MagNA Pure System Application Note No. 7

March 2012

Use of MagNA Pure 96 and LightCycler® Systems for High Throughput Testing of Nail Samples for Dermatophytes Causing Onychomycosis.

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1 Abstract

Dermatophytes are the major cause of onychomycosis. Conventional laboratory diagnosis is labor-intensive, requiring long incubation times and showing poor sensitivity. For routine detection of dermatophytes in nail samples from licensed pedicures, a faster and more accurate detection method is necessary. Molecular testing by PCR has shown in literature to increase sensitivity significantly compared to conventional culture and microscopy methods. In this study, 48 nail samples were collected for validation and tested by direct microscopy, culture and PCR. Using conventional culture, dermatophytes were detected in 40% of the nail samples compared to 83% by PCR detection. In addition, results of 264 nail samples were analyzed after implementation of the new molecular workflow for onychomycosis detection. In the new molecular workflow dermatophyte positivity was found in 56% of these nail samples, followed by direct microscopy for PCR negative nail samples. In summary, PCR detection using the MagNA Pure 96 System and the LightCycler® 480 Instrument was accurate, showing significantly higher sensitivity, reduced time and costs, with suitability for high throughput testing of nail samples.

2 Introduction

The Radboud University Nijmegen Medical Centre (RUNMC) is one of the eight university medical centers in the Netherlands. The RUNMC has three priority areas closely connected to each other namely patient care, education and scientific research. The department of Medical Microbiology has the sections Virology, Bacteriology, Parasitology, Mycology and Molecular Diagnostics. The section Molecular Diagnostics is responsible for the analysis of about 40,000 routine samples yearly involving the full spectrum of infectious diseases. The department of Medical Microbiology of the RUNMC has been appointed in 2011 as the Mycology Reference Centre of the Netherlands. Within the framework of this Mycology Reference Centre, fast and accurate identification of fungi is performed.

Licensed pedicures can send nail samples to our laboratory to diagnose onychomycosis, infection of the nail. Onychomycosis is predominantly caused by dermatophytes. Dermatophytes are keratinophylic fungi comprising three genera, namely *Trichophyton spp.*, *Microsporum spp.* and *Epidermophyton spp.* These genera can infect keratin structures present in nails, skin and hair. Onychomycosis is distributed globally and affects between 2 and 18% of the global population with the most prevalent dermatophyte causing onychomycosis being *Trichophyton rubrum* that accounts for approximately 80-90% of the nail dermatophytes infection [1]. Abnormalities of the nail caused by psoriasis, trauma or other conditions can easily be misdiagnosed as onychomycosis. Costly antifungal treatment requires long periods of intravenous therapy and has many potential side-effects, therefore accurate testing of onychomycosis is essential before treatment can be initiated.

Classical diagnosis of onychomycosis consists of microscopy and culture that require highly skilled laboratory personnel. It has been reported in the literature that detection performed by direct microscopy on nail samples shows lower sensitivities compared to molecular detection. Microscopy can also not discriminate between dermatophytes and other fungi. Culture also shows a reduced sensitivity compared to molecular detection, and cultures can require two to six weeks to become positive [1]. Classical methods for detecting dermatophytes are labor-intensive, requiring long incubation times and showing poor sensitivities. To reduce workload, cost and time for the routine testing of onychomycosis, a molecular detection for dermatophytes on nail samples was implemented using the MagNA Pure 96 System for the extraction of nucleic acids, combined with the LightCycler® 480 Instrument for PCR.

3 Materials and Methods

Sample material

Nail samples were sent to our laboratory by certified pedicures in plastic containers for onychomycosis testing. During the month July 2011, 48 nail samples were collected for validation and tested by culture, direct microscopy and molecular detection. During the months September, October and November 2011, results of 264 nail samples were collected after implementation of the new molecular workflow for onychomycosis detection by using PCR detection and followed by direct microscopy only for PCR negative samples.

Routine microbiology

Nail samples were cut into 6-8 small pieces, using a sharp sterile knife in a disposable petri dish. Five pieces were used for culture, and 1 or 2 pieces were used for direct microscopy. Direct microscopy was performed on the nail samples using Blancophor P dye. Slides were evaluated for yellow to blue fluorescent fungal structures. Culture was performed by incubation of the nail samples on Sabouraud agar slants supplemented with chloramphenicol, Sabouraud agar slants supplemented with chloramphenicol and cycloheximide, and Dixon agar plates. All culture slants were incubated at +29°C for 6 weeks, and the Dixon agar plate was incubated for 1 week. Identification of fungi was done by micro- and macroscopic examination when cultures became positive for fungal growth.

DNA template extraction on the MagNA Pure 96 System

Molecular testing was performed on nail samples that were cut in a size up to 2-3 mm. From each nail sample one 2-3 mm nail piece was incubated overnight at +55 °C, while shaking at 1400 rpm, in 200 µl lysis buffer containing 10 mM Tris–HCl [pH 8.0], 10 mM EDTA, 100 mM NaCl, 1% [w/v] sodium dodecyl sulphate (SDS), 80 mM dithiothreitol (DTT) and 1 mg/ml proteinase K. Then, 200 µl of the lysis buffer were transferred to each well of the MagNA Pure 96 Processing Cartridge, and approximately 3000 copies of phocine herpes virus 1 (PhHV) were added as an internal control to monitor DNA extraction efficiency and PCR inhibition.

3 Materials and Methods continued

For the MagNA Pure 96 System run, the *MagNA Pure 96 DNA and Viral NA Small Volume Kit* was used, in combination with the *Viral NA Plasma SV protocol*. A sample volume of 200 µl was chosen on the software menu, and the elution volume was set to 50 µl. After samples were transferred to the instrument, prefilled kit reagents and disposables were loaded, according to the manufacturer's recommendations. Fully automated robotic isolation of 96 samples was performed in approximately one hour.

LightCycler® 480 PCR analysis

The PCR analysis was performed using the LightCycler® 480 Instrument. A primer and probe set was used from Wisselink et al. (Journal of Microbiological Methods 2011; 85(1): 62-66) to detect the entire dermatophytes group consisting of *Trichophyton spp.*, *Microsporum spp.* and *Epidermophyton spp.* [1]. The dermatophytes primer probe set targets the ITS1 region of the ribosomal DNA and is present in fungi in multiple copies, thereby increasing sensitivity of the molecular detection. In duplex with the dermatophytes PCR, an internal control PCR reaction of PhHV was performed using primers and probes of van Doornum et al. (Journal of Clinical Microbiology 2003; 41(2): 576–80) [2].

Ten microliter aliquots of template DNA, purified using the MagNA Pure 96 Instrument, were added to each of the 40 µl of LightCycler® 480 Probes Master mix (Roche Diagnostics), containing the corresponding primers and probe oligonucleotides for the dermatophytes and PhHV PCR (TIB Molbiol, Berlin). The LightCycler® 480 Instrument was used for qualitative detection of the ITS1 gene using dermatophytes specific primers. The detection of PhHV served as a control for extraction and the absence of PCR inhibition.

4 Results

Internal control PhHV

The detection of PhHV served as a control for the extraction procedure and the absence of PCR inhibition by the nail sample. All PhHV PCR reactions were positive with a mean crossing point (Cp) value of 32.43. In two cases, the standard deviation of these Cp values (32.43 ± 0.87) was calculated to determine the cut-off value for the PhHV PCR. A Cp of ≤ 34.5 cycles for PhHV PCR was regarded as a proven positive internal control reaction. After testing 48 nail samples, one sample required retesting based on the diagnostic cut-off Cp value (its Cp value was 35.6).

An example of the results obtained using the LightCycler® 480 Instrument to perform dermatophyte and PhHV PCR is shown in Figure 1.

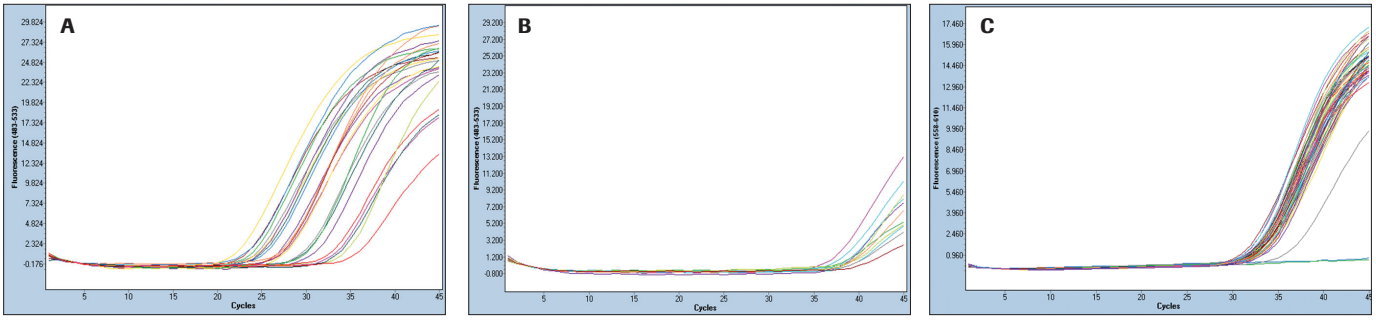

Figure 1: LightCycler® 480 analysis plots of nail samples. A) Positive dermatophytes PCR nail samples (Cp <35) B) Negative dermatophytes PCR nail samples (Cp>35) and C) PhHV PCR of nail samples from which one (in grey) is inhibited with a Cp value >34.5 (Cp = 35.61).

Validation of dermatophytes detection

All 48 nail samples were collected and tested for validation by direct microscopy, culture and PCR. For direct microscopy no differentiation between dermatophytes and other fungi can be made. For culturing of nail samples, only the dermatophyte positive cultures were scored as positive in this study. Other cultured fungi were however also identified to the genera level and included for further analysis. For PCR of dermatophytes, nail samples with a Cp value of <35 were regarded as a positive result. This was determined previously in the study of Alexander *et al.* In this study it was shown that nail samples with a Cp value >36 failed to grow in culture and were negative by direct microscopy. Our results confirmed these results, all 14 out of 15 nail samples with a Cp value of >35 were negative for dermatophytes culture and direct microscopy. One nail sample in the current sample collection showed a discrepant PCR result that was negative by PCR, in contrast to positive using microscopy and culture.

Using the above indicated PhHV cut-off values, the following results were obtained by testing 48 nail samples (see Figure 2). Direct microscopy was positive for fungi in 35 nail samples. From these 35 nail samples, 18 were culture positive for dermatophytes and were all except for one nail sample positive by PCR detection. Seventeen nail samples were negative for dermatophytes by culture although direct microscopy was positive. Two of these nail samples were also PCR negative, and 15 of these nail samples were PCR positive for dermatophytes.

Direct microscopy was negative in 13 nail samples. All of these 13 nail samples were culture negative except for one nail sample in which a dermatophytes was cultured, that was also detected by PCR. One yeast sample was cultured from a nail sample (denoted as culture negative) which was negative by direct microscopy and PCR detection. From 12 direct microscopy negative and culture negative nail samples, 12 were negative by PCR detection but one was however PCR positive.

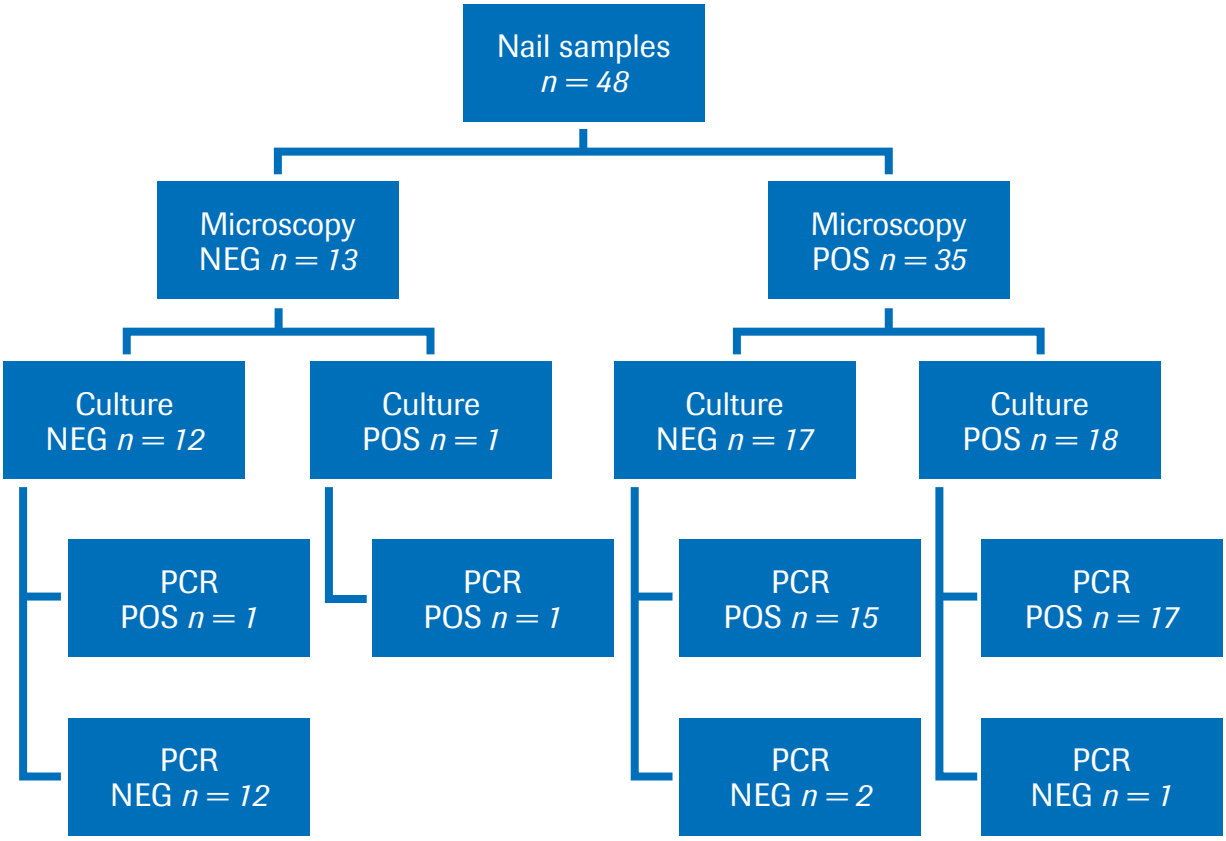

Figure 2: Overview of the results of direct microscopy, culture and PCR detection of 48 nail samples. Microscopy is not able to differentiate dermatophytes from other fungi. PCR detection was specific for all three genera of dermatophytes.

Routine dermatophytes detection

Using a new workflow for molecular detection of onychomycosis, results of 264 nail samples tested during September 2011 and November 2011 were collected (see Figure 3); 147 nail samples were found to be dermatophyte positive by PCR detection (55.7%). Of the 112 nail samples that were found to be negative for dermatophytes by PCR detection, 95 remained fungal negative by direct microscopy; 17 of the dermatophytes PCR detected negative nail samples were detected as fungal positive by direct microscopy; 19 nail samples could not be interpreted due to inhibition of the sample material. These samples produced a Cp value > 34.5 for the PhHV PCR. After repeating the PCR detection by performing a second DNA isolation of the nail sample, five nail samples still could not be interpreted due to Cps > 34.5, and a new nail sample was requested for onychomycosis detection.

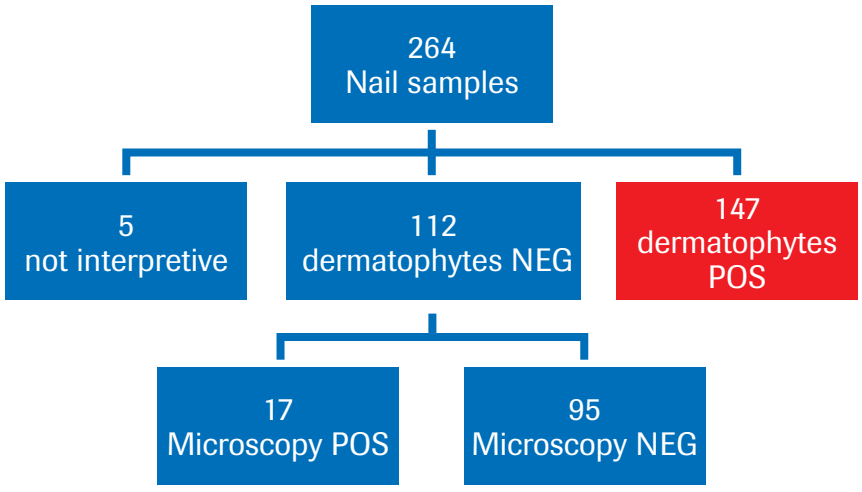

Figure 3: Results of the new molecular workflow for onychomycosis detection during the months September, October and November 2011; 264 nail samples were collected and tested using PCR detection, followed by direct microscopy only for PCR negative samples.

5 Discussion and Conclusions

Recently, different studies have been published about the efficient use of molecular PCR assays to detect dermatophytes in nail samples [1,3]. Molecular detection of dermatophytes by PCR was shown to be accurate, fast and robust in these studies. Important for correct interpretation of onychomycosis is the use of crossing point (Cp) cut-off value in the dermatophytes PCR. Nails are not a sterile material and the presence of dermatophytes detected by real-time PCR does not necessarily correlate with the presence of onychomycosis. It has been shown that PCR Cp values greater than 36 are culture and microscopy negative, and in the nail samples there was no evidence for onychomycosis [3]. This was also confirmed in our currently performed routine dermatophytes detection. Without evidence for infection it is not ethically possible to start costly antifungal therapy due to the many potential side-effects. For this reason, cut-off values for the dermatophytes PCR are of great importance.

The results of the current study of nail samples from licensed pedicures confirmed previously published results. A significant number of nail samples were found to be positive using PCR (83% nail samples), compared to using direct microscopy (73% nail samples) and fungal culture (40% nail samples). One additional dermatophytes positive nail sample was detected in 13 microscopy negative samples, and 17 additional dermatophytes positive nail samples were detected in 35 microscopy positive samples compared to positive culture results. One nail sample showed a discrepant PCR result that was negative by PCR in contrast to a positive microscopy and culture. This could be caused by sampling error of the nail. Each nail is cut in different pieces for all three tests although only local parts of the nail can be infected by the dermatophyte. The large proportion of culture negative and PCR positive nail samples (22 nail samples) underscores the lack of sensitivity of the culture based detection of dermatophytes, confirming previously published results.

5 Discussion and Conclusions continued

Automated DNA isolation performed by the MagNA Pure 96 System, in combination with PCR analysis performed using the LightCycler® 480 Instrument, produced a substantial time reduction for a high throughput method for testing dermatophytes in nail samples. In our mycology laboratory, cultures were incubated up to six weeks before a negative result could be reported. In the current workflow using direct microscopy and PCR detection (see Figure 4) we found that we could reduce this time to a maximum of 48 hours. Direct microscopy is still performed to detect cases in which fungi other than dermatophytes are causing onychomycosis. These findings have allowed us, in our laboratory, to replace conventional culture with molecular PCR testing for the detection of dermatophytes. PCR testing reduced costs and time considerably, at the same time the sensitivity of dermatophytes detection increased significantly. The MagNA Pure 96 System and the LightCycler® 480 Instrument were also shown to be suitable for high throughput testing.

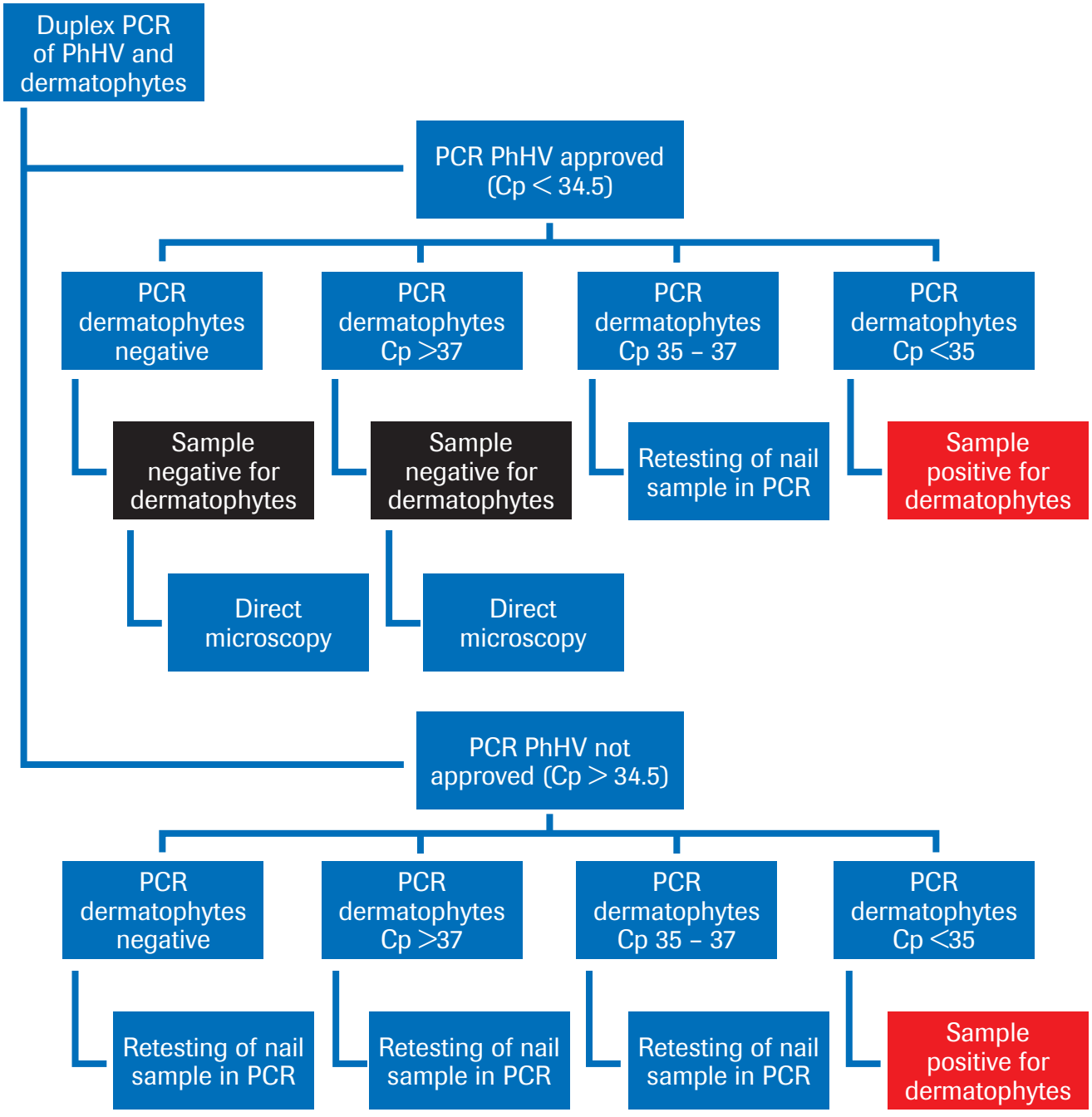

Figure 4: Current workflow of nail samples for onychomycosis detection.

6 References

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(2) van Doornum GJ, Guldemeester J, Osterhaus AD, Niesters HG (2003) Diagnosing herpesvirus infections by real-time amplification and rapid culture. J Clin Microbiol 41: 576-580.

(3) Alexander CL, Shankland GS, Carman W, Williams C (2011) Introduction of a dermatophyte polymerase chain reaction assay to the diagnostic mycology service in Scotland. Br J Dermatol 164: 966-972.

7 Important Note

- Data included in this article are the sole responsibility of the authors that have published them. These authors are responsible for following the respective local regulations for assay setup and validation.
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- 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 System (Cat. Nr. 05 195 322 001) and LightCycler® 480 Real-Time PCR System are not intended for *in vitro* diagnostic use in the U.S.

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Sandhofer Straße 116
68305 Mannheim
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