Abstract

In our diagnostic laboratory, we routinely use the MagNA Pure 96 System for nucleic acid extraction. In this paper, we show that the MagNA Pure 96 System is reliable and convenient for the extraction of viral RNA and/or DNA from a variety of sample materials.

Extraction was very sensitive and efficient, resulting in the detection of 10 – 100 viral copies, depending on sample and virus type. Effects resulting from PCR inhibition were excluded by experiments using Internal Controls. Furthermore, we evaluated one single extraction protocol and one single RT-PCR protocol that can be used generically for all sample and virus types in the same run.

Detection using the Roche LightCycler * 480 Real-Time PCR Instrument revealed that extraction from all 6 materials tested was very efficient. Even with known difficult materials, such as faeces and biopsies, the MagNA Pure 96 System performed well.

In addition, we extracted nucleic acid from 2 different volume inputs (50 and 200 μl) of EDTA whole blood from patients positive for CMV. Higher inputs led to higher sensitivity in 7/8 samples, crossing point (Cp) cycle values were approximately 1.7 cycle lower while Cp values for IC remained similar.
Introduction

The Academic Medical Center (AMC) is one of the eight university medical centers in the Netherlands. The three principal tasks are patient care, research and medical education. The AMC has over 1,000 beds and more than 50,000 clinical and daycare admissions. The Department of Medical Microbiology participates in all three principal tasks of the AMC. Patient care is provided by laboratory diagnosis and consultation. Within the department of medical microbiology, the molecular diagnostic unit (MDU) provides molecular detection of a broad range of microorganisms. Over the last decade, the contribution of molecular techniques to the diagnosis of infectious disease in medical microbiology laboratories has increased significantly. Not only has the amount of samples processed and the choice of targets increased, the nature and variety of the sample materials to be tested have grown.

Testing requires efficient and convenient nucleic acid extraction protocols. Due to the increased size of sample flow, nucleic acid extraction is preferably automated to process many samples, in as little time as possible. For this, there is a need for a fast, automated high-throughput purification system that can handle a variety of materials, preferably in the same extraction run and using the same extraction protocol. To investigate if the MagNA Pure 96 System is able to efficiently extract nucleic acid from various sample materials, we spiked different clinical materials with various amounts of different characterized virus stocks. Internal controls were added to all samples to monitor extraction efficiency of the MagNA Pure 96 System. The readout was performed using real-time PCR with hydrolysis probes in a Roche LightCycler® 480 Real Time PCR Instrument.

Materials and Methods

Virus stocks and patient material

DNA viruses Human Adenovirus (HAV) and Cytomegalovirus (CMV), as well as RNA viruses Human Parechovirus (HPeV) and Influenza A virus, were isolated and cultured from sample materials sent to the laboratory for molecular diagnosis. Virus stocks were TCID50* quantified.

Table 1: Treatment of various human sample materials to be extracted in the MagNA Pure 96 System in the AMC diagnostic laboratory.

<table>
<thead>
<tr>
<th>Material</th>
<th>Pretreatment</th>
<th>Viral Target</th>
<th>Sample Volume**</th>
<th>Elution Volume</th>
<th>Isolation Protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA whole blood</td>
<td>none</td>
<td>CMV, HSV, VZV, EBV</td>
<td>50 μl</td>
<td>50 μl</td>
<td>total NA external lysis</td>
</tr>
<tr>
<td>Plasma</td>
<td>none</td>
<td>BK, Parvo, HHV8</td>
<td>200 μl</td>
<td>50 μl</td>
<td>total NA external lysis</td>
</tr>
<tr>
<td>CSF</td>
<td>none</td>
<td>EV, HPeV, HSV, JC, VZV</td>
<td>200 μl</td>
<td>50 μl</td>
<td>total NA external lysis</td>
</tr>
<tr>
<td>Respiratory material; swabs and lavages</td>
<td>none</td>
<td>EV, HPeV, HRV, InflA, InflB, HAV, RSV, hMPV, PIV 1-4, hCoV, HBoV</td>
<td>200 μl</td>
<td>50 μl</td>
<td>total NA external lysis</td>
</tr>
<tr>
<td>Faeces</td>
<td>pre-lysis of 50 μl faeces in 500 μl MP lysisbuffer; spin down and use supernatant as input</td>
<td>EV, HPeV</td>
<td>50 μl</td>
<td>50 μl</td>
<td>total NA external lysis</td>
</tr>
<tr>
<td>Biopsy</td>
<td>O/N pre-lysis in 200 μl 1% SDS/prot K solution x 56°C; spin down and use 100 μl as input</td>
<td>HAV, CMV</td>
<td>100 μl</td>
<td>50 μl</td>
<td>total NA external lysis</td>
</tr>
<tr>
<td>Culture supernatant</td>
<td>none</td>
<td>any culturable virus</td>
<td>20 μl</td>
<td>50 μl</td>
<td>total NA external lysis</td>
</tr>
<tr>
<td>Vesicle fluid</td>
<td>none</td>
<td>HSV, VZV</td>
<td>20 μl</td>
<td>50 μl</td>
<td>total NA external lysis</td>
</tr>
<tr>
<td>Urine</td>
<td>none</td>
<td>BK, CMV</td>
<td>200 μl</td>
<td>50 μl</td>
<td>total NA external lysis</td>
</tr>
</tbody>
</table>

*Median tissue culture infective dose; the amount of a cytopathogenic agent (virus) that will produce a cytopathic effect in 50% of cell cultures inoculated, expressed as TCID50/ml.

Cytomegalovirus (CMV), Herpes Simplex Virus (HSV), Varicella Zoster Virus (VZV), Epstein Bar Virus (EBV), Human Herpes Virus 8 (HHV8), Enterovirus (EV), Human parECHO Virus (HPeV), Human Rhino Virus (HRV), Influenza A/B (InfA/B), Human Adeno Virus (HAV), Respiratory Syncytial Virus (RSV), Human Metapneumo Virus (hMPV), Para Influenza Virus (PIV), Human Corona Virus (hCoV), Human Boca Virus (HBoV)

** Final input volume of lysed sample in the MagNA Pure 96 System is 500 μl for all materials
Cerebrospinal fluid (CSF), EDTA whole blood, plasma, faeces, throat swabs and adenoid tissue biopsies were taken from the Academic Medical Center clinical virology sample archives. All human sample materials used were collected according to general hospital standards. We have tested the daily routine using the MagNA Pure 96 System in our lab by spiking the samples with viral targets prior to extraction. Sample types analyzed in our laboratory are summarized in Table 1. We used different input volumes of samples, according to the sample type in order to make simultaneous extraction of a variety of sample types possible.

PCR Controls
Positive controls were purified plasmids containing the amplicon sequence of interest. In addition, two different Internal Controls (IC) were used. Phocine Herpes virus (PhoHV plasmid) for DNA targets and Equine Arteritis virus (EAV) for RNA targets.

Sample Pretreatment
- Cerebrospinal Fluid (CSF), EDTA anticoagulated whole blood and plasma were used without additional pretreatment.
- Throat swabs were resuspended in 2 ml of virus transportation medium (UTM Universal Transport Medium, Copan).
- Faeces was pre-treated as follows: 50 μl faeces was added to 450 μl MagNA Pure 96 lysis buffer, mixed, and subsequently incubated for 10 minutes at room temperature (+15 to +25°C). The suspension was centrifuged at 13,000 x g and the supernatant was spiked with target virus and IC prior to extraction.
- Tissue biopsies (ca. 20-40 mm3 in size) were pretreated as follows: Freshly isolated adenoid tissue was submerged in 1 ml of RNAlater (Ambion) and stored at -15 to -25°C. Prior to use for extraction RNAlater was pipetted from the (solid) tissue which was subsequently washed with PBS; 200 μl of a 1 mg/ml Proteinase K/1% SDS solution was added to the tissue, and the mixture was incubated overnight at 56°C. The tube was centrifuged at 13,000 x g and 100 μl of the supernatant was spiked with Internal Control (IC), added to 400 μl MagNA Pure 96 lysis buffer and used for extraction.

DNA/RNA isolation using MagNA Pure 96 System
One single extraction protocol was used for all the different sample types. All extractions were performed using the MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche Diagnostics), in combination with the Viral NA Plasma SV external lysis protocol. For all materials, the input volume of lysed sample was set to 500 μl and elution was set to 50 μl. Unless otherwise stated, the guidelines from the package insert were followed. To assess the MagNA Pure 96 System utility, multiplex PCR using the LightCycler® 480 Instrument was used to analyze MagNA Pure 96-purified DNA/RNA or, alternatively, in case of viral RNA targets, subjected to reverse transcription (RT)-reaction prior to PCR, as described previously. In brief, 40 μl of the MagNA Pure 96 System eluate (80% of the original input) was mixed with 10 μl RT-reaction mix, consisting of 1,500 ng of hexamers, 1x CMB1 buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 0.1% Triton X-100), 0.4 U of reverse transcriptase per μl, 120 μM (each) deoxynucleoside triphosphate, 0.08 U of RNAsin per μl, and 5 mM MgCl2. The mixture was incubated for 30 min at 42°C.

Real-Time PCR Amplification and Detection (using TaqMan® Probes)
As input in PCR, either 5 μl of the MagNA Pure 96 System eluate (10% of the original input) for DNA or 5 μl of the RT-reaction (8% of the original input) for RNA was used in a final volume of 20 μl. In all PCRs performed, the Roche LightCycler® 480 Probes Master (containing a hot start Taq enzyme) was used supplemented with 1U/ml Uracil-DNA Glycosylase (UNG), 900 nM of each primer and 200 nM of each TaqMan® probe. Sequences of the specific primers and probes used are published elsewhere. Detection was done using the Roche LightCycler® 480 Real-Time PCR Instrument. For all targets the same PCR program was used with the following cycling conditions: 2 min x 50°C (activation UNG), 10 min x 95°C (denaturation template and activation polymerase), followed by 45 rounds consisting of 15 sec x 95°C and 1 min x 60°C.

For data analysis with the LightCycler® 480 Software, the Second Derivative Maximum analysis method was used.
Results

Figure 1a. Shows the amplification curves for CMV (FAM), PhoHV IC (HEX) in inset.

Plasma. 200 μl plasma was spiked with CMV (dilution series 1 x 10^4 to 1 x 10^2 copies and negative control) and PhoHV IC (2.5 x 10^3 copies). After extraction the DNA was directly analyzed in real-time PCR.

Figure 1b. Shows the amplification curves for CMV(FAM), PhoHV IC (HEX) in inset.

EDTA whole blood. 50 μl EDTA whole blood was spiked with CMV dilution series (1x 10^4 to 1 x 10^2 copies and negative control) and PhoHV IC (2.5 x 10^3 copies). After extraction the DNA was directly analyzed in real-time PCR.

Figure 1c. Shows the amplification curves for HPeV (FAM), EAV IC (HEX) in inset.

CSF. 200 μl CSF was spiked with HPeV dilution series (1 x 10^5 to 1 x 10^1 copies and negative control) and EAV-IC (2 x 10^3 copies). After extraction the RNA was subjected to RT-reaction and real-time PCR.

Figure 1d. Shows the amplification curves for HAV (LC670), HPeV (FAM; upper inset) and PhoHV IC (HEX; lower inset) are also shown.

Throat swabs. A 200 μl throat swab sample was spiked with Human Adeno Virus dilution series (1 x 10^8, 1 x 10^7, 1 x 10^5, 1 x 10^3 and 1 x 10^1 copies and negative control), HPeV fixed concentration(1 x 10^3 copies) and PhoHV IC (2.5 x 10^3 copies). After extraction the nucleic acid was subjected to RT-reaction and real-time PCR.
Discussion

We show in this paper that the MagNA Pure 96 System is able to extract nucleic acid, RNA and/or DNA, from different kinds of clinical samples. Furthermore, a variety of sample types could be extracted efficiently in the same run using the same extraction protocol. To perform these experiments, we selected samples that had been tested as negative in our diagnostic department. These samples were spiked with various loads of different viruses, and subsequently extracted (external lysis protocol) and used for detection. To all samples, an internal control was added to exclude inhibition effects and monitor extraction and PCR efficiencies.

Discussion

Detection using the Roche LightCycler® 480 Real-Time PCR Instrument revealed that extraction from all six different types of sample materials tested was very efficient. Even with known difficult materials, such as faeces and biopsies, the MagNA Pure 96 System performed well.

In addition, we extracted nucleic acid from 2 different volume inputs (50 and 200 μl) of EDTA whole blood from patients positive for CMV. Higher inputs led to higher sensitivity in 7/8 samples, crossing point (Cp) cycle values were approximately 1.7 cycle lower while Cp values for IC remained similar (data not shown).
The MagNA Pure 96 System, in combination with the MagNA Pure 96 DNA and Viral NA Small Volume Kit, is a highly efficient and fast system for the extraction of nucleic acid from a variety of clinical sample materials. Remarkably, a single extraction protocol combined with a single downstream real-time RT-PCR protocol can be used. The MagNA Pure 96 System allows for high-throughput and fast turnaround times (it takes less than 60 minutes to extract nucleic acid from 96 samples). Due to the use of a single generic extraction protocol different materials can be extracted together simultaneously, thereby adding to the flexibility of the system. This is especially useful for molecular diagnostic laboratories that handle large and heterogeneous sample workflows.

References

1. Van Doornum et al. Journal of Clinical Microbiology, Februari 2003, Vol. 41 no.2; 576
2. Scheltinga et al., Journal of Clinical Virology, August 2005, Vol. 33 no. 4; 306

Important Note: Regarding the specific assay(s) described, Roche was neither involved in establishing the experimental conditions nor in defining the criteria for the performance of the specific assays. Roche therefore cannot take any responsibility for performance or interpretation of results obtained for the biological target parameter(s) described by the authors or other users using a similar experimental approach.

Potential users are informed to be aware of and in accordance with local regulations for assay validation and the scope of use for the involved Roche products, and to ensure that their use is valid in the countries where the experiments are performed.

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.