DNA Microarray-Based Detection and Identification of Fungal Specimens

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Abstract

Novel DNA-based molecular methods can be used to detect the fungal species faster than with the conventional methods. Combination of PCR and microarray provides rapid, sensitive, and reliable detection of pathogenic fungi. The advantages over other DNA-based methods are that microarray technologies allow broader coverage of detectable targets and simultaneous detection of multiple targets in a single assay. Furthermore, microarray technologies have the potential to discriminate between closely related fungal species. Although the use of microarray technologies in clinical diagnostics is still rare, the microarray-based approaches are believed to have great clinical potential in the field of infectious diseases.

Keywords

DNA • Polymerase chain reaction • Microarray • Pathogen • Fungi • Identification

Introduction

Conventional microbiological diagnostics of a fungal infection mainly rely on microscopic and cultural techniques that are time-consuming, labor-intensive, and require expertise. These methods usually yield diagnostic results in days or in some cases up to weeks after sampling. Furthermore, cultivation of fungi is not always successful under laboratory conditions. Such failures may occur due to unsuitable culturing media and conditions for the fungal species in question. Molecular methods based on detection of nucleic acid (NA) from clinical samples aim to circumvent these problems. In addition, they aim to improve the diagnosis of fungal infections by shortening time to result and increasing sensitivity and accuracy. Polymerase chain reaction (PCR)-based assays can amplify and detect minute quantities of DNA isolated from a pathogenic fungus in few hours, having a limit of detection of only a few genome copies per reaction. Although the multiplex PCR is slowly gaining ground in fungal diagnostics, most of the tests are still amplifying only one or few fungal targets in

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a single reaction [1, 2]. Multiplex or broad-range PCR in combination with microarray allows rapid detection of microbial DNA and species identification of multiple microbial targets in a single assay [3–7]. The simple, array-type technologies with broad target coverage have especially been believed to have great clinical potential in the field of infectious diseases [8–12]. Rapid clinical diagnostics reduces the use of antimicrobials in addition to allowing a faster switch to the most optimum treatment, which improves patient outcome and reduces both side-effects and treatment costs [13–17].

Breakspear and Momany have reviewed the use of fungal microarray in research settings, including studies of fungal metabolisms, development, pathogenesis, symbiosis, and industrial fermentations [18]. Recently, several publications have also demonstrated the applicability of fungal microarray in clinical diagnostic purposes [7, 19–22]. These publications described the use of a multiplex/broad-range PCR with oligonucleotide probe array, targeting highly conserved and variable species-specific regions of the internal transcribed spacers (ITS) of rRNA gene complex of clinically relevant fungal pathogens. More often the ITS regions have been chosen as targets in fungal microarrays due to their presence in numerous copies in the fungal genome, which enables highly sensitive amplification by PCR. The high level of sequence variability of the ITS regions also allows reliable differentiation of closely related fungal taxa and species. Moreover, the comprehensive rRNA gene complex database is rapidly expanding and, thus, supporting the in silico design of primers and oligonucleotide probes.

Three commercial PCR and microarray-based products for fungal diagnostics stand out from the rest: CLART® SeptiBac+ (Genomica, Madrid, Spain), MycArray™ (Myconostica, Manchester, UK), and Prove-it™ Sepsis, v2.0 (Mobidiag, Helsinki, Finland). All of these assays use similar methodologies for detection of pathogenic fungal species from clinical samples. The assays involve the use of PCR as an amplification method prior to microarray phase, where the actual identification of fungal species occurs. The ArrayTube™ or an analog microarray is used as a platform for the oligonucleotide probes. The ArrayTube™ has been demonstrated to detect and identify viral and bacterial pathogens or bacterial pathotypes with a high degree of sensitivity [23–27] and to be capable of detecting antimicrobial resistance genes [28–30] from an isolated DNA sample. Also, Monecke et al. have published a case report of peritonitis where the ArrayTube™ harboring the fungal content was used to detect the causative agent, *Rhizopus microspores* [31].

In contrast to the previously mentioned fungal microarray publications, in these three commercial platforms, the principle behind the visualization of a positive hybridization on the microarray is based on a colorimetric reaction instead of fluorescent-based methods. In the workflow, biotin labeled amplicons are first hybridized with the specific oligonucleotide probes pre-printed on the microarray surface and then streptavidin-horseradish peroxidase (HRP) conjugate is attached to the biotin label. Finally, the presence of the HRP is visualized in the precipitation reaction by which HRP catalyzes the conversion of 3,3′,5,5′-Tetramethyl Benzidine (TMB) substrate or an analog into a precipitate thus forming a colored spot on the specific microarray position. An image is then captured from the microarray by dedicated reader device. The image is analyzed, and the result of the analysis, typically the name of the causative agent and signal intensities of each oligonucleotide probes, are reported by the software.

The fungal panels of the CLART SeptiBac+, MycArray, and Prove-it Sepsis assays vary, but all of them target the clinically relevant *Candida* species, that is, *C. albicans*, *C. krusei*, and *C. glabrata*. The assays aim at identification of fungal species from the positive blood culture used in sepsis diagnostics. Sepsis necessitates rapid and accurate diagnostics to improve the chances of a positive outcome for the patient. The fungal sepsis is associated with significant mortality and morbidity, especially when *Candida* spp. is the causative agent. Fluconazole is the choice for first-line therapy in candidemia; therefore, rapid differentiation between fluconazole-sensitive and

Fig. 53.1 The images of the Prove-it™ Sepsis TubeArray reader and Prove-it™ TubeArray, which is a plastic microreaction tube containing a microarray at the bottom

potentially fluconazole-resistant *Candida* species is of the essence. Recent studies have shown that appropriate and early antifungal therapy (treatment started within the 48 h after the onset of candidemia) is a major factor associated with a good prognosis in fungal infection [16, 17].

The performance of Prove-it Sepsis assay in routine clinical settings for sepsis diagnostics has been recently published [32]. In the multicenter study, the definitive identification of bacterial species with the Prove-it microarray platform and the corresponding assay protocol was considered highly sensitive (95%) and specific (99%). It was concluded that the assay was faster than the gold-standard culture-based methods and it could thus enable earlier evidence-based management for clinical sepsis. Furthermore, it was also stated that the microarray platform's robust nature, ease of implementation, software-controlled decision support for results, and portability has potential for successful strategic implementation in low resource settings (Fig. 53.1). The current generation of the Prove-it Sepsis v2.0 assay consists of a pathogen panel that covers the majority of sepsis-causing pathogens, including over 60 g-negative and gram-positive bacterial species, the methicillin resistance marker together with 13 fungal species. The fungal detection is realized by broad-range PCR primers that originate from the conserved regions of ITS together with specific oligonucleotide probes located at hyper-variable regions flanked by the primers. Each probe on the array matches either a particular pathogen species or higher-level taxon. The turnaround time of the assay is three hours, excluding DNA extraction. The fungal pathogen panel of the assay covered the following clinically relevant species: *C. albicans, C. glabrata, C. parapsilosis, C. tropicalis, C. guilliermondii, C. lusitaniae*, *C. dubliniensis*, and *C. krusei* and pan-yeast identification covering *C. pelliculosa, C. kefyr, C. norvegensis, C. haemulonii,* and *Saccharomyces cerevisiae*. The protocol below is based on the procedure of Prove-it Sepsis StripArray (Figs. 53.2 and 53.3).

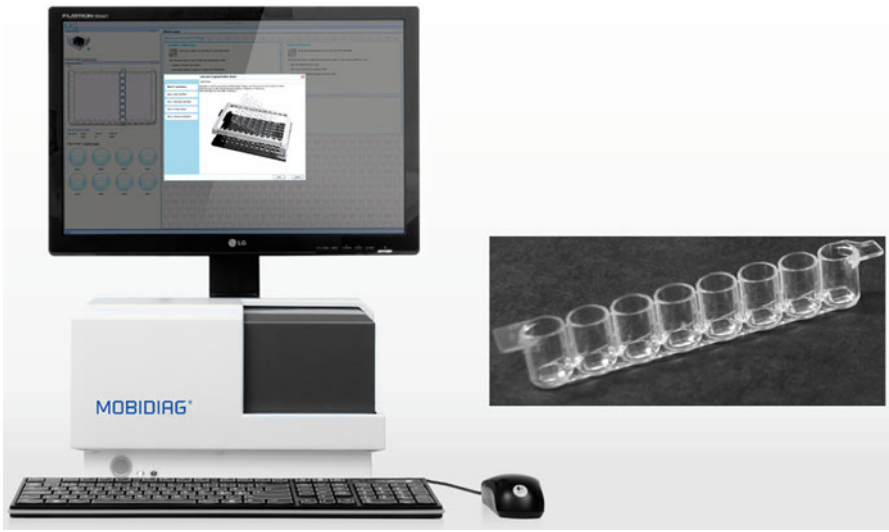

Fig. 53.2 The images of the Prove-it™ Sepsis StripArray system and Prove-it™ StripArray, which consists of eight plastic microreaction vials containing a microarray at the bottom of each well

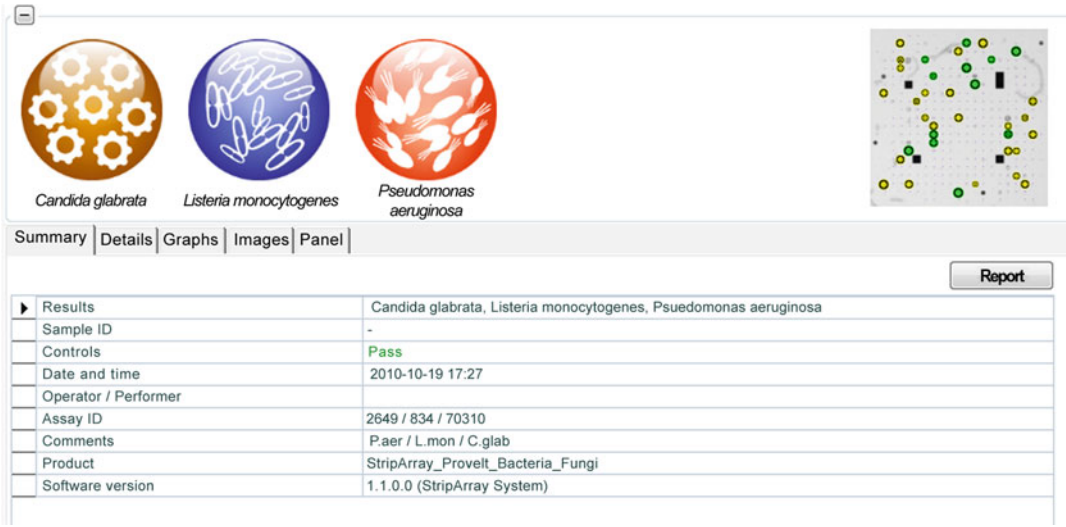

Fig. 53.3 The image of the Prove-it™ Advisor result. The top section of the result view presents the end result of the assay including icons for the identified target(s) and the analyzed microarray image. The results view has the following tabs: Summary, Details, Graphs, Images, and Panel, from which the details can be viewed. Also, the detailed result of bacterial content and bacterial controls (pass/fail), the detailed result of fungal content and fungal controls (pass/fail), and other assay information (from Sample ID to Software version) that is common for all contents are shown

Materials

1. Prove-it Sepsis v2.0 kit.
2. Prove-it StripArray System.
3. Distilled water.
4. Nucleic acid and nuclease-free, aerosol-resistant pipette tips.
5. Sterile, nucleic acid-free 1.5-mL microfuge tubes.

6. Sterile, nucleic acid-free PCR tubes suitable for the PCR instrument.
7. Racks for tubes.
8. Disposable gloves and laboratory coats.
9. PCR Thermal Cycler. The performance of Prove-it Sepsis has been evaluated using Eppendorf Mastercycler® epGradient S. The selection of the PCR cycler instrument may affect the assay protocol duration and the assay sensitivity.
10. At least two thermal mixers capable of 25, 30, and 66 °C with microtiter plate adapter.
11. A vortex mixer.
12. A spin microfuge.
13. Adjustable micropipettes for pre-PCR and post-PCR areas.
14. A vacuum suction system.

Methods

Detection and Identification of Fungal Species Using PCR- and Microarray-Based Methods

The protocol of the commercial Prove-it Sepsis v2.0 assay is modified from the protocol published by Järvinen et al [3]. and Aittakorpi et al. [33].

Preparing the Fungal PCR

1. Take PCR reagents, except for polymerase, to room temperature.
2. Vortex and spin down all reagents.
3. Prepare the fungal master mixture to a clean laboratory tubes. Add 3.1 μL of PCR water (Mobidiag), 1.5 μL of 10× Buffer (Qiagen, Hilden, Germany), 1.1 μL of BSA (Mobidiag), 0.3 μL of $MgCl_2$ (Qiagen), 2.3 μL of dNTP-mix (Mobidiag), 0.8 μL of Prove-it Fungi Primer-F (Mobidiag), and 2.0 μL of Prove-it Fungi Primer-R (Mobidiag) to the master mixture. Make 10% more of the master mixture than needed.
4. Add polymerase to the master mixture, 0.4 μL of HotStarTaq® DNA Polymerase (Qiagen) per reaction. Always store polymerase at −20 °C.
5. Vortex and spin down the master mixture. Aliquot it to PCR tubes or strips (11.5 μL of master mixture per tube).
6. Add 2 μL of the internal PCR control to each tube. It is not recommended to store or handle the PCR controls in the same facilities where the PCR master mixes and primers are handled. The PCR control can be added into the mixture in the same facilities with the DNA template.
7. Add 1.5 μL of DNA sample.
8. Place all the tubes/strips to PCR machine and start PCR program: a denaturation step at 95 °C for 15 min, 36 cycles of 10 s at 96 °C, 35 s at 52 °C, 10 s at 72 °C, 5 cycles of 5 s at 96 °C, 30 s at 65 °C, 5 cycles of 5 s at 96 °C and finally 30 s at 68 °C.

Preparing Fungal Hybridization onto Prove-it Sepsis StripArrays

1. Pick up the number of microarrays and seals needed.
2. Take all hybridization reagents and distilled water to room temperature and make sure that they are equilibrated to RT.
3. Switch on the thermal blocks and make sure that they are at right temperatures of +30 and +66 °C. Also check correct agitation speed of 550 rpm.
4. Prepare a fresh hybridization buffer by mixing together the 2× hybridization buffer and Hybridization buffer diluents (1:2).
5. Prepare a fresh conjugate solution by mixing together conjugate stock and conjugate diluent (1:80).
6. Prewashing.
   (a) Add 200 μL of distilled water into microarray wells.
   (b) Incubate at +30 °C for 10 min with 550 rpm agitation.
   (c) After incubation, carefully remove all liquid from microarray wells.
7. Hybridization.
   (a) Add 97 μL of fresh hybridization buffer to the microarray wells.
   (b) Add into the same microarray well 3 μL of fungal PCR product.

(c) Incubate at +66 °C for 20 min with 550 rpm agitation.
(d) After incubation, carefully remove all liquid from microarray wells.

8. Washing.
   (a) Add 200 μL of washing buffer into microarray wells.
   (b) Incubate at +30 °C for 1 min with 550 rpm agitation.
   (c) After incubation, carefully remove all liquid from microarray wells.
9. Conjugation
   (a) Add 100 μL of freshly mixed conjugate solution into microarray wells.
   (b) Incubate at +30 °C for 10 min with 550 rpm agitation.
   (c) After incubation, carefully remove all liquid from microarray wells.
10. Washing
   (a) Add 200 μL of washing buffer into microarray wells.
   (b) Incubate at +30 °C for 5 min with 550 rpm agitation.
   (c) After incubation, carefully remove all liquid from microarray wells.
11. Precipitation staining
   (a) Add 100 μL of substrate into microarray wells.
   (b) Incubate at +25 °C for 10 min. NO agitation for this incubation!
   (c) After Incubation, carefully remove all liquid from microarray wells.
12. Analysis with the Prove-it StripArray reader and Prove-it Advisor software.

DNA Extraction

A prerequisite for a successful DNA-based analysis of fungal specimens is the efficiency of cell wall disruption step and subsequent recovery of fungal DNA without putative PCR inhibitors originated from the specimen. Hence, the most appropriate sample preparation and DNA extraction method for any particular application depends also on the specimen type and quantity used. Khot and Fredericks have reviewed both in-house and commercial DNA extraction methods used with various clinical specimens in PCR-based fungal diagnostics [1]. A variety of in-house methods are available. Also, many manufacturers are providing DNA purification kits that are suitable for preparation of DNA from fungal specimens [34]. However, when adapting a protocol to be used in clinical diagnostics, it is of high importance that the used reagents and materials are free of fungal bioburden. Any risks for false-positive result reporting due to the fungal bioburden should be avoided. Since the contamination of DNA extraction reagents with fungal DNA is common [35], the upstream methods to be used in conjunction with PCR and microarray-based analysis should always be evaluated carefully. When blood culture samples is used as a specimen type, it should also be taken into account that blood culture media contains a common additive polyanetholesulfonate (SPS), which is a potent inhibitor of PCR and resistant to removal by some DNA purification methods [36].

No traces of fungal bioburden have been observed from the current production versions of three commercially available DNA extraction methods. These methods are also efficient regarding the disruption fungal cell wall and the removal of SPS from positive blood culture samples. The protocols for automated solution of NorDiag Arrow (Nordiag, Oslo, Norway) and Nuclisens®easyMAG® (bioMérieux, Marcy l'Etoile, France), and manual solution of MycXtra Fungal DNA Extraction Kit (Myconostica, Manchester, UK) are introduced below.

Extraction of DNA from Blood Culture Using NorDiag Arrow

The NorDiag Arrow pipetting instrument is recommended to be used according to the manufacturer's instructions and recommendations with the Arrow Viral NA kit and Viral NA v.1.0 program (www.nordiag.com). Shortly, NorDiag Arrow is an automated extraction instrument for NAs, using a magnetic bead-based method and running 1–12 samples simultaneously. Arrow provides cost-efficient purchasing and running

costs. The following protocol is to be used in conjunction with Prove-it Sepsis assay:

1. Switch the NorDiag Arrow instrument on and on the select protocol menu, select the protocol dedicated for Arrow VIRAL NA kit to be run.
2. Load the instrument with the required consumables, i.e., pumps and pump-tips.
3. Place the cartridge containing the extraction reagents onto the Arrow rack and place the Arrow rack to the instrument. Note: The foil on the cartridges must be peeled off prior to starting a run.
4. For the DNA eluate, place a clean microcentrifuge tube to the appropriate place in the Arrow rack.
5. Mix 240 μL of blood culture and 10 μL of proteinase K in a microcentrifuge tube.
6. Place the sample solution to the appropriate place in the Arrow rack.
7. From the protocol touch screen, choose the sample input volume of 250 μL.
8. From the protocol touch screen, choose the sample elution of 100 μL.
9. Start the protocol. The run is carried out automatically.
10. Run is finished within ~50 min, after which DNA is ready to be used in PCR applications.

Extraction of DNA from Blood Culture Using NucliSENS easyMAG

NucliSENS® easyMAG® instrument is recommended to be used according to the manufacturer's instructions and recommendations (www.biomerieux.com). NucliSENSeasyMAG is an automated system for total nucleic acid extraction from a variety of sample types and volumes, capable of running 1–24 samples simultaneously. NA extraction method is based on the magnetic silica particles. The target NAs bind to the magnetic silica particles during the incubation of lysed sample. The magnetic device is then introduced to the silica particles, enabling the system to purify the NAs trough several washing steps. After washing, the heating step releases DNA from the silica, after which it is ready to be used in PCR applications. The following protocol is to be used in conjunction with the Prove-it Sepsis assay:

1. Switch the NucliSENS easyMAG instrument on and select the protocol Generic 2.0.1 to be run.
2. Start the off-board lysis extraction protocol Generic 2.0.1 and set the sample material; i.e., blood culture media and the elution volume of 55 μL www.biomerieux.com.
3. Add 100 μL of blood culture to 2 mL of NucliSENS Lysis buffer. Vortex thoroughly. Incubate 10 min at room temperature.
4. Insert aspiration tip sets into the instrument.
5. Load the lysed sample into the 1 well of the 8-well sample vessel.
6. Mix 550 μL of distilled water and 550 μL magnetic silica together.
7. Add 100 μL of silica mixture to the well of the sample vessel containing the lysed sample and mix thoroughly.
8. Insert the vessel into the instrument.
9. Start the run which is carried out automatically. The instrument automatically verifies if there are sufficient amount of the on-board reagents.
10. After the run of 40 min, the extraction protocol is completed and the eluted DNA can be moved from the vessel to a clean laboratory tube for the use in PCR applications.

Extraction of DNA from Clinical Specimen Using MycXtra Fungal DNA Extraction Kit

MycXtra kit is recommended to be used according to the manufacturer's instructions and recommendations (www.myconostica.com). The principle of the kit is to lyse the fungal cells in the sample by combining the use of a detergent and a mechanical force against specialized beads. The cellular components are lysed by a mechanical action on a vortex. From the lysed cells, the released DNA is bound to a silica spin filter. The filter is washed and DNA is harvested in a buffered solution. The protocol is manual.

1. Centrifuge the sample for 20 min at 3,000 × *g*. Decant the supernatant and retain it.
2. Resuspend the pellet in 800 μL of the retained supernatant and transfer it to microcentrifuge tube.
3. Centrifuge for 2 min at 10,000 × *g* and discard the supernatant. Resuspend the pellet in the solution remaining in the tube and transfer the entire amount to a 2-mL Bead Solution tube.
4. Gently invert to mix.
5. Add 60 μL of Solution S1 to the Bead Solution tube and invert several times.
6. Add 200 μL of Solution IRS to the Bead Solution tube.
7. Vortex at maximum speed for 10 min.
8. Centrifuge the Bead Soltuin tube at 10,000 × *g* for 30 s.
9. Transfer 450 μL of supernatant to a clean microcentrifuge tube taking care not to disturb the beads. Discard the Bead Solution tube.
10. Add 250 μL of Solution S2 to the supernatant and vortex for 5 s. Incubate at 4–8 °C for 5 min.
11. Centrifuge tubes for 1 min at 10,000 × *g*.
12. Avoiding the pellet, transfer the entire volume of the supernatant to a clean microcentrifuge tube.
13. Add 1.1 mL of Solution S3 to the supernatant. Mix by inverting.
14. Load approximately 650 μL on to a spin filter and centrifuge at 10,000 × *g* for 30 s. Discard the flow trough. Repeat this step until all supernatant has passed through the spin filter.
15. Add 300 μL of Solution S4 to the spin filter and centrifuge for 30 s at 10,000 × *g*.
16. Discard the flow through.
17. Centrifuge again for 1 min to remove the last traces of S4, which will inhibit the PCR reaction.
18. Carefully place spin filter in a new microcentrifuge tube and add 40 μL of Solution S5 to the center of the white spin filter membrane. Leave at room temperature for 2 min.
19. Centrifuge for 30 s at 10,000 × *g*.
20. Discard the spin filter.
21. DNA in the tube is now ready for use in a PCR application.

Notes

1. Specimens should always be considered as potentially infectious.
2. Store and extract DNA from a specimen separately from the reagents and the pre-PCR area.
3. The procedure should be performed in physically separated areas (pre-PCR area and post-PCR area) to avoid contamination with microbial organisms or nucleic acids or any other extraneous material or agents; e.g., amplicons from previous PCR runs. In the pre-PCR area, the preparation of the PCR mixture should be conducted in an area separate from where the addition of the DNA sample takes place.
4. Each pre/post-PCR area should have its own dedicated working materials assigned; e.g., pipettes, spin microfuge, and disposable gloves. Any material in the post-PCR area must never come into contact with that of the pre-PCR area.
5. Always follow the unidirectional workflow from the pre-PCR area to the post-PCR area. Never reverse the direction.
6. Always wear suitable protective clothing and gloves during the procedure.
7. Follow the recommendation of the manufacturer of thermal cycler regarding to the quality of PCR plastic ware.
8. Avoid scratching the microarrays in the bottom.
9. Avoid bubble formation on the microarray surface. Reverse pipetting is recommended to avoid bubble formation.
10. Keep the microarray bottom clean at all times to avoid any interference when detecting the assay result.
11. Be careful not to let the microarray wells dry for longer than necessary between the hybridization steps.
12. TMB-based substrate must be protected from light.
13. Proceed to the PCR step immediately after the DNA extraction step. Also, proceed to the microarray step after the PCR step. Storing the DNA extract or PCR product may affect the assay result.

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