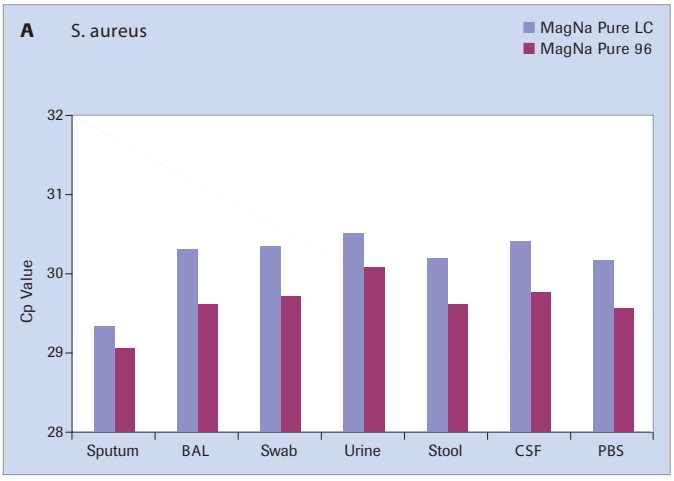
Evaluation of Sample Pretreatment using the MagNA Pure 96 System

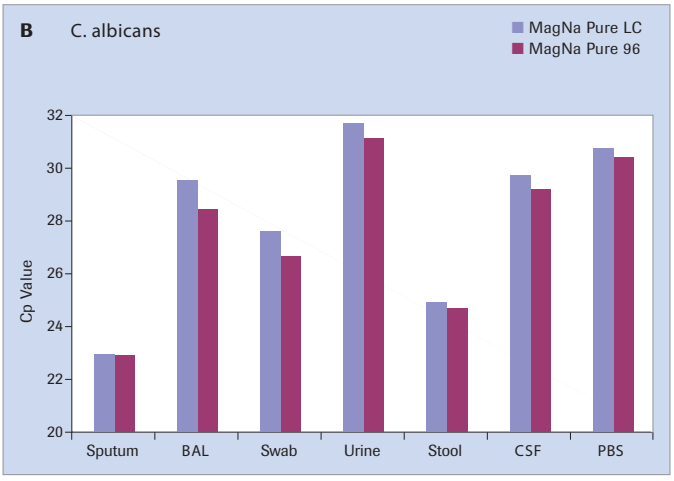
For samples used directly without pretreatment, we obtained for CSF and PBS and for most of the sample materials (BAL, urine) under study, slightly lower Cp values than with pretreatment. For all samples, including the difficult ones, all targets were detectable without pretreatment.However, pretreatment had positive effects on the CP valuesof the difficult sample materials sputum and swabs, reflecting improved analytical sensitivity. This was particularly obvious for 1000 µl input volume (see Figure 2). For extremely viscous samples, we observed reduced clumping and more robust processing with pretreatment, resulting in more consistent and reliable relationship between input volume and CP value compared to without pretreatment. For these samples, the reduction in target input amount due to pretreatment was compensated by the enhanced lysis and extraction procedure. Based on these results, pretreatment is typically not required for routine samples, but may be advantageous for difficult samples. Pretreatment yields reliable results when inactivating highly infectious pathogens prior to MagNA Pure 96 Instrument loading.

Comparison of MagNA Pure 96 and MagNA Pure LC Systems

We extended the evaluation of the MagNA Pure 96 Pathogen Universal 200 protocol by comparing it to the MagNA Pure LC procedure. Representative Cp values for a bacterial, a fungal and a viral target are shown in Figures 3A and 3B.

In most cases results using the MagNA Pure 96 System were superior or comparable to those obtained using the MagNA Pure LC System. Similar results were obtained for Enterococcus spp., HAV und EBV. To be able to compare results, pretreatment was used for both methods.





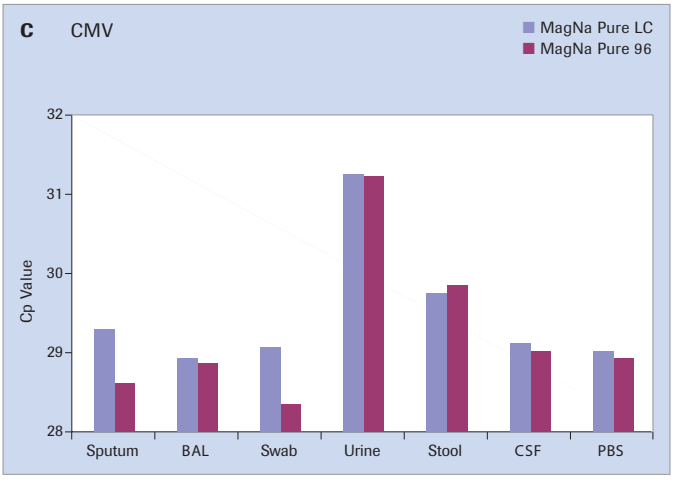


Figure 3: Results for nucleic acid isolation and detection form different sample materials using the MagNA Pure 96 Pathogen Universal 200 protocol in comparison to the MagNA Pure LC procedure; Panel 3A: S. aureus; **Figure 3B:** C. albicans; Panel 3C: CMV. Lower Cp values indicate higher yield.

Results and Discussion

Another comparison of the MagNA Pure 96 and MagNA Pure LC Systems is presented in Figures 4A and 4B, showing the LightCycler® 480 amplification profiles of urine spiked with E. coli and serum spiked with defined amounts of Influenza A virus.

Analyses of quadruplicates for each target yielded homogeneous and reproducible curves for each method. For both targets, we observed slightly lower Cp values using the MagNA Pure 96 Systems, indicating improved NA isolation by this system.

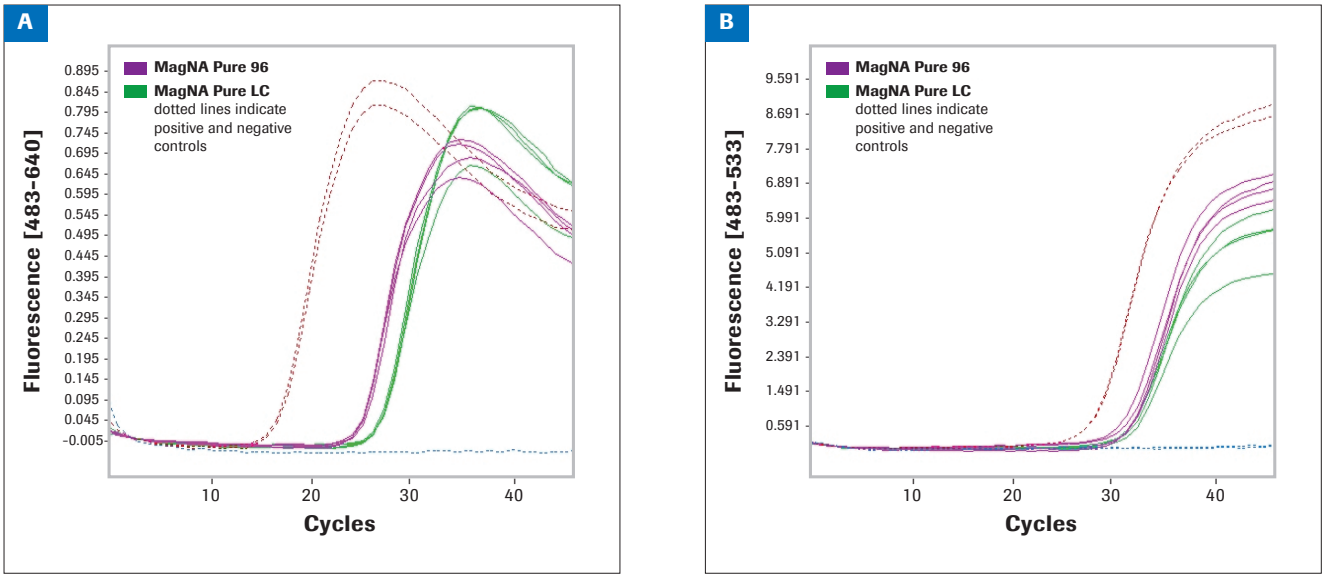


Figure 4, LightCycler® 480 amplification/detection profile of NA isolated by the MagNA Pure 96 Pathogen Universal 200 protocol in comparison to the MagNA Pure LC procedure; Panel 4A: E. coli isolated from urine; Panel 4B: Influenza A isolated from serum.

Suitability of the MagNA Pure 96 Pathogen Universal 200 protocol for blood samples

In order to evaluate the Pathogen Universal protocol also with virus and related typical sample materials, we performed experiments to examine the performance of the MagNA Pure 96 Pathogen Universal 200 protocol with different blood samples. Here we used two pretreatment protocols, one including, and the other lacking the heating step at +95°C (see Figure 1). Using the direct procedure without pretreat-ment, we obtained reliable target detection in whole blood, EDTA-plasma, citrate-plasma and serum samples (data not shown). Using pretreatment, the procedure with the +95°C incubation step yielded inferior results, especially for serum, particularly for RNA virus. This indicates that the analyses of blood samples can be performed using the MagNA Pure 96 Pathogen Universal 200 Protocol without any pretreatment. When a heat inactivation of pathogens is required, it appears that pretreatment, in particular the +95°C incubation step, in combination with this protocol can exert negative effects on the NA isolation.

Discussion

In the present study, we show that the Pathogen Universal Protocol used with the MagNA Pure 96 Instrument produces efficient and sensitive isolation of high quality nucleic acid for reproducible and reliable results with LightCycler® System detection of even very low amounts of DNA and RNA target sequences. Compared to the established and in our lab extensively used MagNA Pure LC System, the MagNA Pure 96 System shows comparable or superior performance. We expect a similar performance of the MagNA Pure 96 System that we previously evaluated and established for the MagNA Pure LC System (see Table 1).

We demonstrate here that the MagNA Pure 96 Pathogen Universal protocol is optimized for a variety of sample materials, including difficult samples and also to blood specimens that are typical for samples of viral nature. Compared to the Viral NA protocols it has more extensive washing steps, related to the difficult texture of typical

sample materials of bacterial and fungal nature. To support this, it also changes/uses one tip more per sample. According to our evaluation, the protocol permits the purification and analysis of blood samples with other material types in the same run. As this allows management of laboratory workflows in a convenient and cost-effective way, the use of the MagNA Pure 96 Instrument supports an improved workflow and obtaining analysis results in shorter times.

For improved sensitivity needs, with regard to low amounts of target nucleic acid in a sample material, the Pathogen Universal protocol enables flexible sample input volumes of up to 500 µl and 1000 µl. We found that 1000 µl input volume produced suboptimal recovery for a few sample types with richer texture, probably due to inhibitory effects and/or overloading. This could be largely prevented by pretreatment.

With regard to pretreatment for most difficult sample materials, our study showed the robustness of the MagNA Pure 96 Pathogen Universal protocol with its prolonged washing cycles. In general, pretreatmentwas not required for pathogen detection. These additional time-consuming hands-on steps can thus be avoided.

For very difficult and viscous sample matrices, standard pretreatment may enhance the robustness of the nucleic acid isolation procedure and results in improved target detection.

When inactivation of pathogens is required for safety reasons, the standard pretreatment described here, will yield consistent nucleic acid isolation from most sample types and does not produce disadvantageous effects. Depending on bacteria type, additional pretreatment may be necessary, such as specific enzymatic lysis with lysostaphin or lysozyme or a freezing step (see pack insert of bacterial lysis buffer). For some blood sample (esp. serum)/target nucleic acid combinations (esp. RNA virus) especially the heat deactivation might cause higher CP values. In case external pathogen deactivation is required and a low amount of target nucleic acid in such combinations is to be investigated, rather the more specialized Viral Nucleic Acid protocol and its external lysis function should be used.

Our findings here confirm that the MagNA Pure 96 Instrument is engineered for high throughput applications in microbiology laboratories.

Conclusion

Compared to the established MagNA Pure LC procedure, the newly established MagNA Pure 96 Pathogen Universal Protocol yields similar reliable results for the molecular detection of pathogens from a broad range of sample types, including blood samples and so-called difficult-to-process matrices, such as sputum, stool or swabs. Sensitive and successful real-time PCR-based detection of pathogenic microorganisms is dependent on efficient isolation of high quality NA. According to our study results the MagNA Pure 96 Instrument fulfills these requirements combined with user convenience and fully automated processing of up to 96 samples in one hour, making it ideal for routine high throughput workflows purifying heterogeneous samples in the microbiology laboratory.

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Important Note:

The MagNA Pure 96 Instrument (06 541 089 001) is for *in vitro* diagnostic use.

The LightCycler® 480 Instrument is for life science research only. Not for use in diagnostic procedures.

Abbreviations

BAL	Broncho Alveolar Lavage
BLB	Bacteria Lysis Buffer
CMV	Cytomegalovirus
CP	Crossing Point
CSF	Cerebrospinal Fluid
DTT	Dithiothreitol
EBV	Epstein-Barr Virus
HAV	Hepatitis A Virus
PBS	Phosphate Buffered Saline

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Roche Molecular Diagnostics
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