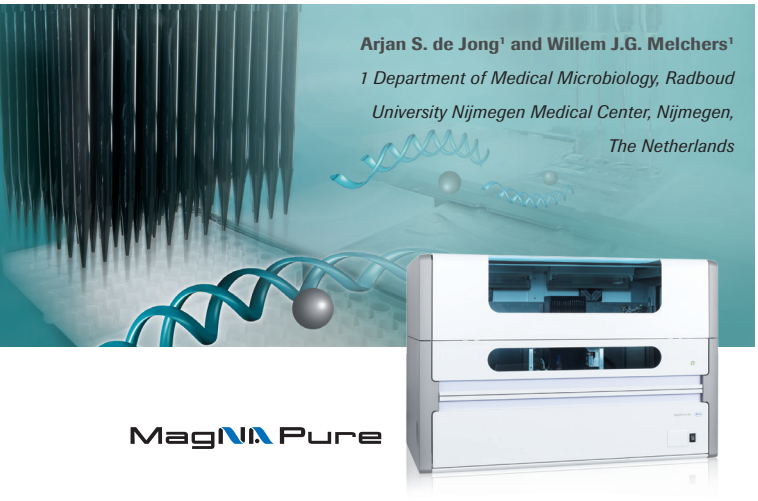
MagNA Pure System Application Note No. 8 January 2015

Utility of the MagNA Pure 96 System and LightCycler® 480 Instrument for the Detection of Respiratory Pathogens.



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Abstract

Acute respiratory disease (ARD) is a major cause of morbidity in developed countries and accounts for a large number of hospitalizations of especially young children. In addition, in developing countries, it constitutes an important cause of death. Viruses were shown to be a major cause of ARD. The last decade has seen the growing importance of molecular techniques in not only the discovery of novel viral respiratory pathogens, but also in routine testing of viral respiratory disease, replacing traditional virus detection methods in many laboratories. Also in our laboratory, a multiplex real-time PCR respiratory panel was introduced a number of years ago, leading to faster analysis and detection. In 2010, the MagNA Pure 96 System was introduced into the laboratory, where it is combined with the LightCycler® 480 Instrument to yield an easy-touse, efficient set-up for molecular research. This paper describes our results obtained with this setup in analysis of viral respiratory samples in 2010 and 2011.

Introduction

The Radboud University Nijmegen Medical Center (RUNMC) is one of eight university medical centers in The Netherlands. The primary tasks are patient care, research and medical education. The RUNMC has approximately 1,000 beds and more than 30,000 clinical admissions annually. The department of Medical Microbiology of the RUNMC consists of the sections Bacteriology, Virology, Parasitology, Mycology and Molecular Diagnostics. The Molecular Diagnostics section is responsible for the detection of a wide range of microorganisms, covering the entire field of medical microbiology. In addition to commercial CE-IVD marked diagnostic tests, lab-developed or in-house tests constitute an important part of the routine molecular diagnostics of infectious diseases.

Introduction continued

Increasing sample numbers and increasing diversity in molecular testing demand for efficient and automated extraction platforms, combined with flexible nucleic acid amplification systems. To address this demand, we have tested here the combination of the MagNA Pure 96 System and Roche LightCycler® 480 real-time PCR Instrument in our laboratory.

Acute respiratory disease is associated with high morbidity, in developing as well as developed countries. Viral respiratory tract infections are the leading cause of ARD, and in developed countries they account for a large portion of hospitalizations of especially young children, moreover, ARD is a major cause of death in developing countries [1]. In the last decade, molecular techniques have played an important role in the discovery of novel respiratory viruses like SARS coronavirus [2], human metapneumovirus [3], and the swine-origin pandemic H1N1/2009 influenza virus [4]. Moreover, the application of molecular techniques has revolutionized the routine testing of viral respiratory disease; in many laboratories multiplex real-time PCR assays have partially or fully replaced conventional virus identification methods, such as virus culture and commercially available rapid tests. Molecular testing usually increases the yield of detected pathogens, as a result of both increased sensitivity and the possibility to screen for viruses that can hardly or not at all be grown in tissue culture cell lines.

The MagNA Pure 96 System was implemented for the isolation of nucleic acids from all kinds of samples, including respiratory specimens. Together with the LightCycler® 480 real-time PCR instrument, the MagNA Pure 96 System provides a setup that allows for easy-to-use as well as efficient handling of large numbers of different materials with a variety of microbial pathogens, using a single isolation protocol and a limited number of different PCR reaction set-ups. This paper describes the results of our two year experience with the current setup for the research of viral respiratory pathogens.

Materials and Methods

Sample Material

All samples described in this paper were from 2010 and 2011, and brought to the microbiology department for testing for the presence or absence of respiratory pathogens. Samples were from different sites of the respiratory tract, predominantly throat swabs. Nasopharyngeal aspirates that were sent into the laboratory with a specific request for respiratory syncytial virus (RSV) detection were subjected to routine rapid testing using direct immune fluorescence. When tested inconclusive or negative for RSV, these samples were subjected to respiratory PCR analysis.

Respiratory PCR Panel

The respiratory PCR panel initially tested for the presence or absence of viral respiratory pathogens only, namely RSV, influenza A (FluA), influenza B (FluB), human rhinovirus (HRV), human metapneumovirus (HMPV), parainfluenzaviruses 1, 2, 3 and 4 (PIV1, PIV2, PIV3, PIV4), human coronaviruses OC43 and 229E (HCoV), enteroviruses (EV), human parechoviruses (HPeV), human bocavirus (HBoV) and human adenoviruses (AdV). Starting July 2010, mycoplasma pneumonia (MP) and chlamydophila pneumonia/psittachi (CP) were added to the panel. The respiratory panel is an internally controlled (equine arthritis virus, EAV) real time PCR panel that consists of 8 duplex PCR's (RSV/EAV, FluA/FluB, HRV/hMPV, PIV1/PIV3, PIV2/PIV4, hCoV43/hCoV229, EV/HPeV, Adv/HBoV) and two single target PCR's (MP and CP).

MagNA Pure 96 Nucleic Acid Isolation

Respiratory samples were checked for viscosity. Viscous/mucous materials likely to yield pipetting errors were pretreated by bead-beating using the MagNA Lyser Instrument (Roche) for 20 seconds at 6500 rpm. Samples were spiked with 5 µl of the isolation/inhibition control EAV [5], and used for total nucleic acid isolation on the MagNA Pure 96 System, using the MagNA Pure 96 DNA and Viral NA Small Volume Kit(Roche Diagnostics) and the Viral NA Plasma SV protocol. The input volume was 200 µl and the elution volume was set at 50 µl. Each isolation run was controlled by a negative control sample (virus transport medium, VTM).

Reverse transcription PCR (RT-PCR)

RNA in the total nucleic acid isolates was reverse transcribed to cDNA using TaqMan® Reverse Transcription Reagents Kit (Applied Biosystems), in a 50 µl reaction mix containing 20 µl of nucleic acid isolate and random hexamers as primers, according to the manufacturer's instructions. All reverse transcription reactions were controlled by a negative RT control (PCR grade water instead of template RNA) and a positive RT control (EAV RNA). Real-time PCR mixes (50 µl)

Materials and Methods continued

consisted of 25 µl of 2x LightCycler® 480 Probes Master (Roche), 5 µl of cDNA or extracted DNA and either 0.5 µM of each primer and 0.1 µM of each probe, or 5 µl of primer/probe premix. Real-time PCR was performed on the Roche LightCycler® 480 Instrument using the following conditions for all viral targets: 10 min denaturing and hot-start at +95°C, followed by 50 cycles of 15s at +95°C, 15s at +55°C, and 20s at +72°C. The conditions for the CP and MP targets were 5 min at +95°C, followed by 50 cycles of 15s at +95°C, 15s at +50°C, and 20s at +72°C. All real-time PCR reactions were controlled by one negative PCR control sample (5 µl of PCR grade water), and one positive control samples (purified plasmid preparations of the respective PCR products). Primers and probes were either from TIB Molbiol (Berlin, Germany) or obtained as premixes from Diagenode Diagnostics (Liege, Belgium) (see Table 1).

Target	Forward primer	Reverse primer	Hydrolysis/ TaqMan® probes	Target gene	Reference
HCoV OC43	CATACTATCAACCCATTCAACAAG	CACGGCAACTGTCATGTATT	ATGAACCTGAACACCTGAAGCCAATCTATG	Membrane protein	Gaunt
HCoV 229E	CATACTATCAACCCATTCAACAAG	CACGGCAACTGTCATGTATT	ATGAACCTGAACACCTGAAGCCAATCTATG	Membrane glycoprotein	Gaunt
HBoV	GGAAGAGACACTGGCAGACAA	GGGTGTTCCTGATGATATGAGC	ATGAACCTGAACACCTGAAGCCAATCTATG	NP1	Allander
AdV	CATGACTTTTGAGGTGGATC CATGAATTTCGAAGTCGACC TATGACATTTGAAGTTGACC	CCGGCCGAGAAGGGTGTGCGCAGGTA	AGCCCACCCTKCTTTAT GAGTCYACCCTTCTCTATGT	Hexon	Claas
EV	TCCTCCGGCCCCTGA	AATTGTCACCATAAGCAGCCA GATTGTCACCATAAGCAGCCA	CGGAACCGACTACTTTGGGTGTCCGT CGGAACCGACTACTTTGGGTGACCGT	5'UTR	Nijhuis
HPeV	TGCAAACACTAGTTGTAAGGCC	TTGGCCCACTAGACGTTT	CGAAGGATGCCCAGAAGGTACCCG	5'UTR	†
MP	ATTCCCGAACAAAATAATG	GTTTGACAAAGTCCGTGAAG	CAAAGCCACCCTGATCACCC	P1 Cytadhesin	Templeton
CP	GCGGAAGGGTTAGTAGTACA	ATCGCATAAACTCTTCCTCA	AAGGGATCTTCGGACCTTTCGG	16S rRNA	†

Figure 1: Sequences and target genes of primers and probes used for real-time detection of the specific viruses.

* Primers and probes for FluA, FluB, RSV, EAV, PIV1, PIV2, PIV3, PIV4, HRV and HMPV were obtained as premixes from Diagenode Diagnostics, Belgium

† Personal communication with Dr. E. Claas, LUMC Leiden, The Netherlands

For data analysis using the LightCycler® 480 Software, the AbsQuant/2nd Derivative Maximum analysis method was used; raw data were subjected to color compensation to correct for fluorescence cross-talk.

Results

Workflow

The protocol used in our laboratory for respiratory samples suspected for the presence of a viral etiological agent can be found in Figure 1. The respiratory panel consists of 8 duplex PCR's (RSV/EAV, FluA/FluB, HRV/hMPV, PIV1/PIV3, PIV2/PIV4, hCoV43/hCoV229, EV/HPeV, Adv/HBoV). Starting July 2010, two single target PCR's for MP and CP were added to the panel. Samples are spiked with 5µl of the isolation/inhibition control EAV, and nucleic acids were extracted using the MagNA Pure 96 System. Samples that are viscous/mucous and likely to produce pipetting errors using the MagNA Pure System were pretreated by bead-beating with the MagNA Lyser Instrument for 20 seconds at 6500 rpm. RNA is reverse transcribed to cDNA using random hexamer primers and the PCR reactions are performed on the LightCycler® 480 Instrument using either cDNA or total nucleic acid isolates, depending on whether the viral genome is RNA or DNA. From January 2010, when the MagNA Pure 96 System was implemented in the routine testing, to December 2011, a total of 2,255 respiratory samples were analyzed.

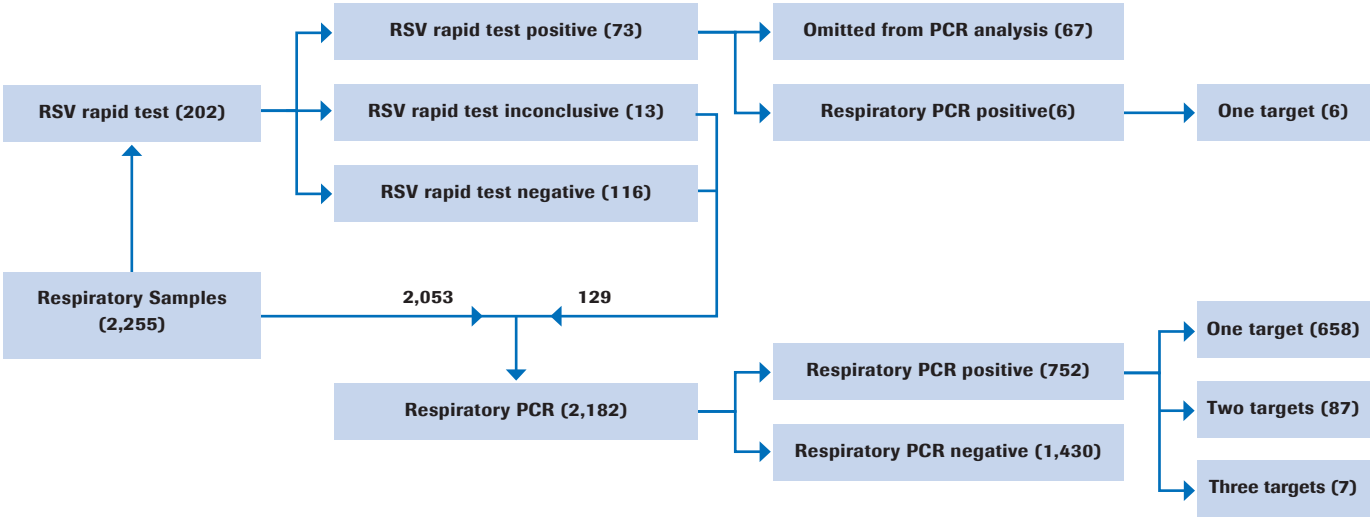

Figure 1: Schematic showing how all the respiratory samples were handled.

RSV rapid testing.

Upon specific request, 202 samples (mostly nasopharyngeal aspirates) were analyzed using the RSV rapid test (see Figure 1 and Table 2). Seventy-three (36.1%) tested positive for RSV and 67 of these were therefore excluded from respiratory PCR analysis. The remaining 6 samples were analyzed by respiratory PCR and all tested positive for RSV only. All other samples, yielding either negative results (116 samples, 57.4%) or inconclusive results (13 samples, 6.4%), were subjected to PCR analysis. Of the 13 samples that yielded inconclusive results in the RSV rapid test, 4 (30.8%) were positive by respiratory PCR, of which two were RSV positive. Of the RSV rapid test negative samples, 34 (29.3%) yielded a positive PCR result, of which 20 were HRV positive and 4 were positive for RSV despite the RSV rapid test negative result. In the 10 remaining samples, a variety of respiratory pathogens was detected. Of note, the PCR results of the samples that were subjected to respiratory PCR analysis following RSV rapid testing are included in the analysis of all respiratory PCR results.

RSV rapid test result	Number (%)	Tested in PCR	PCR positive for any pathogen number (%)	PCR positive for RSV number
Undetermined	13 (6.4)	13	4 (30.8)	2
Negative	116 (57.4)	116	34 (29.3)	4
Positive	73 (36.1)	6	6 (100.0)	6
Total	43/44	135	45 (33.3)	12

Table 2: Results of specimens that were subjected to RSV rapid testing.

Samples that were subjected to respiratory PCR analysis were collected from the entire respiratory tract, ranging from nose swabs to bronchoalveolar lavages (B.A.L.); see Table 3. More than half of the 2,188 specimens were throat swabs (1163 samples; 53.1%), followed by combined throat/nose swabs (222 samples; 10.1%). The overall positivity rate was 34.6% (738/2188 samples). Taking into account that for some specimen types the numbers are low, our results showed that the highest positivity rates were found for bronchial washes (29/45; 64.4%), sputa (49/88; 55.7%), nasopharyngeal secretions (69/130; 53.1%) and nose swabs (24/46; 52.2%), whereas biopsies, pleural fluids (both 0/4) and postmortem samples (2/37; 5.4%) had the lowest positivity rates.

Patient material	Total Number (%)	Positive number (%)
Throat swab	1163 (53.1)	393 (33.8)
Throat/nose swab	222 (10.1)	84 (37.8)
Bronchoalveolar lavage	197 (9.0)	48 (24.6)
Nasopharyngeal secretion	130 (5.9)	69 (53.1)
Swabs from other sites	107 (4.9)	25 (23.3)
Sputa	88 (4.0)	49 (55.7)
Nose swab	46 (2.1)	24 (52.2)
Bronchial wash	45 (2.1)	29 (64.4)
Mouth and throat wash	42 (1.9)	7 (16.7)
Postmortum materials	37 (1.7)	2 (5.4)
Nasopharyngeal swab	13 (0.6)	5 (38.5)
Liquids	11 (0.5)	6 (54.5)
Mouth swab	10 (0.5)	3 (30.0)
Biopsy	4 (0.2)	0
Pleural fluid	4 (0.2)	0
Other materials	69 (3.2)	14 (21.9)
Total	2188 (100.0)	758 (34.6)

Table 3: Types of respiratory samples used, the total number of samples per sample type and the number and percentage of positive samples per sample type.

PCR results

Fifty-eight (2.7%) of 2188 samples showed PCR inhibition (table 4), as demonstrated by the Cp value of the isolation/inhibition control EAV being above the cut-off value of 31. This cut-off was determined as the mean + two times the standard deviation of more than 300 respiratory samples of various collection types (data not shown). In our hands, repeating the isolation and reverse transcriptase-PCR procedures did not yield significant numbers of valid EAV values for respiratory samples that initially were inhibited (data not shown); therefore all samples that showed PCR inhibition were reported as 'undetermined due to PCR inhibition', accompanied with a request to send in a new sample for PCR analysis. A total of 758 samples (34.6%) were positive by respiratory PCR, yielding a total number of 859 PCR positive targets (table 4). The most encountered respiratory pathogen was HRV (264/859 targets; 30.7%), followed by RSV (137/859; 15.9%) and FluA (74/859; 8.6%). For most pathogens, the positivity rates were similar between the years 2010 and 2011. However, there were a few notable exceptions. The percentage of HRV positive samples differed between 2010 and 2011 (37.5% versus 21.9% respectively), whereas FluA and FluB were much more prevalent in 2011 than in 2010 (2.7% and 1.4% in 2010 versus 16.3% and 9.9% in 2011, respectively).
Finally, the increase in MP positive samples in 2011 is remarkable (1.0% in 2010 and 5.1% in 2011).

Results continued

Ninety-four samples were positive for more than one target (see Table 4), yielding a co-infection frequency of 12.4%. Table 5 shows that the two viruses that were detected at the highest frequency (HRV and RSV) were also the viruses that were most often detected together with one or more other pathogens (52/264 detections; 19.7%, and 29/137; 21.2%, respectively). In HRV co-infections, RSV and HBoV were most frequently detected as additional pathogens; in RSV co-infections, HRV was most frequently detected as additional pathogen, with HPeV and HCoV following at distance. In terms of percentage, HpeV, AdV and HBoV were the viruses most often detected in co-infections (45.2%, 43.3%, 37.8% co-infections, respectively). All are most often detected together with HRV. FluB and HMPV were the viruses that were detected the least often together with other pathogens, both absolute and in terms of percentage. Interestingly, although both HRV and FluA were detected at high frequencies, these viruses were not detected together in co-infections. In 2010, the relative frequency of co-infections was higher than in 2011 (see Table 4).

	Total Number (%)	2010 number (%)	2011 number (%)
Number of respiratory PCR tests	2,188	1137	1051
Number of inhibited samples	58 (2.7)	21 (1.8)	37 (3.5)
Number of positive samples	758 (100.0)	419 (100.0)	339 (100.0)
Samples positive for 1 target	664 (87.6)	357 (85.2)	307 (90.5)
Samples positive for ≥ 1 target	94 (12.4)	62 (14.8)	32 (9.5)
Samples positive for 2 targets	87 (11.5)	58 (13.8)	29 (8.6)
Samples positive for 3 targets	7 (0.9)	4 (1.0)	3 (0.9)
Number of positive targets	859	485	374

Table 4: Number of detected targets and number of positive samples. Positive samples are subdivided to depict the number and percentage of samples positive for one, two or three targets.

Discussion

We show in this paper that the MagNA Pure 96 System is able to efficiently extract DNA and RNA from a variety of routine respiratory samples, ranging from swabs in virus transport medium to B.A.L. Our isolation protocol is standardized in such a way that, except for the optional MagNA Lyser treatment of difficult-to-pipet materials, all specimens are isolated using one single isolation method, using the MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche Diagnostics) and the Viral NA Plasma SV protocol with fixed input and elution volumes. This was the workflow irrespective of sample type and DNA or RNA target. Using EAV as the isolation/inhibition control target, we found that no more than 2.7% of all samples showed PCR inhibition.

Using multiplex real-time PCR on the LightCycler® 480 Instrument, we are able to analyze the samples for the presence of 17 different respiratory pathogens, including both RNA and DNA targets, from one isolation extract obtained from the MagNA Pure 96 System. We found that from January 2010 to December 2011 in 34.6% of all samples, one or more respiratory pathogens were detected, with HRV and RSV being the most prevalent (264 and 137 positive samples, respectively). When the results of the RSV rapid test are taken into account, the prevalence of RSV is even higher (67 additional rapid test positives).

In summary, our data on the analysis of respiratory samples demonstrate that the MagNA Pure 96 System and the LightCycler® 480 Instrument are very suitable for efficient nucleic acid isolation and amplification in several different respiratory samples using a single generic isolation protocol, irrespective the type of nucleic acid or pathogen.

Discussion continued

	Total number (%)[1]	2010 number (%)[1]	2011 number (%)[1]	> 1 target detected number (%)[2]	Most detected additional target[3]
HRV	264 (30.7)	182 (37.5)	82 (21.9)	52 (19.7)	RSV (11) HBoV (10)
RSV	137 (15.9)	72 (14.8)	65 (17.4)	29 (21,2)	HRV (11) HPeV (4) HCoV (4)
FluA	74 (8.6)	13 (2.7)	61 (16.3)	10 (13,5)	RSV (5) †
EV	54 (6.2)	35 (7.2)	19 (5.1)	10 (18,5)	HRV (6)
HBoV	45 (5.2)	33 (6.8)	12 (3.2)	17 (37.8)	HRV (10)
FluB	44 (5.1)	7 (1.4)	37 (9.9)	2 (4.5)	
HMPV	39 (4.5)	17 (3.5)	22 (5.9)	3 (7.7)	
HCoV	37 (4.3)	30 (6.2)	7 (1.9)	9 (24.3)	HRV (4)
PIV3	35 (4.1)	21 (4.3)	14 (3.7)	6 (17.1)	HRV (3)
HPeV	31 (3.6)	23 (4.7)	8 (2.1)	14 (45.2)	HRV (7) RSV (4)
AdV	30 (3.5)	22 (4.5)	8 (2.1)	13 (43.3)	HRV (6)
MP	24 (2.8)	5 (1.0)	19 (5.1)	3 (12.5)	HRV (2)
PIV2	20 (2.3)	11 (2.3)	9 (2.4)	2 (10.0)	total NA external lysis
PIV1	13 (1.5)	10 (2.1)	3 (0.8)	2 (15.4)	HRV (2)
PIV4	9 (1.0)	3 (0.6)	6 (1.6)	2 (22.2)	HRV (2)
CP	3 (0.3)	1 (0.2)	2 (0.5)	0	
Total	859 (100.0)	485 (100.0)	374 (100.0)		

† no FluA/HRV coinfections detected

Table 4: Number of detected targets and number of positive samples. Positive samples are subdivided to depict the number and percentage of samples positive for one, two or three targets.

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