

## Utility of the MagNA Pure 96 System and LightCycler® 480 Instrument for Testing of Stool Samples on Toxin-producing *Clostridium difficile*

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MagNA Pure

### Abstract

*Clostridium difficile* infection (CDI) is an important cause of hospital diarrhea. Conventional CDI testing by enzyme immunoassays is fast yet shows poor sensitivity and specificity, whereas the ‘gold standard’ toxigenic culture is laborious and time consuming. Molecular tests for CDI are fast and highly sensitive and specific. Our laboratory has developed a molecular procedure that allows for rapid detection of CDI directly in stool samples. It is based on the isolation of highly pure DNA extracts from feces using the MagNA Pure 96 System, followed by detection of the *C. difficile* toxins *tcdA* and *tcdB* using real-time PCR on the LightCycler® 480 Instrument. The easy to use pretreatment of feces combined with nucleic acid isolation on the MagNA Pure 96 System resulted in only 0.5% of inhibited samples. The positivity rate for CDI detection increased from 7.3% using conventional methods to 13.7% using real-time PCR. Nearly all PCR positive samples were confirmed by toxigenic culture, demonstrating the high specificity.

With the MagNA Pure 96 System, handling steps were minimized and a high throughput and standardization were gained, causing less complexity, user interference and costs.

## Introduction

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The Radboud University Nijmegen Medical Center (RUNMC) is one of eight university medical centers in The Netherlands. The principle tasks are patient care, research and medical education. The RUNMC has close to 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 provides 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, in-house or home-brew tests constitute an important part of the routine molecular diagnostics of infectious diseases. Increasing sample numbers and increasing diversity in the diagnostic pallet 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 the LightCycler 96 Instrument. *Clostridium difficile* infection (CDI) is the major cause of hospital diarrhea. The

laboratory analytics of CDI is based on the demonstration of toxin A/B directly in stool samples or in culture after isolation of the pathogen. The direct cytotoxicity test has been the gold standard in laboratory diagnosis for many years, but more recently toxigenic culture has been used for this purpose [1]. As these methods are both labor intensive and have a long time-to-result, enzyme immunoassays (EIAs) are now used widely in routine laboratories [2]. However, the performance of these EIAs have been described to be poor, with sensitivity and specificity ranging from 60% to 99% and 70% to 100%, respectively [3,4]. Real-time PCR detection of *C. difficile* toxins is fast and has been shown to be both sensitive and specific [4, 5]. To this end, stool samples are processed to yield 10% (w/v) fecal suspensions which after an easy-to-use pretreatment procedure are directly processed using the MagNA Pure 96 System. Real-time PCR targeting both *C. difficile* toxins A and B (*tcdA* and *tcdB*) is performed using the LightCycler® 480 Instrument to detect the presence or absence of CDI.

## Materials and Methods

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### Sample material

For this study, 45 culture positive stool samples were studied retrospectively. In addition, 150 consecutive stool samples requested over a period of 2 months were analyzed prospectively. Samples were from individual, hospitalized subjects, and were the first sample of each subject [6].

Subsequently, a total of 1,254 stool samples were tested for the presence of *C. difficile* toxin genes by real-time PCR from January 2011 until December 2011.

### Nucleic Acid Isolation on the MagNA Pure 96 System

Stool specimens were collected in plastic containers without additives. Upon arrival in the laboratory, 10% (w/v) fecal suspensions were prepared by adding (dependent on the consistency) approximately 100 mg or 100 µl stool sample to 1 ml of sterile PBS. The remainder of the stool specimens was stored at +2 to +8°C for subsequent culture in case of a positive PCR result. Alternatively, the S.T.A.R. buffer (Roche) can be used for transport and storage of fecal samples. This might be advantageous for some applications; however, in our

hands the use of S.T.A.R. buffer as compared to PBS did not improve results in terms of yield or PCR inhibition.

Fecal suspensions were homogenized by vortexing for 1 minute, left to rest for 10 minutes at room temperature (+15 to +25°C) and again vortexed for 1 minute. Suspensions were centrifuged for 30 s at 1,000 x g, and 195 µl of the supernatant was spiked with 5 µl of the isolation control Phocine Herpes Virus (PhHV) [7], and used for DNA isolation on the MagNA Pure 96 System. DNA was isolated using the MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche) and the Viral NA Plasma SV protocol. This isolation procedure is used routinely in our setting, and by using the same isolation protocol for all different types of clinical samples, we are able to combine all samples that need to be processed on one day in one MagNA Pure 96 run.

The input volume was 200 µl and the elution volume was set at 50 µl. Each isolation run was controlled using a negative control sample (195 µl PBS).

**Real-Time PCR Amplification and Detection**

A multiplex real-time PCR assay was developed, in which the *tcdA* and *tcdB* *C. difficile* toxin genes were detected together with the gB polymerase gene of phocine herpesvirus (PhHV-1), which was included as internal control. Primers and TaqMan® probes for *tcdB* and PhHV detection were as described ([5, 7], respectively). For *tcdA* detection, primers were as described [8], whereas the anti-sense molecular beacon [8] was replaced by a sense TaqMan® probe (5'-CTACTAgAggAAgAgA-TTCAAATCCTCA-3'). As it is not clinically significant to discriminate between *tcdA*, *tcdB* or *tcdA/tcdB* positive specimens, both *tcdA* and *tcdB* probes are 6FAM labeled. PhHV was detected using a LightCycler® Red 610 labeled TaqMan® probe.

All primers and probes were from TIB Molbiol, Berlin. Real-time PCR mixes (50 µl) consisted of 25 µl of 2x LightCycler® 480 Probes Master (Roche), 0.5 µM of each primer and 0.1 µM of each probe, and 5 µl of extracted DNA. Real-time PCR was performed using the Roche LightCycler® 480 System using the following conditions: 10 min denaturing and hot-start at +95°C, followed by 50 cycles of: 10 s at +95°C and 60 s at +60°C. All real-time PCR runs were controlled by using one negative PCR control sample (5 µl of PCR grade H<sub>2</sub>O), and two positive control samples of purified plasmid preparations containing *tcdA* and PhHV or *tcdB* and PhHV PCR products, respectively.

For data analysis with the LightCycler® 480 Software, the AbsQuant/2nd Derivative Maximum analysis method was used.

**Results****Study set-up**

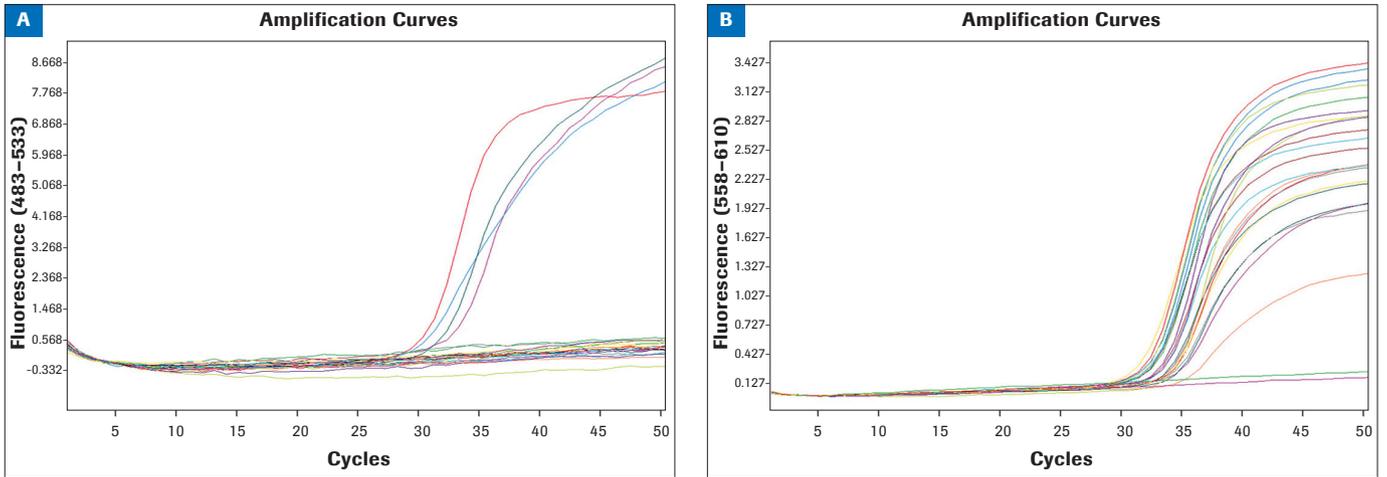
For this study, real-time PCR procedure performed retrospectively on 45 stool samples that were tested positive by toxigenic culture, and prospectively on 150 consecutive stool samples [6]. All 45 toxigenic culture positive samples were positive by real-time PCR. Of the 150 prospective samples, 18 (12.0%) tested positive by real-time PCR, of which 17 were positive by toxigenic culture. This discrepancy is most likely the result of the higher sensitivity of the PCR compared to toxigenic culture. All PCR negative samples were negative by toxigenic culture. Compared to the toxigenic culture gold standard, sensitivity and specificity of the real time PCR test were 100% and 99.2%, respectively [6].

**MP96 Utility by PCR Detection**

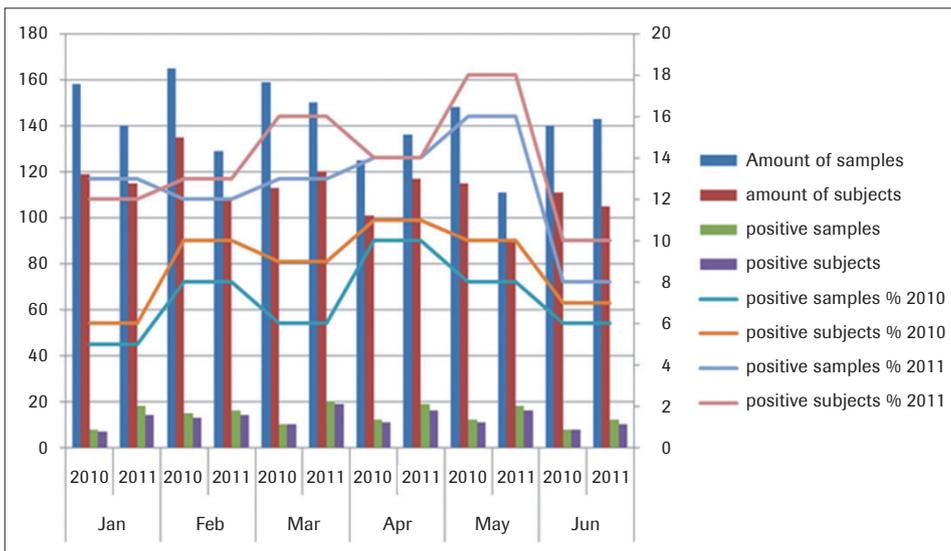
From January 2011 until December 2011, a total of 1,254 stool specimens were screened for the presence of *C. difficile* toxin genes by real-time PCR. No discrimination between *tcdA*, *tcdB* or *tcdA/tcdB* positive specimens was made, as both detection probes were 6-FAM labeled. Samples were collected and pretreated during day 1, DNA isolation using the MagNA Pure 96 System and real-time PCR were performed on day 2, and results were made available that same day.

To monitor for PCR inhibition, samples were spiked with PhHV isolation control. The mean crossing point (C<sub>p</sub>) value for PhHV of 100 processed stools was 34.65, with a standard deviation (SD) of 1.19. Based on these data, samples were regarded as inhibited when the C<sub>p</sub> value of the PhHV PCR was > 37 (i.e., the mean + 2x SD). Forty samples (3.2%) showed PCR inhibition in their initial PCR runs. Of these inhibited samples, stool suspensions were diluted 1:5 and extraction and real-time PCR were repeated. Six samples (0.5%) remained inhibited after retesting and were reported as 'not interpretable due to PCR inhibition'.

A total of 172 stool samples (13.7%) tested positive for *C. difficile* toxins by real-time PCR. An example of the results that were gained using the LightCycler® 480 System are shown in Figure 1. In the year 2010, the positivity rate was 7.3% using enzyme immunoassay and toxigenic culture for confirmation, which represents an almost 90% increase as the result of PCR-based screening. Figure 2 shows the number of subject samples and individual subject tested and the number and percentage of positive samples and subjects in the years 2010 and 2011. Two real-time PCR positive samples were not available for culture. Of the remaining positive samples, all but one were confirmed by culture.



**Figure 1: LightCycler® 480 results of a typical *C. difficile*/PhHV duplex real-time PCR run.** Amplification plots are shown of *tcdA/tcdB* (6FAM-label, panel A) and PhHV (LCred610-label, panel B). Two positive controls (*tcdA* and PhHV, and *tcdB* and PhHV), and two negative controls (one isolation and one PCR control) are included in each run. Panel A shows *tcdA/tcdB* amplification curves of the two positive controls and two positive routine samples. In panel B, all samples except the two negative controls show amplification of PhHV.



**Figure 2: Comparison of CDI test results from routine testing in the years 2010 (EIA and toxigenic culture) and 2011 (PCR).** Bars represent the absolute numbers of subject samples and individual subjects as well as the numbers of positive samples and positive subjects. Lines represent the percentages of positive samples and subjects.

## Discussion

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We show in this note that the MagNA Pure 96 System is suitable for use in DNA isolation from stool specimens. The combination of a simple pretreatment protocol together with the MagNA Pure 96 DNA and Viral NA Small Volume Kit and the Viral NA Plasma SV protocol shows excellent performance, resulting in PCR inhibition in only 3.2% of all samples in the initial run and 0.5% upon retesting of 1:5 diluted stool suspensions of the inhibited samples. This percentage is low compared to what is shown in other reports concerning PCR inhibition of stool samples, where PCR inhibition is found in 1.8% up to 15% of the samples tested [9 and references therein].

### Minimization of handling steps with high throughput

This isolation protocol does not require additional substances or specific buffers, cycles of freezing and thawing, nor prolonged off-board incubation in lysis buffer to avoid PCR inhibition, and is therefore both user-friendly and suitable for high-throughput screening of stool samples.

### Real-time PCR method increased find-rate

Implementation of this real-time PCR approach demonstrated increased both the sensitivity and specificity of *C. difficile* detection. Using real-time PCR, sample positivity increased by almost 90% compared to traditional testing using enzyme immunoassay. Nearly all PCR positive samples were confirmed by toxigenic culture.

Conventional CDI testing by enzyme immunoassay is fast yet shows poor sensitivity and specificity, whereas the 'gold standard' toxigenic culture is laborious and time consuming.

### Application reduces complexity and cost

The LightCycler® 480 Instrument offers the possibility to detect both *C. difficile* and the DNA isolation control PhHV in one multiplex reaction, reducing costs. The LightCycler® 480, with the ability to run 96 or 384 samples in one batch, allows the possibility of running multiple assays which increases a laboratory's flexibility and efficiency. This is an absolute requirement in our university hospital setting.

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## Important Note

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- The LightCycler<sup>®</sup> 480 Instrument is for life science research only. Not for use in diagnostic procedures.



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