TESTING FUNGAL DNA ON LIGATION DETECTION REACTION MICROARRAY

Leverage from the EU 2007–2013

European Union
European Regional Development Fund

LAHTI UNIVERSITY OF APPLIED SCIENCES
Faculty of Technology
Degree Program in Environmental Technology
Environmental biotechnology
Bachelor's thesis
Spring 2009
Ossi Tonteri

Lahden ammattikorkeakoulu
Tekniikan laitos

TONTERI, OSSI: Testing fungal DNA on ligation detection reaction microarray

Ympäristöbiotekniikan opinnäytetyö, 33 sivua, 8 liitesivua

Kevät 2009

TIIVISTELMÄ

Tämän opinnäytteen tavoitteena oli testata kuinka hyvin LDR- mikrosiru (Ligation Detection Reaction) kykenee havaitsemaan sienilajien puhdasviljelmästä eristettyä DNA:ta. Opinnäytteessä käytettyjä koettimia oli testattu aikaisemmin vain polyakryyliamidigeelielektroforeesilla (PAGE), mutta tässä työssä näitä koettimia testattiin ensimmäistä kertaa mikrosirulla. Opinnäyte oli osana YMLI A30175 -projektia, joka on rahoitettu EU:n aluekehitysrahastosta.

Opinnäyte koostui kolmesta vaiheesta. Ensimmäisessä vaiheessa LDR sirujen testauksessa käytettävät DNA-templaatit, jotka koostuivat sienten ribosomaalisen DNA:n välialueista (ITS-alue), monistettiin polymeraasiketjureaktion (PCR) avulla. PCR-tuotteiden laatu varmistettiin elektroforeesigeelillä. Toinen vaihe koostui ligaatiosta ja hybridisaatiosta. Ligaatiossa PCR-tuotteet ja alukeparit liitettiin yhteen ligaatioreaktion avulla. Hybridisaatiossa ligaatio-tuotteet hybridisoitiin mikrosirun pinnalla olevien alukkeiden kanssa ja syntyneet fluoresenssitasot mitattiin skannerilla. Kolmannessa vaiheessa tuloksia analysoitiin käyttämällä Bioconductor- ohjelmaa.

Opinnäytteessä testatuista 24 alukeparista 7 antoi selkeästi positiivisen tuloksen. Kaksi alukepareista voitiin luokitella toimivan heikosti, koska vain toinen alukkeiden templaateista antoi heikosti tai vahvasti positiivisen tuloksen. Viisitoista aluketta antoivat negatiivisen tuloksen. Näistä neljä oli antanut aikaisemmin negatiivisen tai epäselvän tuloksen PAGE-geelillä tai niitä ei ollut aikaisemmin testattu. Lisäksi yhden alukkeen sekvenssissä huomattiin virheitä. Käytettyjen näytteiden konsentraatio oli alhainen ja saattoi osaltaan vaikuttaa tuloksiin.

Tulokset osoittivat, että vaikka osa käytetyistä koettimista ei toiminut, osa kuitenkin antoi positiivisen tuloksen sirulla. Huonosti toimineet alukkeet tarvitsivat enemmän kokeita, jotta saataisiin varmuus niiden toiminnasta.

Avainsanat: DNA-mikrosirut, ligaatiotunnistusreaktio (LDR), sisäilman homeet, *Aspergillus, Penicillium*

Lahti University of Applied Sciences

Faculty of Technology

TONTERI, OSSI: Testing fungal DNA on ligation detection reaction microarray

Bachelor's Thesis in Environmental Biotechnology, 33 pages, 8 appendixes

Spring 2009

ABSTRACT

The aim of this thesis was to test how LDR (Ligation Detection Reaction) microarrays could detect fungal DNA isolated from pure cultures. The DNA probes for fungal detection had been tested before by polyacryl amide gel electrophoresis (PAGE), but in this study they were tested on the microarray for the first time. This project was part of the project YMLI A30175 funded by EU Regional Fund and aiming for example to further develop DNA microarrays as a product and service for environmental analysis.

The practical part of the thesis project consisted of three steps. First, the templates were prepared for LDR testing by polymerase chain reaction (PCR) amplification of the fungal nuclear ribosomal internal transcribed spacers (ITSs) region and to ensure by agarose gel electrophoresis that the PCR has been successful. In the second step, the probes (altogether 24 common and discriminating probes) were ligated together with the PCR product and then hybridized to LDR array. The fluorescent levels of the hybridized microarray spots were read with a scanner. In the third step the data was analyzed using Bioconductor, which is open source software.

Seven of the tested probes worked well on microarray. Two of the probes were classified as faintly working on microarray, because only one of their two samples gave faint or positive signal. From the 15 probes that gave negative result, 4 probes had already shown unclear results on PAGE gel or had not been tested before. One of negative probes was found to have errors in their sequence. Also, the quality of the used samples might have influenced the results.

The results of this thesis show that some of the probes did not work properly on microarray, but however some of the probes gave positive result. To confirm that the probes are not working, more testing would be needed.

Keywords: DNA microarrays, Ligation Detection Reaction, indoor fungi, *Aspergillus, Penicillium*

CONTENTS

Acknowledgment

I will thank for staff in the Institute of Biotechnology and Almalab for all the help during the laboratory work. Dr Helena Rintala and MSc Päivi Kärkkäinen I will thank for providing the samples and probes used in this work. Special thanks to Dr Jenni Hultman for patience, help and advice given during this work. Thanks to Dr Silja Kostia for giving this work and advice during the work.

1 INTRODUCTION

Microarrays are diagnostical technology developed quite recently and mostly used in biomedical and molecular biology analysis, for example in genotyping, transcript profiling and in studying gene-expression (Hoheisel 2006). Microarrays allow simultaneous detection of multiple samples in a single experiment. Generally, a DNA microarray consists of microscopic spots attached on a solid support (usually glass slide). These spots (also referred to as DNA-probes) are designed to recognise and react with specific fluorescently labelled DNA sequences during the hybridisation process. The amount of binding between the probes and the target is quantified by scanning the emitted fluorescent. Because microarray experiments produce a huge amount of data from the spots, resulting data is analysed using statistical tools (Hoheisel 2006, Coppe´e 2008).

Different types of microarrays have already been used in monitoring biological processes and diagnosing various environmental samples, and the number of different applications is increasing. For example, microarrays have been used to diagnose the norovirus from drinking water (Brinkman et al. 2008), cyanobacteria from lake water (Rudi et al. 2000), landfill methanogens (Gebert et al. 2008), waste water pathogens (Lee et al. 2007), a uranium bioremediation site (He et al. 2007) and microbes from oil reservoir formation waters (Bonch-Osmolovskaya et al. 2003). The possibility to detect thousands of genes and species at one assay makes it an interesting choice for diagnosing environmental samples.

Ligation detection reaction (LDR) was combined with microarray technology to increase detection specificity and sensitivity (Gerry et al. 1999; Busti et al 2002). LDR technique microarrays have been used in detecting and characterising microbes for example from cyanobacterial blooms (Rantala et al 2008), and microbial community in the composting process (Hultman et al. 2008). A sensitivity of 0,04 % of the total sample DNA has been reported in addition to species level differentiation (Hultman et al. 2008).

The moisture damage in buildings is a relatively common problem causing severe health problems and economic loss. The fungal species are found from buildings with high moisture, for example allergenic molds from *Aspergillus* and *Penicillium* genera that grow indoors. The present analysis methods for fungal species are slow and require expertise, i.e. the identification of cultured species. The DNA microarrays could offer a fast analysis method for screening harmful species in a moisture damaged buildings. Diagnosing samples with microarrays could be done much faster and more efficient than conventional methods, like culturing. (Jaakkola et al. 2002, Nevalainen 2007, Rintala 2005)

The aim of this study is to test how well LDR microarrays can detect fungal DNA isolated from pure cultures. The biological knowledge, i.e. the information of DNA probes is developed by Dr Helena Rintala's research group in the National Institute for Health and Welfare, except the probes for *Penicillium* genus, which are developed by Jarmo Ritari (Institute of Biotechnology, University of Helsinki). Most of the DNA probes for fungal detection used in this thesis were tested earlier on PAGE gel, but in this study they were tested on the microarray platform for the first time. The technology platform is developed by Dr Petri Auvinen's research group in the Institute of Biotechnology in Viikki. This co-operation is part of the project YMLI (A30175) funded by EU regional fund. One aim of the project is to further develop microarray technology to be a commercially available analysis method in environmental analytics.

2 LDR MICROARRAYS

Ligation detection reaction (LDR) was first used in detecting single base mutations in genetical diseases in the late 80's. LDR technique was combined with microarray technology in 2002 (Busti et al 2002). LDR microarray experiment can be divided to ligation and hybridization steps. In the ligation step two probes are ligated adjacent to each other on the target DNA (FIGURE 1). The discrimating probe (D-probe) has a fluorescently labelled stain on its 5'-end. The other probe, called the common probe (C-probe), has a special short sequence called complementary Zip-code attached to its 5'-end. These two probes will anneal adjacently with the target DNA only if their base-pairs have a perfect complementary with the target DNA. Even a mismatch of a single base pair will lead to unsuccessful ligation of the probe pair. This makes the LDR method highly sensitive and makes it possible to be used in detecting single nucleotide polymorphism. A successful ligation reaction produces ligation product, which has a fluorescent label on its other end and a cZip-code on its other end. The ligation reaction is thermally cycled in a similar way as in a PCR (Barany 1991). This makes it possible to produce more ligation products to detect on microarray. The probes are designed so that their junction point distinguishes the target DNA from other species' DNA. The target DNA is PCR amplificated DNA from the gene of interest. (Busti et al 2002, Hultman 2009)

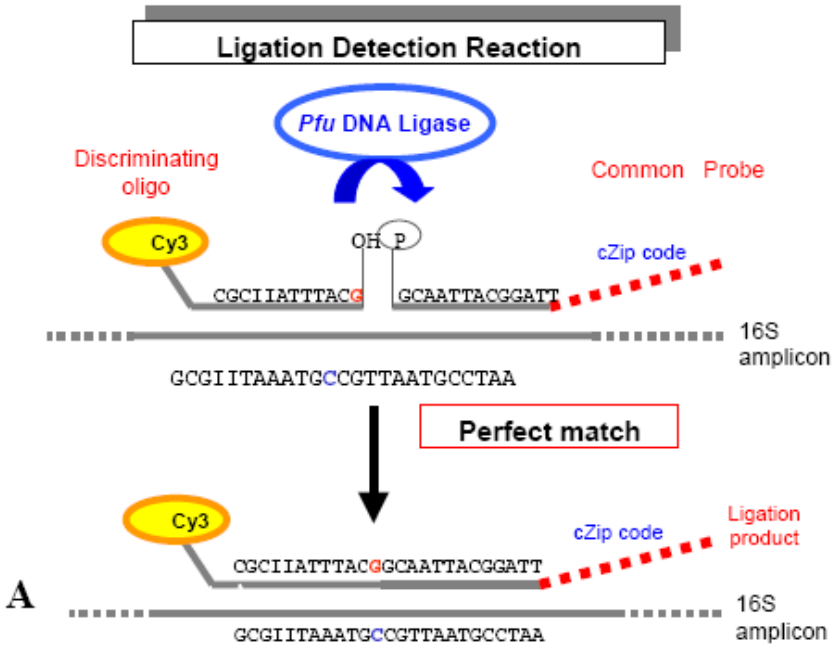


FIGURE 1. Schematic representation of ligation steps of LDR. (Busti et al 2002).

In hybridization, the ligation product is applied to the surface of the microarray's printed glass. On the surface of the printed glass the Zip-codes, which are complementary to cZip-codes are attached. The Zip-codes hybridize with the cZip-codes in certain thermic conditions, but only if they have an exact match. This means, that only the positive hybridization spots will have cZip codes attached on them. Because successfully ligated cZip codes are carrying fluorescent labels, positive spots can be detected because they produce fluorescent emissions, which can be detected with a scanner. In the LDR microarray application developed by Hultman et al 2008, negative and positive hybridization spots are discriminated using control probe named B3, which binds to all spots, even the ones where the cZip code was unable bind. Because the B3-probes emit a different wavelength compared to the cZip codes, hybridization can be normalized through comparison of the ligation probe and control probe intensities. (Hultman 2009).

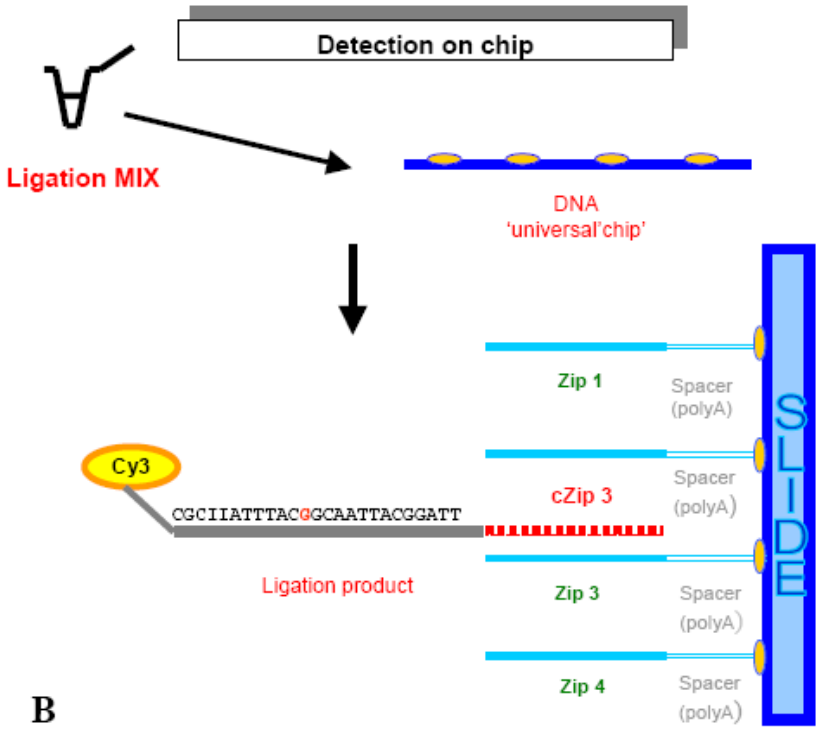


FIGURE 2. Schematic representation of hybridization steps of LDR. (Busti et al. 2002)

3 FUNGI AND MOISTURE DAMAGED BUILDINGS

Fungi are multicellular, eukaryotic organisms that are heterotrophic, meaning that they need external organic substances to live. Therefore, fungi usually live as saprophytes, parasites or symbionts of plants or animals. Fungi forms branched filaments called hyphae or mycelium. Most fungi that grow indoors form mycelium and are generally called molds. (Samson 1994, Simon-Nobbe et al. 2008)

Many fungal species produce mycotoxins, which are non-volatile secondary metabolites which have toxic response on humans. The severity of the toxic effects depends on the duration of exposure, the type of mycotoxin and the health of the invidual. Humans are most often exposed to mycotoxins by inhaling the spores from air or being exposed to mold contaminated material. Examples of mycotoxins are aflatoxin (*Aspergillus flavus and A. parasiticus*), ergot alkaloids (*Claviceps spp., A. fumigatus and Penillicum chermerium*), ochratoxins (*A. ochraceus, A.*

alliaceus, A. terreus, P. niger and P. viridicatum) and tricothenes (*Fusarium sporotrichioides, Microdochium nivale and Stachybotrys atra*) (Bhatnagar 2002, Samson 1992). Fungi also produce volatice organic compounds, such as alcohols and ketones. These often cause the musty odour associated with mold. Other compounds that cause irritation include glucans, which are components of the fungal cell wall. (King & Auger 2002, Simon-Nobbe et al. 2008)

Moisture damage in buildings has proven to be a quite common problem. In Finland, 50 percent of the houses are considered to need some kind of remedy and at least 40 percent of the school buildings have damage (Nevalainen 2007). The reasons for frequent moisture problems in cold climates are the construction of tight buildings with lack of ventilation for better energy efficiency, and insufficient maintenance. Errors made in designing and during the construction process can also be a reason for water damage. Other reasons are technical aging of the building material and the structure. Water and heat damages are also a common reason for moisture problems. A building's source of moisture is often from the rain and melting water, the moisture from the soil, the moisture from indoor and outdoor air or household water, and the moisture in the building material. Moisture gets to building material from leaks, condensation and capillation. (National Public Health Institution 2009, Jaakkola et al. 2002).

The most important requirements for molds to grow indoors are moisture, nutrients and temperature. Nutrients (carbon, natrium, potassium, phosphorus) are usually available in building material (Dix and Webster 1994). Usual temperature in buildings is 20-25 º C, which is optimal for mesofilic bacteria (20-<45) (Ingold and Hudson 1993, Atlas and Bartha 1993). The pH of building material is usually in the range of 5-6.5, which is also the optimal pH for fungi (Ingold and Hudson 1993). As indoor environments usually have enough oxygen and light, moisture becomes the most critical condition for fungal grow in indoor environments. (Hyvärinen 2002).

Sources of molds found in indoor environments are outdoor air, plants, handling of root vegetables, firewood or biological waste, and other activities of occupants,

but also moisture damage of the building. To survive longer times and grow indoors, molds require a surface, moisture and external nutrients. Suitable substrates usually found indoors include cellulose rich material, such as s wood, paper, and gypsum board. However, any material can support mold growth, if it is moist long enough. Nutrients are also found even in water and house dust (Hyvärinen 2002). Buildings with frequent water damage and high moisture have a higher risk for fungal contamination. Molds that most commonly are found indoors include species from the genera *Aspergillus* and *Penicillium*. Presence of certain fungi species has been linked with possible moisture and mold damage of a building (TABLE 1). (Samson 1994, King & Auger 2002)

TABLE 1. Fungi indicating mold problems based on air and surface samples (Samson 1994).

Water activity	Fungal taxon
High water activity (a_w>0,90-0,95)	*Aspergillus fumigatus* *Trichoderma spp.* *Exohiala spp.* *Stachybotrys chartarum*[a] *Phialophora spp.* *Fusarium spp.* *Ulocladium spp.* *Yeasts, eg. Rhodotorula*
Moderate wateractivity (0,85< a_w<0,90)	*Aspergillus versicolor*[a]
Low water activity (a_w<0,85)	*Aspergillus versicolor*[a] *Eurotium spp.* *Wallemia sebi* *Penicillium chrysogenum* *Penicillium aurantiogriseum*

[a]Important toxine producing species

Health effects associated with mold or water damaged buildings are well known, but the causative agents and mechanism causing the symptoms are still poorly known (Bornehag et al 2001). However, molds have been shown to affect human health by numerous ways. Respiratory symptoms and illnesses, irritation of skin, eyes and mucosal membranes as well as general symptoms, such as fatigue and headache are the most commonly reported (Husman 1996, Peat 1998).

4 MATERIALS AND METHODS

4.1 Samples, probes and methods

4.1.1 DNA samples and probes

All together 46 different fungal DNA samples were included into this thesis. The samples were obtained from Dr Helena Rintala, National Institute for Health and Wellfare (Kuopio). Most of the probes had been tested before with the same samples on polyacryl amide (PAGE) with the C-and D-probes with varying results (APPENDIX 1).

Thirty two (32) of the samples represented different *Aspergillus* and *Penicillium* species and the rest of the samples (14) were duplicates from a different strain. All together 23 different common (C)- and 24 discriminate (D)-probes were tested with their correspondent samples, meaning that when duplicate samples were included, all together 37 DNA samples were expected to have their correspondent probes. D-probes for *Eurotium amstelodami/chevalieri* and *Eurotium herbariorum* (eur_ams_d100 and eur_her_d100) had the same corresponding C-probe (eur_ams_c100_A80), therefore the number of D and C probes were different (24 and 23). For testing false positive on microarray, 9 samples were expected not to have a corresponding probe and thus to not produce a positive reaction on the mi-

croarrays. The probeset also included a panfungal probe that was included in all reactions as a positive control, and it shoud give a positive result, if any fungal DNA from orders *Peziza myceta* was present in the reaction. A complete list of the samples and the probes is shown in APPENDIX 2.

Total of 22 samples had not been earlier sequenced to confirm that their DNA did match with the probes. These samples were sequenced in DNA sequencing and Genomics laboratory at Viikki, Institute of Biotechnology.

4.1.2 Polymerase chain reaction (PCR) amplification

The fungal nuclear ribosomal internal transcribed spacers ITS region was PCR amplified using primers ITS4 (5´TCCTCCGCTTATTGATATGC-3) (White et al. 1990) and Fun18F (5'-TTG CTC TTC AAC GAG GAA T-3) (Hultman et al. in press). The amplified fragment size was ca. 700-800 bp. The used primers were ordered from Oligomer. PCR was carried out in a 50 µl volume using 0,5 µl (1 U) of Dynazyme II-enzyme (Finnzymes, Helsinki, Espoo), 5 µl of Dynazyme Buffer F-516S (Finnzymes, Helsinki, Espoo), 0,4 µl of 25 mM dNTP (Finnzymes), 0,5 µl (0,75 U) Pfu-polymerase, 25 pmol of both primers and 20 - 34 ng of template DNA (APPENDIX 3) and various amounts of distilled water. PCR termocycling was carried out in MJ Research thermal cycler (GMI, Minnesota, USA) under following conditions: denaturation of 5 min at 94°C, followed by 25 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 45 s, with a final extension of 10 min at 72°C (Hultman 2008).

PCR products were run in agarose gel (1 %) to check the success of PCR reaction and the quality of PCR products. For each run a 5 µ PCR product was used, 5 µ water and 2 µl of 6x Loading Dye. Eleven µl of this mix was pipetted in one cave. In each gel run, one sample was run without template as a negative control. No positive control was used in the gel runs. Each sample was run for 2 hours using 80 V and 400 mA.

PCR products were purified using QIAquick® PCR Purification Kit (Qiagen, Venlo, Netherlands). The purification was done following the instructions found in a handbook and using the reagents included in the purification kit.

4.1.3 Ligation and hybridization

The ligation mix (20 µl) for each reaction included 2 µl of 1xTaq ligase buffer (New England Biolabs, Massachusetts, USA), 1 µl of 600 mM tetramethylammonium chloride (TMAC), 1 µl of 250 fmol C- and D-probes (Oligomer, Helsinki, Finland), 5 units of 1xTaq ligase (New England Biolabs), water and purified PCR product of varying amounts. The reaction was first heated in 94 ºC for 2 minutes before adding the 1xTaq ligase. The ligation program was run cycled in the following conditions: initially 2 minutes in 94 ºC, followed with 30 seconds in 67 ºC (step 1), 4 minutes in 94 ºC (step 2), steps 1 and 2 were then cycled for 40 rounds and finally 30 min in 4 ºC. The concentrations of PCR products were measured using Nanodrop (Thermo Fisher Scientific, Waltman, USA) The molarity of PCR products were calculated using Oligocalc (Northwestern University Medical School) using the measured concentrations of PCR products.

The slides used in this thesis were manufactured mainly by SCHOTT's Nexterion Slide A MPX (Mainz, Germany) and ArrayIT (Sunnyvale, USA). The SCHOTT Nexterion glass slides were used in testing the single templates. SCHOTT glass slides are made from low auto-fluorescent borosilicate glass. SCHOTT microarrays were printed in Biomedicum (Helsinki, Finland). A single ArrayIT Flex chip was used in testing the different template mixes. These arrays were printed by ArrayIT.

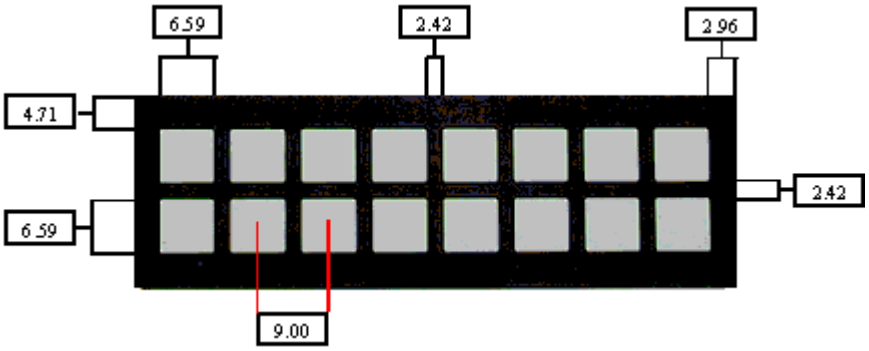


FIGURE 3. SCHOTT's Nexterion Slide A MPX and its dimensions (SCHOTT 2009).

Before hybridization, the used LDR arrays were crosslinked with 1000 mJ using the UV Stratalinker 2400 (Stratagene, CA, USA). In crosslinking, the array is exposed to ultraviolet irradiation. Crosslinking binds the oligos better to the surface of the glass.

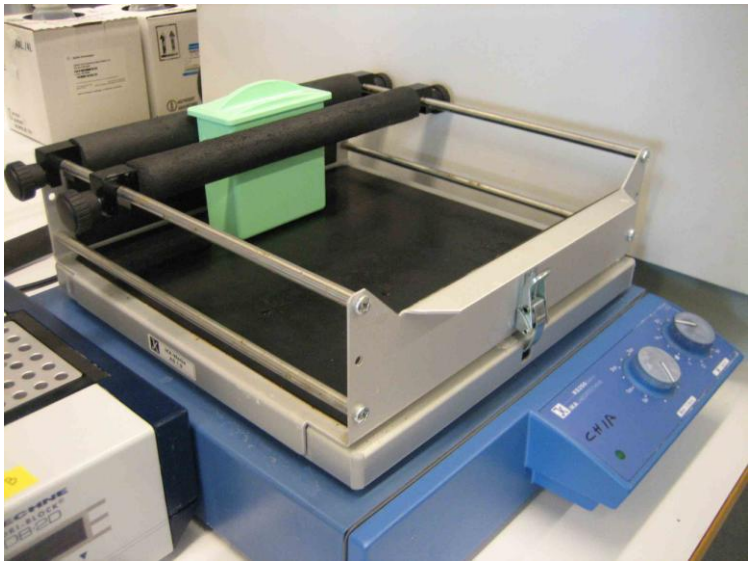


FIGURE 4. The washes in the hybridization were done in a shaker with a beaker full of hybridization buffer or distilled water.

First, the prehybridization washing mixture consisting of 20 X SSC and 20% SDS was preheated for 5 minutes in a 42º C temperature water bath. After preheating

the LDR arrays, the slides were placed in 50 ml Falcon tubes filled with thr prehybridization washing mix and heated in a water bath at 42º C. After 42 minutes of prehybridization, the slides were washed for three times for 5 minutes with room temperature MQ water in shaker. After the washes, the slides were dried in a table centrifuge. The slides were then placed on the hybridization chambers. Small drops of water were applied to the hybridization chambers to prevent the slides from drying. The superstructures were applied to the slides.

FIGURE 5. Picture of SCHOTT 16 MPX Slide placed on hybridization chamber with superstructure attached.

The hybridization mix was done mixing 10 µl of 20X SSC, 1 µl of 10 mg/ml herring sperm DNA, 9 µl of MQ-water and 19 µl of ligation product. This mixture was divided into PCR strips, and denaturated using a PCR machine in 94 ºC for 2 minutes. After the denaturiaton, the PCR strips were chilled on ice, and the control probe (B3) was added to reaction. The hybridization mix was then applied to the subarrays. The subarrays were sealed with a Biotool tape. The slides were placed in a dark oven and incubated in 50 º Cfor 2 hours.

After the incubation, the slides were first rinsed with a washing buffer (0.1X SSC, 0.1% SDS) and then washed three times in the same buffer for 15 minutes. The slides were then washed three times in MQ water for 5 minutes. Finally, the slides were dried in table centrifuge.

The ligation step was done using the facilities of Almalab (the Department of Ecological and Environmental Sciences, University of Helsinki, Lahti) but the hybridization step was done in the Institute of Biotechnology, University of Helsinki.

4.1.4 Scanning and data-analysis

Scanning was done using GenePix Autoloader 4200A and GenePix program version 6.1. The wavelengths for ligation products and B3 were 532 and 488.

FIGURE 6. GenePix Autoloader 4200A used in the scanning the microarray chips. The rack where the microarray is inserted is seen halfway out.

For the testing of invidual probes, the results were collected by visually observing the positive spots on the arrays. In the test with different mixes of templates, the result data was analysed using the R statistical environment. Script used to produce the boxplots was obtained from MSc Jarmo Ritari. (R development core team 2007 and Gentleman et al. 2004).

4.2 Testing scheme

4.2.1 Testing probes only with their invidual target DNA

The aim of the first tests was to determine, if the probes could detect their target DNA, and if there were false positive spots. For these tests, PCR amplified template DNA only from the target species was included in the ligation reaction with all of the 24 probepairs. The results were examined by visually confirming the positive spots from the scanned image. The amount of each 46 templates used in the invidual tests was 100 fmol.

The first 5 of the 46 samples were tested on SCHOTT glass slides that had badly printed oligonucleotides, and only few of the subarrays per glass were good enough to be used. The quality of the printed oligonucleotides was visually examined with a scanner before the hybridization. Forty one (41) of the samples were tested on better SCHOTT glass slides with quite good oligonucleotide prints. Still, only 14 of the expected 16 subarrays had oligonucleotids printed on them, and two of the subarrays had no printings. All the used glasses were printed in Biomedicum, University of Helsinki.

4.2.2 Testing specificity and multiple samples in one subarray

In this experiment, a varying amount of DNA from different templates was applied to the same ligation reaction. The aim of these tests was to determine how small amounts of DNA could be detected on the microarray, and also to find out if

"competing" DNA would affect the detection. The templates for this step were chosen on the basis of the first step tests, so that they all had already given positive results. These tests were done on ArrayIT Flex-chip having 16 subarrays with good oligonucleotide prints.

In the subarrays from 1 to 5 in glass slide (TABLE 2), a mixture of five different DNA templates was tested. In the ligation reaction, one of the templates was used a in a consentration of 10 fmol and the rest of the templates in a consentration of 50 fmol. On subarray 14, a similar test was done using different samples. On subarrays 6-9, one template was tested with a concentration of only 1 fmol, while the other 4 templates had a concentration of 50 fmol.

On subarrays 10-13, four templates were tested with large amounts of competing DNA from samples EPA 364 (*P. variabile*, 300 fmol) and RE 060 (*P. viridicatum*, 200 fmol) that did not have probes and thus should not be detected in microarray. On subarray 16, duplicate samples (EPA 427 and HT 561) of *A. ustus* were tested with a large amount of template (550 fmol). This test was to find out if too much of DNA would affect the detection.

On subarray 15, a single template was tested, because on the previous test this sample gave a strong false positive result.

TABLE 2. Different mixes of templates used in the last slide, subarrays 1-9 and 14. The templates with the smallest amounts in the tests are highlighted with yellow.

TEMPLATES						
Test	Subarray	RE 004 (*A. fumigatus*)	RE 009 (*A.versicolor*)	HT 445 (*A. flavus*)	EPA 427 (*A. ustus*)	EPA 231 (*A. terreus)*
10 fmol	14	50 fmol	50 fmol	10 fmol	50 fmol	50 fmol
		EPA 231 (*A. terreus*)	EPA 427 (*A. ustus*)	EPA 526 (*A. fumigatus*)	RE 015 (*P. variotii)*	RE 059 *(P. corylophilum)*
	1	50 fmol	50 fmol	50 fmol	10 fmol	50 fmol
	2	50 fmol	50 fmol	50 fmol	50 fmol	10 fmol
	3	10 fmol	50 fmol	50 fmol	50 fmol	50 fmol
	4	50 fmol	10 fmol	50 fmol	50 fmol	50 fmol
	5	50 fmol	50 fmol	10 fmol	50 fmol	50 fmol
1 fmol	6	50 fmol	1 fmol	50 fmol	50 fmol	50 fmol
	7	50 fmol	50 fmol	50 fmol	1 fmol	50 fmol
	8	50 fmol	F50 fmol	50 fmol	50 fmol	1 fmol
	9	1 fmol	50 fmol	50 fmol	50 fmol	50 fmol

TABLE 3. Different mixes of templates used in the last slide, subarrays 10-13 and 15-16. The templates with the smallest amounts in tests are highlighted with yellow.

TEMPLATES							
Test	Subarray	EPA 231 *(A. terreus)*	EPA 526 *(A. fumigatus)*	RE 015 *(P. variotii)*	RE 059 *(P. corylophilum)*	EPA 364 (no probe) *(P. variabile)*	RE 060 (no probe) *(P. viridicatum)*
”100 fmol + DNA with no probe”	10	-	-	100	-	300 fmol	200 fmol
	11	100	-	-	-	300 fmol	200 fmol
	12	-	100	-	-	300 fmol	200 fmol
	13	100	-	-	-	300 fmol	200 fmol
”Single template”	Subarray	HT274 *(P.citreonigrum)*					
	15	100 fmol					
”Too much DNA”	Subarray	EPA 427 *(A. ustus)*	HT 561 *(A. ustus)*				
	16	50 fmol	550 fmol				

5 RESULTS AND DISCUSSION

5.1 Probes tested only with their invidual target DNA

All the invidual templates were tested with 100 fmol of the PCR product used in the ligation. Seven of the 24 (including a panfungal probe) different probes gave either a strong or a moderate signal in the chip, which was in accordance with the results from the PAGE gel tests. One exeption was *A. flavus* (EPA 532), which gave only a faintly positive spot on chip, although it had given a moderate spot on the PAGE gel. The other strain for *A. flavus* (HT 445) gave clearly positive results, so the probe can be regarded as positive.

Two of the tested probes *(A. restrictus* and *E. nidulans)* were classified as working faintly, with only one of the two DNA samples giving a positive or a faintly posi-

tive signal, and the other sample giving a negative signal. Probes for *E. nidu*lans strains (EPA 527 and RE 062) had given unclear results in the PAGE gel tests and although sample EPA 527 produced a strong positive signal on microarray, it had a mismatch when aligned with a BLAST. From the *A. Restrictus* strains, one had no positive signal from the PAGE gel, but showed a faint signal on the microarray.

Fifteen of the tested probes were not working as expected. However, four of the DNA samples that gave a negative signal, *P. glaubrum* (RE 083), *P.citrinum* (EPA 458), *P. fellutanum* (HT 193) and *P. italicum* (EPA 059), had not been tested on the PAGE gel before. In addition, three of the negative samples, *A. sydowii* (EPA 421), *A. versicolor* (RE 009) and *A. restrictus* (EPA 458), had also given negative results earlier on the PAGE gel, so their negative result in test was consistent with the previous test. Two of these (HT 651 and EPA 421) also had mismatches in BLAST aligning, which is most probably the reason for the negative result.

The panfungal probe (Zip-code A96) that was included in every reaction, did not give a positive result on any of the tested subarrays.

On the SCHOTT array, the slides used in the invidual tests, signal A88 was positive on almost all of the subarrays with all of the tested samples. Due to this *P.citrinum's* (EPA 448) positive result is uncertain, because it also had the same zip-code A88. Also the spot for A93, originally allocated to *Penicillium expansum*, seemed to be always positive regardless of the sample. This happened both on the SCHOTT as well on the ArrayIT slides. It was later discovered, that the probe with zip-code the A93 (*A. italicum*) had errors in sequence and therefore did not work properly.

Sample HT 274 (*P. citreonigrum*) gave a strong positive signal on spot A87, although its zip-code is A89. This sample was tested again invidually in the last experiment on an ArrayIT array, but in this test it did not give any positive spots.

From the 9 samples that did not have their corresponding probes, and therefore should not hybridize and give positive results, 4 positive spots were detected.

Sample EPA 611 (*A. candidus*) gave a positive signal on spot A76 (*A. sydowii*). This was strange, because the actual sample of *A.sydowii* (EPA 421) did not give positive results. Sample HT 620 *(A. wentii)* gave a positive signal in spot A78 *(A. ustus)*. One possibility for these false positive results could be that probes were unspecific to their target species and hybridized also when DNA was from close relative to their target species.

With some of the subarrays, uxepected positive spots were detected. These falsely positive spots were found with EPA 434 (faint signal on spot A78), RE 062 (A92 faint), EPA 088 (A72), RE 025 (A78 faint), HT 274 (A89 strong), HT 193 (A69 strong). The reason for these faintly positive results could be contamination during pipetting or handling the samples. The positive signal in spot A72 could be misinterpretation of the scanner image, because none of the probes had the zip-codes allocated to spot A72.

Other possibility for false positive spots could also be misinterpretation with the scanner images, because when the spots were detected visually, usually only the image of ligation probe signals was viewed, unless spots were really bad. Normally, control signal (B3) is used to compare the signal intensities with the ligation probe signal (Cy3) to recognize badly working spots and false positives. Because of this, the use of only the ligation probe signal image might have caused some of the false positive results.

From the sequenced samples, some were short or had bad quality. The sequences were aligned with BLAST by Dr. Jenni Hultman.

TABLE 4. The results from testing single templates with probes.

QUALITY	PROBE/ZIP CODE	SPECIES (ID)	CHIP	PAGE	CONTRADICTION	BLAST RESULT	REMARKS	RATING
GOOD	asp_fum_c100_A69	Aspergillus fumigatus (EPA 526)	SS	SS	Nc			+++
		Aspergillus fumigatus (RE 004)	SS	MS	Nc			++
	asp_och_c100_A74	Aspergillus ochraceus (EPA 426)	MS	SS	Nc	Ok		++
	asp_ter_C100_A77	Aspergillus terreus (EPA 231)	SS	SS	Nc			+++
	asp_ust_c100_A78	Aspergillus ustus (HT 561)	SS	MS	Nc			++
		Aspergillus ustus (EPA 427)	SS	SS	Nc			+++
	pae_var_C100_A95	Paecilomyces variotii (RE 015)	SS	SS	Nc			+++
		Paecilomyces variotii (EPA 075)	MS	MS	Nc	Ok		++
	pen_cor_c100_A89	Penicillium corylophilum (RE 059)	SS	SS	Nc	Ok		+++
		Penicillium corylophilum (EPA 434)	SS	SS	Nc		A78 FS	+++
	asp_fla_c100_A68	Aspergillus flavus (HT 445)	SS	MS	Nc	Ok		++
		Aspergillus flavus (EPA 532)	FS	SS	Nc	Ok		+
FAINT	asp_nid_c100_A70	Emericella nidulans (=A.nidulans) (EPA 527)	SS	U	?	Mismatch		-
		Emericella nidulans (=A.nidulans) (RE 062)	NS	U	?	Ok	A92 FS	-
	asp_res_C100_A75	Aspergillus restrictus (EPA 458)	FS	NS	?	Ok		-
		Aspergillus restrictus (RE 057)	NS	SS	C	Ok		:::
NO SIGNAL/ CONTRADICTORY	asp_nig_c100_A71	Aspergillus niger (EPA 088)	NS	SS	C		A72 SS	:::
		Aspergillus niger (RE 056)	NS	MS	C	No match		:::
	asp_syd_C100_A76	Aspergillus sydowii (EPA 421)	NS	NS	Nc	Mismatch		-
	asp_ver_c100_A79	Aspergillus versicolor (EPA 524)	NS	SS	C			:::
		Aspergillus versicolor (RE 009)	*NS*	*NS*	*Nc*			-
	eur_arms_c11_A80	Eurotium chevalieri (EPA 530)	NS	SS	C			:::
		Eurotium herbariorum (HT 538)	NS	SS	C			:::
		Eurotium sp. (HT 651)	*NS*	*NS*	*NC*	2 mismatches		-
	pen_bio_C100_A81	Penicillium brevicompactum (EPA 435)	NS	U	?			-
		Penicillium brevicompactum group (RE 017)	NS	U	?			-
	pen_chr_C100_A82	Penicillium chrysogenum (EPA 467)	NS	SS	C			:::
		Penicillium chrysogenum complex (RE 025)	NS	MS	C		A78 FS	:::
	pen_cit_c102_A87	Penicillium citreonigrum (HT 274)	NS	MS	C		A89 SS	:::
	pen_ctr_c101_A88	Penicillium citrinum (EPA 448)	MS	NT	?		A88 *	-
	pen_ita_c100_A93	Penicillium italicum (EPA 059)	FS	NT	?		A93 *	
		Penicillium |talicum (HT 018)	FS	SS	Nc		A93 *	
	pen_fel_c100_A91	Penicillium fellutanum (HT 193)	NS	NT	?		A69 SS	-
	pen_gal_c100_A92	Penicillium glabrum (HT 084)	NS	SS	C			:::
		Penicillium glabrum (RE 083)	NS	NT	?	No match		-
	pen_exp_c101_A90	Penicillium expansum (EPA 054)	NS	SS	C			:::
	pen_ros_C101_A94	Penicillium roseopurpureum (HT 146)	NS	SS	C			:::

TABLE 5. The results from testing single templates with samples with no probe

	ID	SPECIES	SPOT	REMARKS	PAGE GEL RESULT
NO PROBE	EPA 322	*Penicillium variabile*	NS		-
	EPA 364	*Penicillium variabile*	NS		-
	EPA 447	*Penicillium spinulosum group*	NS		-
	EPA 453	*Penicillium sclerotium*	NS		-
	EPA 611	*Aspergillus candidus*	A76 Positive		-
	EPA 616	*Penicillium islandicum*	NS		-
	HT 620	*Aspergillus wentii*	A78 Positive		-
	RE 060	*Penicillium viridicatum*	NS		-
	RE 064	*Aspergillus clavatus*	NS		-

*Positive on all subarrays

SS= Strongly positive

MS= Moderately positive

FS= Faintly positive

NS= No signal

NT= Not tested

Color codes and rating:

Green: The probe works as expected, moderately or strongly positive spot, no controversies with the PAGE tests or the BLAST results (rating from + to +++)

Yellow: Negative or faint spot on at least one of the samples, but not necessarily tested or unclear result on the PAGE gel (rating -)

Red: No positive spot, controversies with the PAGE and/or the BLAST results (rating --)

Blue: Probe had an error in the zip-sequence.

5.2 Testing with multiple samples on one subarray

In the last experiment varying amounts of DNA from different templates was applied to the same ligation reaction. The goal for these tests was to determine how small amounts of DNA could be detected on the microarray, and also to find out how "competing" DNA would affect the detection. Boxplots from the results are in APPENDIX 4. The results are also collected to TABLE 6 to make reading the results easier. For the boxplots results, the logarithmic value 2,5 was considered as a detection limit for a positive spot.

From the samples tested with 10 fmol, *P. variotii* and *A.terreus* showed an ok spot, *A. ustus* were slightly above the detection limit and *A. fumigatus* and *P. corylophium* showed to be below the detection limit. Especially on subarray 2 and also on subarray 5 there were some problems, because some of the samples that gave a positive signal with 50 fmol on other arrays gave negative results.

With 1 fmol *P. corylophilum* and *P.variotii* gave positive results, *A. ustus* gave a faint result and *A. terreus* and gave a negative result. The positive result for *P. corylophilum* is surprising, because with 10 fmol it did not give a positive result. When testing with mixes consisting of large amount of DNA that had no corresponding probes and DNA that had corresponding probes, only *P. corylophilum* was gave positive signal, but *A. terreus* was close to the detection limit. *P. varioitii* and *A.fumigatus* did not show a positive result. With *P. variotii* and *A. terreus* there was a faint hint in spot A78 (*A. ustus*).

The single template tested on the array (HT274, *P. citreonigrum*, zip-code A87) did not give a positive result, so it did not give a positive result in spot A89, like when tested on the previous array.

In the last test, a large amount of DNA from both strains of *A. ustus* (EPA 427 and HT 561) was tested. As a result, only a faint spot was detected.

On the all of the subarrays, spot A121 gave a positive result. This is because the probe pair A93 (*P. italicum*) hybridizes in ligation with the zip-code A121. It was also later found ou that probe pair A93 had errors in the sequence as it was ordered in wrong orientation. On two subarrays, spot A48 was positive with visual observation, although there was no probe with such a zip-code.

Probe with zip-code A69 (*A. fumigatus*, strains EPA 526 and RE 004)) did not give a clearly positive result on any of the subarrays, even when 100 fmol was used. On most of the tests, sample EPA 526 was used without a positive result, but on subarray 14, an alternate sample RE 004 was used, and it did also gave a negative result. In the previous tests on this study, both these samples gave clearly positive results on the chip, and on the PAGE gel they also had showed positive or moderate results. One of the reasons for the negative results could be that the DNA from other samples might interfere the detection. Another reason could be that something went wrong in the ligation phase, for example a pipetting error, although it is more unlikely that it could happen with both of the strains.

Sample *A.ustus* (strains EPA 427 and HT 561) gave only faint results on almost every array, even when 550 fmol was used. This sample also gave strongly positive results on previous the chip and the PAGE gel tests.

TABLE 6. The results from the tests with multiple templates (subarrays 1-9 and 14).

TEMPLATES							
Test	Subarray	RE 004 (*A. fumigatus*)	RE 009 (*A.versicolor)*	HT 445 (*A. flavus)*	EPA 427 (*A. ustus)*	EPA 231 (*A. terreus)*	
10 fmol	14	No	Faint/Ok	Faint/No(10)	No	No	
	Subarray	EPA 231 (*A. terreus)*	EPA 427 (*A. ustus)*	EPA 526 *(A. fumigatus)*	RE 015(A95) *(P. variotii)*	RE 059 *(P. corylophilum)*	Remarks
	1	No	Faint	No	Ok (10)	Ok	*
	2	No	Faint	No	No	No (10)	*
	3	Ok (10)	Faint	No	Ok	Faint	A48,*
	4	No	Faint (10)	No	Ok	Ok	*
	5	Faint	Faint	No (10)	No	No	*
1 fmol	6	Ok	Faint (1)	No	Ok	Ok	*
	7	Ok	Ok	No	Ok (1)	Ok	A48,*
	8	Ok	Faint	No	Faint	Ok (1)	*
	9	No (1)	Faint	No	Ok	Ok	*

TABLE 7. Results from the tests with multiple templates (subarrays 10-16).

TEMPLATES						
Test	Subarray	EPA 231 *(A. terreus)*	EPA 526 *(A. fumigatus)*	RE 015 *(P. variotii)*	RE 059 *(P. corylophilum)*	Remarks
”100 fmol + DNA with no probe”	10	-	-	No (100)	-	*, A78 faint
	11	-	No (100)	-	-	*
	12	-	-	-	Faint/Ok(100)	*
	13	No/faint	-	-	-	*, A78 faint
”Single template”	Subarray	HT274 *(P. citreonigrum)*				
	15	No				*
”Too much DNA”	Subarray	EPA 427 *(A. ustus)*	HT 561 *(A. ustus)*			
	16	Faint	Faint			*

*A93 and A121 positive

5.3 Problems

In the PCR amplification of the DNA templates, there were difficulties in getting good PCR products with some of the samples. With some of the templates, large amounts had to be added to the reaction to produce a clear band on the electro-phoresis gel. However, some of the templates showed good bands with small amounts (1-2 µ). The concentrations of PCR products were quite low, most of the templates had the concentration of around 3-5 ng/µl. Also, the template DNA was

measured and it also showed low concentrations. Low concentrations of the templates and the PCR products may have affected the detection of the templates on the microarray, but some of the templates gave positive spots even with low concentrations. For example, the sample EPA 434 (*P. corylophilum*), that had a concentration of only 2,91 ng/µl and RE 004 (*A. fumigates*), that had a concentration of 3,78 ng/µl, gave positive results.

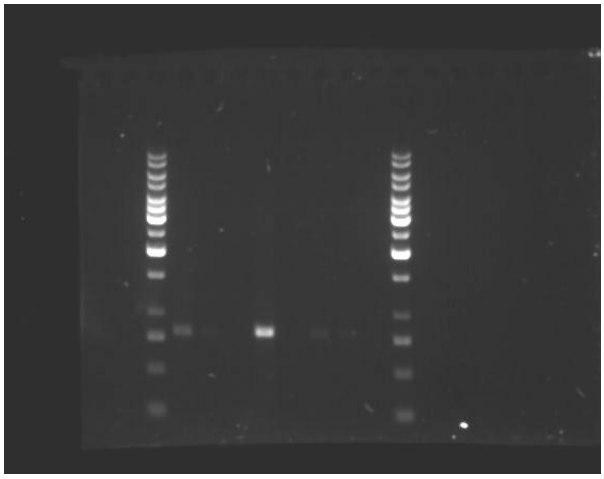


FIGURE 7. PCR products run on electrophoresis gel. PCR products in this gel had templates from 1 µ to 18 µl.

In the first hybridization attempt, no positive spots were found on the microarray. The problem was found in the D-probes, which did not have the fluorescent stains, and therefore did not show positive spots on the microarray. New D-probes with fluorescent stains were then ordered and used in the following tests.

On the second attempt of the hybridization, the results were again bad because no clearly positive spots were observed. For the next test the annealing temperature in the ligation cycle was reduced to 60^{o} degrees. Also, to make sure that there were no problems in the protocol itself, a positive control was added along with samples EPA 527 and on later tests with EPA 421 and EPA 322. Positive controls included a mix of the different samples and the probes known to work on the microarray. Lowering the annealing temperature seemed to help, because the samples started

to produce positive spots on the array. The positive control showed very clear spots on the microarray, much clearer than most of the tested samples gave, so there were no problems with the protocol itself.

6 CONCLUSIONS

The aim of this study was to test how previously designed probes could detect their target DNA on an LDR microarray platform. Two probe's single sample and two probe's alternate samples had not been tested before on PAGE gel. Rests of the probes were tested before with at least one sample on the PAGE gel. The results show that only 7 of the total 24 probes gave a clearly positive /signal, 2 samples were classified as faint and altogether 15 probes gave negative results (including panfungal).

From the 15 probes that gave a negative result, the results of five probes could be explained on the basis of the previous PAGE gel tests. Two of the probes had not been tested before on the PAGE gel, two had given negative results and one had given an unclear result on PAGE gel. With one of the probes, errors in the sequence were found. All in all, 10 probes gave negative results although they had worked well on previous PAGE gel tests.

On the tests with multiple samples on the same ligation, the results are a bit unclear. Some of the probes were detected with 10 and 1 fmol, but some of the results contradict with each other, such as with *P. corylophilum*, which gave a positive result with 1 fmol, but a negative result with 10 fmol. When testing the DNA from the samples that had no probes, and therefore should not be detected, only one probe (*P. corylophilum*) gave result that could be regarded as a positive.

Although some of the probes did not work as expected, the LDR microarray showed to be a promising platform for diagnosing and characterizing environmental samples. To confirm that the tested probes are not working on microarray, fur-

ther tests are probably needed. Some of the probes had errors and need to be re-designed. Also, the quality of the samples might have affected some of the results.

One of the most critical factors affecting the specificity and ability to characterize the samples in the microarrays is the design of the probes. If probes have a bad design and have errors in their sequence, they might give negative results or a falsely positive signal. In the designing of the probes, sequence information of the target species is crucial. In the future, producing sequence information will become faster because of new technologies (i.e. pyrosequencing). This will help in designing the probes.

One of the problems with developing microarrays to a commercial product is the quality of printed oligos on the microarray. Some of the microarrays used in this study had bad quality oligo prints that had smeared, or bad spots. These technical problems with the printing quality would have to be solved if the platform is to be used as a commercial product.

The use of microarrays in diagnosing environmental samples will most probably increase in the future. Microarrays have many advantages over the conventional diagnostics methods with environmental samples. Microarrays offer a much faster and easier method for characterizing samples, especially when compared to incubation and microscoping. For example, samples taken from buildings with a suspected mold damage are traditionally incubated, and the growth of molds is inspected by microscoping. The incubation usually takes about a week to complete and microscoping needs experienced staff. Microarrays also allow the detection of hundreds or even thousands of species from a single sample simultaneously. Microarrays also can detect the DNA from dead microbes. Other advantages are that microarrays can recognize molds on the genus level and the detection can be automated. (Rintala 2005)

REFERENCES

Atlas R.M. & Bartha R. 1993. Microbial ecology. Fundamentals and applications. Thirdedition. The Benjamin/Cummings Publishing Company. P. 215-220.

Barany, F. 1991. Genetic diseasedetection and DNA amplification using cloned thermostable ligase. Proc Natl Acad Sci U S A88: 189-193.

Bhatnagar D., Yu J. & Ehrlich K.C. 2002. Toxins of filamentous fungi. Chemical Immunology. 81: 167–206.

Bonch-Osmolovskaya E.A., Miroshnichenko M.L., Lebedinsky A.V., Chernyh N.A., Nazina T.N., Ivoilov V.S., Belyaev S.S., Boulygina E.S., Lysov Y.P., Perov A.N., Mirzabekov A.D., Hippe H., Stackebrandt E., L'Haridon S. & Jeanthon S. 2003. Radioisotopic, culture-based, and oligonucleotide microchip analyses of thermophilic microbial communities in a continental high-temperature petroleum reservoir. Applied Environmental Microbiology. 69:6143-6151.

Bodrossy L. & Sessitsch A: Oligonucleotide microarrays in microbial diagnostics.2004.Current Opinion in Microbiology. 7(3):245-254.

Bornehag C-G., Blomquist G., Gyntelberg F., Järvholm B., Malmberg P. 2001. Dampness in buildings and health. Indoor Air 11:72-86

Brinkman E.B., Fout G.S. 2007. Development and evaluation of a generic tag array to detect and genotype noroviruses in water. Journal of Virological Methods 156 (2009). P. 8–18.

Busti, E., Bordoni, R., Castiglioni, B., Monciardini, B. & Sosio, M. 2002. Bacterial discrimination by means of a universal array approach mediated by LDR (ligase detection reaction). BMC Microbiology, 2:27, pages 1-13.

Coppe´e, J-Y. 2008. Do DNA microarrays have their future behind them?, Mi-

crobes and Infection, number 10, s. 1067-1071.

Dix N.J. & Webster J. 1995. Fungal ecology. Chapman & Hall. Cambridge. Great Britain. P. 26.

Garrido P, Gonzalez-Toril E, Garcia-Moyano A, Moreno-Paz M, Amils R. & Parro V. 2008. An oligonucleotide prokaryotic acidophile microarray: its validation and its use to monitor seasonal variations in extreme acidic environments with total environmental RNA. Environ Microbiol 2008, 10(4):836-850.

Gerry, N., Witowski, N., Day, N., Hammer, R., Barany, G. & Barany, F.1999. Universal DNA Microarray Method for Multiplex Detection of Low Abundance Point Mutations. Journal of Molecular Biology. Vol 292. p. 251-262.

Gebert J., Stralis-Pavese N., Alawi M. & Bodrossy L. 2008. Analysis of methanotrophic communities in landfill biofilters using diagnostic microarray. Environmental Microbiology. 10(5):1175-1188.

Gentleman, R. C., Carey, V. J., Bates, D. M., Bolstad, B., Dettling, M., Dudoit & S. et al. (2004) Bioconductor: open softwaredevelopment for computational biology and bioinformatics. Genome Biol 5: R80.

He Z., Gentry T.J., Schadt C.W., Wu L., Liebisch J., Chong S.C., Huang Z., Wu W., Gu B., Jardine P., Criddle C.& Zhou J. 2007. GeoChip: a comprehensive microarray for investigating biogeochemical, ecological and environmental processes. The ISME Journal (2007) 1, p. 67–77.

Hoheisel, Jörg D. 2006. Microarray technology: beyond transcript profiling and genotype analysis. In publication Nature , Vol 7, p. 21-210.

Hultman, Jenni 2008. Conversation 25.06.2008.

Hultman, Jenni. 2009. Microbial diversity in the municipal composting process

and development of detection methods. Doctoral dissertation. University of Helsinki, Faculty of Biosciences, Department of Ecological and Environmental Sciences Institute of Biotechnology.

Hultman, J., Vasara, T., Partanen, P., Paulin, L., Auvinen, P. & Romantschuk, M. In press. Fungal succession during municipal solid waste composting using a cloning-based analysis.

Husman T. 1996. Health effects of indoor-air microorganisms. Scandinavian Journal of Work, Environment & Health. 2/1996, 22:11. P. 5-13.

Hyvärinen A.M. 2002. Characterizing Moisture Damaged Buildings – Environmental and Biological Monitoring. Publications of National Public Health Institute A8/2002. 121 p.

Ingold C.T. & Hudson H.J. 1993. The biology of fungi, Sixth Edition. Chapman & HaLondon. P. 7-24.

Jaakkola, M. S., Nordman. H., Piipari E R., Uitti J., Latitinen J., Karjalainen A., Hahtola P. & Jaakkola J.K. 2002. Indoor Dampness and Molds and Development of Adult-Onset Asthma: A Population-Based Incident Case–Control Study. Environmental Health Perspectives .Vol 110. Number 5. P.543-547.

Aino Nevalainen .2007. Kosteusvauriot: kansanterveys- vai rakennusongelma? Kansanterveys 04/2007. P. 5.

King N. & Auger P. 2002. Indoor air quality, fungi, and health. Canadian Family Physician. Vol 48 pages 298-302.

Lee D-Y., Laudera H., Cruwysa H., Fallettaa P. & Beaudette L.A. 2007. Development and application of an oligonucleotide microarray and real-time quantitative PCR for detection of wastewaterbacterial pathogens. Science of the total environment 398 (2008), p. 203 – 211.

Loy A., Lehner A., Lee N., Adamczyk J., Meier H., Ernst J., Schleifer K.H., & Wagner M. 2002.Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment. Appl Environ Microbiol 2002, 68(10):5064-5081.

National Public Health Institute. 2009. Kosteusvaurion tutkiminen ja korjaaminen; Kosteusvaurioiden yleisyys ja syyt . [Visited 10.3.2009]. Available: [http://www.ktl.fi/portal/suomi/tietoa_terveydesta/elinymparisto/hometalo_ja_kosteusvaurio/kosteusvaurion_tutkiminen_ja_korjaaminen/#navi0]

Peat J.K., Dickerson J., & Li J. 1998. Effects of damp and mould in the home on respiratory health: a review of the literature. Allergy 53(2): 120-128.

Rintala H. 2005. DNA-siru sisäilman mikrobien tunnistuksen välineenä. LIMIT - Liiketoimintaa mittauksista –seminar. Presentation.

Rudi K., Skulberg O.M., Skulberg R. & K.S. Jakobsen. 2000. Application of sequence-specific labeled 16S rRNA gene oligonucleotide probes for genetic profiling of cyanobacterial abundance and diversity by array hybridization. Applied and environmental microbiology. Sept. 2000. Vol. 66. No. 9. P. 4004–4011.

Samson R.A. 1992. Mycotoxins: a mycologist's perspective. Medical Mycology. Volume 30, Issue 6, supplement 1. P. 9–18.

Samson R.A., Flannigan B., Flannigan M.E., Verhoeff A.P., Adan OCH. & Hoekstra E.S. 1994. Health implications of fungi in indoor environments. Elsevier Publications, Amsterdam. Air Qaulity monographs. Vol. 2. P. 529-538.

Schena, M., Shalon, D., Davis, R.W. & Brown, P.O. 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 270 p. 467-470.

Schott AG. 2009. Protocol Nexterion Slide A Star MPX 16 DNA-application (April 2009), p. 2.

Simon-Nobbe B., Denk U., Pöll V., Rid R. & Breitenbach M. 2008. The Spectrum of Fungal Allergy. International Archives of Allergy and Immunology 2008;145:58–86.

White T.J, Bruns T, Lee S. & Taylor J.1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: A guide to methods and applications. Academic Press. P. 315-322.

APPENDICES

Results from previous PAGE gel tests APPENDIX 1

List of templates, species and zip-codes APPENDIX 2

Amount of templates used in PCR amplification. APPENDIX 3

Boxplot figures from the last test generated with Bioconductor. APPENDIX 4

APPENDIX 1. Page-gel test results.

Species	Probe	Tested strains	
		Strain 1	Strain 2
Aspergillus flavus	asp_fla_d100	EPA532 / NRRL16883	HT445
Aspergillus fumigatus	asp_fum_d100	EPA526 / NRRL163	RE4
Aspergillus (Emericella) nidulans	asp_nid_d100	EPA 527	RE 62
Aspergillus niger	asp_nig_d100	EPA88 / ATCC16888	RE 56
Aspergillus ochraceus	asp_och_d100	EPA426 / NRRL398	
Aspergillus restrictus	asp_res_d100	EPA458 / FRR3689	RE 57
Aspergillus sydowii	asp_syd_d100	EPA421 / NRRL250	RE 91
Aspergillus terreus	asp_ter_d100	EPA231 / ATCC1012	
Aspergillus ustus	asp_ust_d100	EPA427 / NRRL275	HT561
Aspergillus versicolor	asp_ver_d100	EPA524 / NRRL238	RE9
Eurotium amstelodami/chevalieri	eur_ams_d100	EPA 530	HT651
Eurotium herbariorum	eur_her_d100	HT538	
Penicillium brevicompactum group	pen_bio_d100	EPA435 / FRR862	RE17
Penicillium chrysogenum complex	pen_chr_d101	EPA467	RE25
Penicillium citreonigrum	pen_cit_d102	HT274	
Penicillium citrinum	pen_ctr_d101	EPA448 / FRR1841	
Penicillium corylophilum	pen_cor_d100	EPA434 / FRR802	RE 59
Penicillium expansum	pen_exp_d101	EPA54 / ATCC7861	RE90
Penicillium fellutanum	pen_fel_d100	HT193	
Penicillium glabrum	pen_gla_d100	RE 83	HT84
Penicillium italicum	pen_ita_d100	EPA59 / ATCC48114	HT18
Penicillium roseopurpureum	pen_ros_d101	HT146	
Paecilomyces variotii	pae_var_d100	EPA75 / ATCC22319	RE15
Panfungal probe	panfungal01_d100		

Color code
Has worked well on gel
Has worked moderately on gel
Result from gel unclear
Not tested on gel
Hasn't worked on gel

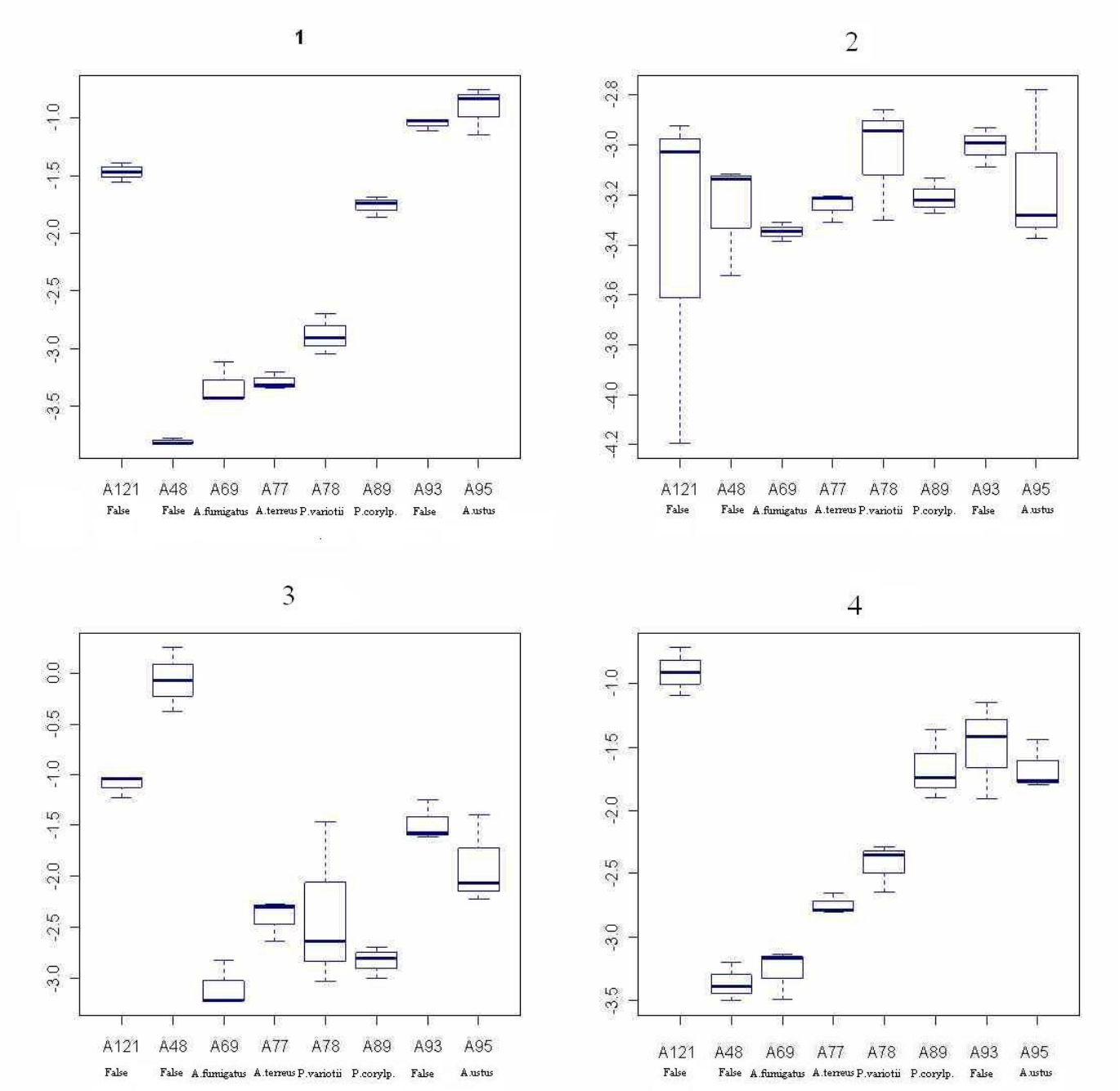
APPENDIX 2. Amount of templates used in PCR amplification.

Number	ID	Template	Number	ID	Template
1	EPA 054	13	24	EPA 616	10
2	EPA 059	7	25	HT 018	1
3	EPA 075	0,5	26	HT 084	3,5
4	EPA 088	4,4	27	HT 146	6,5
5	EPA 231	11	28	HT 193	1,5
6	EPA 322	13	29	HT 274	6
7	EPA 364	10	30	HT 445	1
8	EPA 421	12,5	31	HT 538	1
9	EPA 426	17	32	HT 561	3
10	EPA 427	15	33	HT 620	6,5
11	EPA 434	2	34	HT 651	14
12	EPA 435	2	35	RE 004	8
13	EPA 447	20	36	RE 009	11
14	EPA 448	8	37	RE 015	13
15	EPA 453	9,5	38	RE 017	7,5
16	EPA 458	7	39	RE 025	6
17	EPA 467	6	40	RE 056	8
18	EPA 524	9	41	RE 057	13
19	EPA 526	18	42	RE 059	1
20	EPA 527	2	43	RE 060	1
21	EPA 530	3,3	44	RE 062	3
22	EPA 532	0,5	45	RE 064	5
23	EPA 611	17	46	RE 083	3

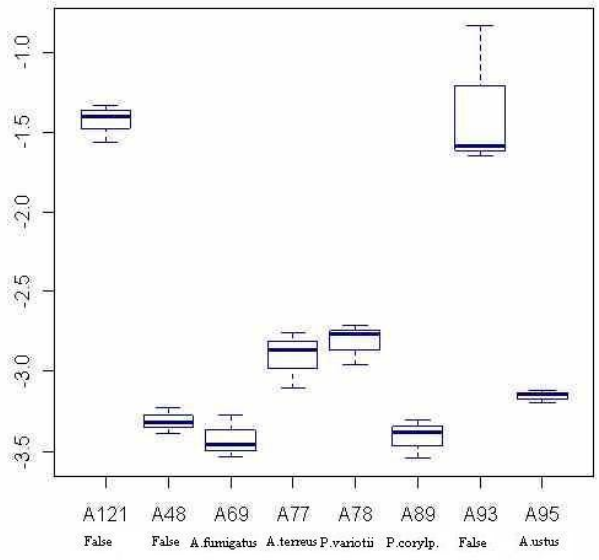
APPENDIX 3. List of templates, species and zip-codes.

	ID	Species	Kantakokoelma	Zip-code
1	EPA 054	Penicillium expansum	ATCC 7861	pen_exp_c101_A90
2	EPA 059	Penicillium italicum	ATCC 48114	pen_ita_c100_A93
3	EPA 075	Paecilomyces variotii	ATCC 22319	pae_var_C100_A95
4	EPA 088	Aspergillus niger	ATCC 16888	asp_nig_c100_A71
5	EPA 231	Aspergillus terreus	ATCC 1012	asp_ter_C100_A77
6	EPA 322	Penicillium variabile		
7	EPA 364	Penicillium variabile		
8	EPA 421	Aspergillus sydowii	NRRL 250	asp_syd_C100_A76
9	EPA 426	Aspergillus ochraceus	NRRL 398	asp_och_c100_A74
10	EPA 427	Aspergillus ustus	NRRL 275	asp_ust_c100_A78
11	EPA 434	Penicillium corylophilum	FRR 802	pen_cor_c100_A89
12	EPA 435	Penicillium brevicompactum	FRR 862	
13	EPA 447	Penicillium spinulosum group	FRR 1750	
14	EPA 448	Penicillium citrinum	FRR 1841	pen_ctr_c101_A88
15	EPA 453	Penicillium sclerotium		
16	EPA 458	Aspergillus restrictus	FRR 3689	asp_res_C100_A75
17	EPA 467	Penicillium chrysogenum	uusi kanta	pen_chr_C100_A82
18	EPA 524	Aspergillus versicolor	NRRL 238	asp_ver_c100_A79
19	EPA 526	Aspergillus fumigatus	NRRL 163	asp_fum_c100_A69
20	EPA 527	Emericella nidulans (=A.nidulans)	NRRL 2395	asp_nid_c100_A70
21	EPA 530	Eurotium chevalieri	NRRL 78	
22	EPA 532	Aspergillus flavus	NRRL 16883	asp_fla_c100_A68
23	EPA 611	Aspergillus candidus		
24	EPA 616	Penicillium islandicum	NRRL 10127	
25	HT 018	Penicillium expansum		pen_exp_c101_A90
26	HT 084	Penicillium glabrum		pen_gal_c100_A92
27	HT 146	Penicillium roseopurpureum		pen_ros_C101_A94
28	HT 193	Penicillium fellutanum		pen_fel_c100_A91
29	HT 274	Penicillium citreonigrum		pen_cit_c102_A87
30	HT 445	Aspergillus flavus		asp_fla_c100_A68
31	HT 538	Eurotium herbariorum		
32	HT 561	Aspergillus ustus		asp_ust_c100_A78
33	HT 620	Aspergillus wentii		
34	HT 651	Eurotium sp.		eur_arms_c11_A80
35	RE 004	Aspergillus fumigatus		asp_fum_c100_A69
36	RE 009	Aspergillus versicolor		asp_ver_c100_A79
37	RE 015	Paecilomyces variotii		pae_var_C100_A95
38	RE 017	Penicillium brevicompactum group	ATCC 58606	
39	RE 025	Penicillium chrysogenum com-plex		pen_chr_C100_A82
40	RE 056	Aspergillus niger		asp_nig_c100_A71
41	RE 057	Aspergillus restrictus		asp_res_C100_A75
42	RE 059	Penicillium corylophilum		pen_cor_c100_A89
43	RE 060	Penicillium viridicatum	DSM 62878	
44	RE 062	Emericella nidulans (=A.nidulans)	DSM 820	asp_nid_c100_A70
45	RE 064	Aspergillus clavatus	DSM 816	
46	RE 083	Penicillium glabrum		pen_gal_c100_A92
		Panfungal probe		panfungal_C100_A96

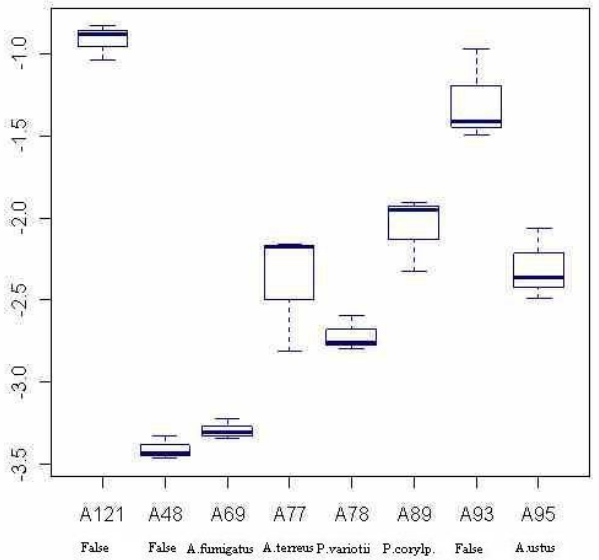
APPENDIX 4. Boxplot figures from the last test generated with Bioconductor.



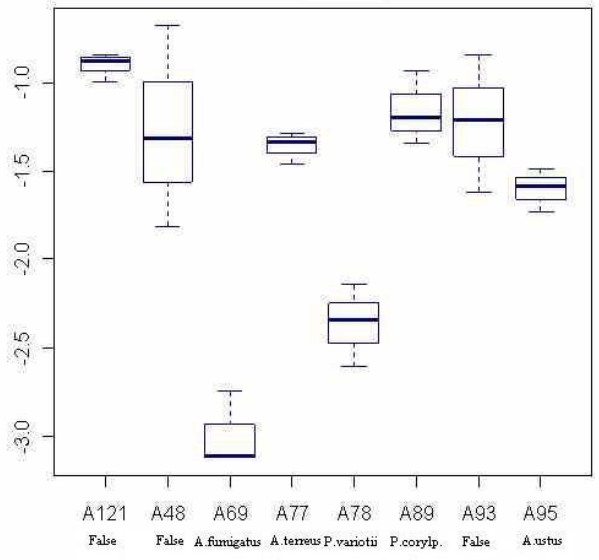
5


-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
A121 A48 A69 A77 A78 A89 A93 A95
False False A.fumigatus A.terreus P.variotii P.corylp. False A.ustus

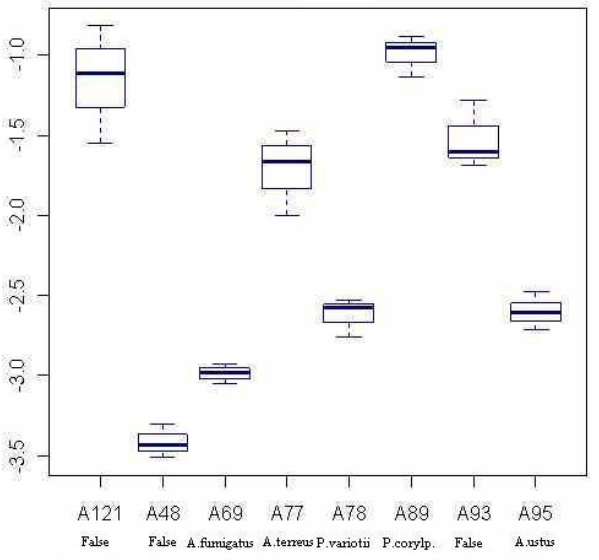
6


-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
A121 A48 A69 A77 A78 A89 A93 A95
False False A.fumigatus A.terreus P.variotii P.corylp. False A.ustus

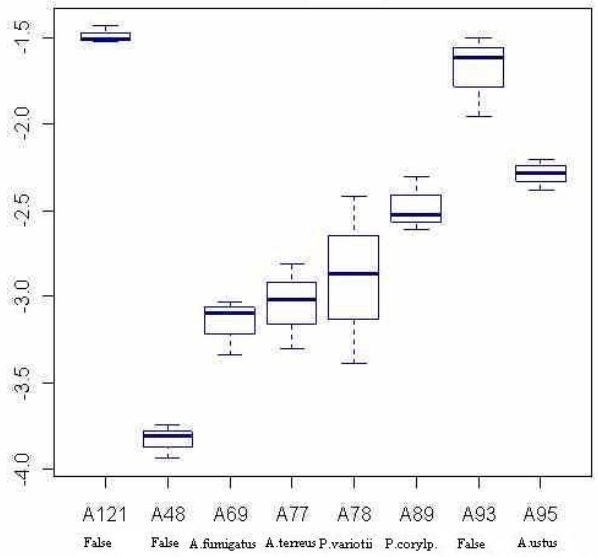
7


-1.0
-1.5
-2.0
-2.5
-3.0
A121 A48 A69 A77 A78 A89 A93 A95
False False A.fumigatus A.terreus P.variotii P.corylp. False A.ustus

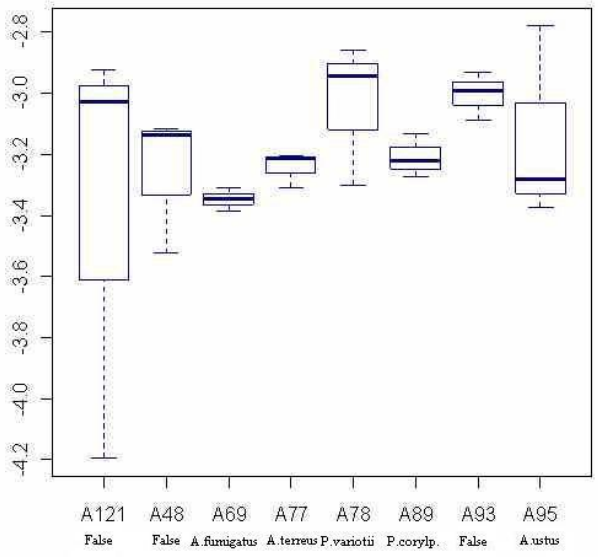
8


-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
A121 A48 A69 A77 A78 A89 A93 A95
False False A.fumigatus A.terreus P.variotii P.corylp. False A.ustus

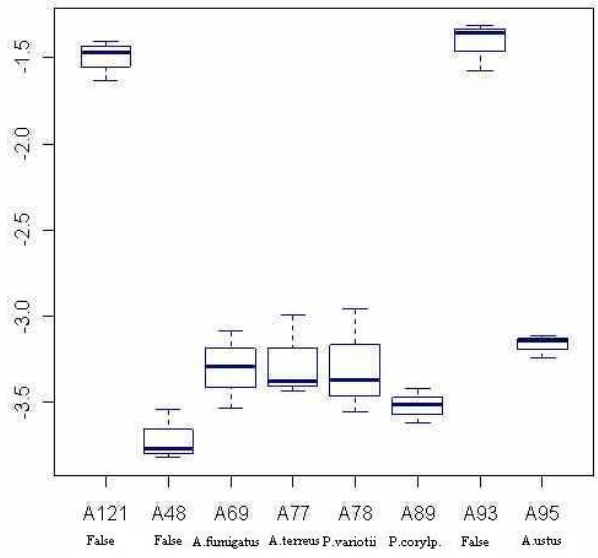
9


-1.5
-2.0
-2.5
-3.0
-3.5
-4.0
A121 A48 A69 A77 A78 A89 A93 A95
False False A.fumigatus A.terreus P.variotii P.corylp. False A.ustus

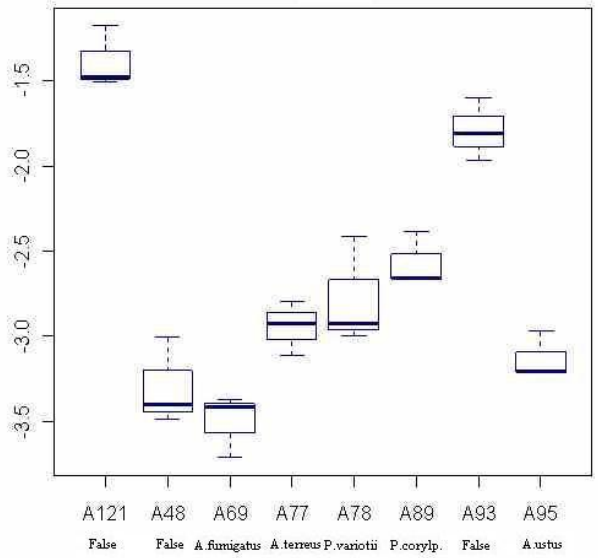
10


-2.8
-3.0
-3.2
-3.4
-3.6
-3.8
-4.0
-4.2
A121 A48 A69 A77 A78 A89 A93 A95
False False A.fumigatus A.terreus P.variotii P.corylp. False A.ustus

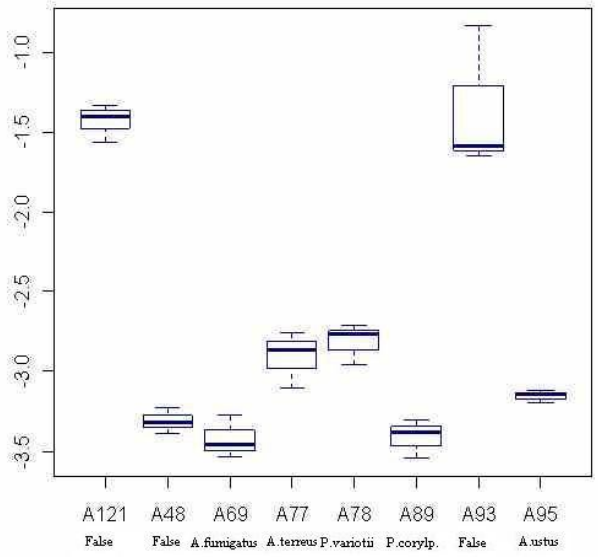
11


-1.5
-2.0
-2.5
-3.0
-3.5
A121 A48 A69 A77 A78 A89 A93 A95
False False A.fumigatus A.terreus P.variotii P.corylp. False A.ustus

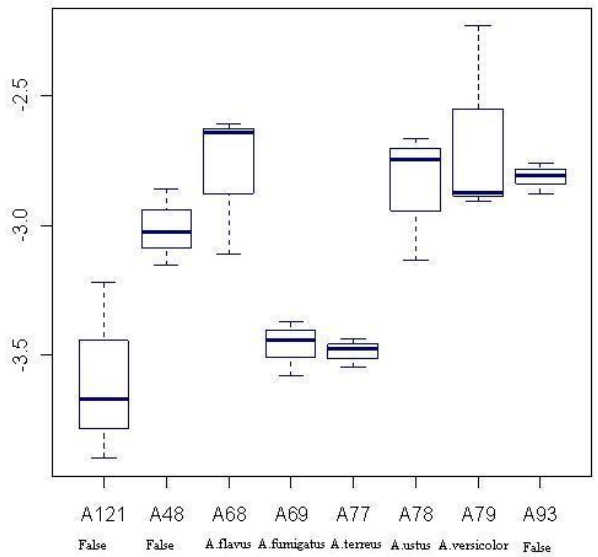
12


-1.5
-2.0
-2.5
-3.0
-3.5
A121 A48 A69 A77 A78 A89 A93 A95
False False A.fumigatus A.terreus P.variotii P.corylp. False A.ustus

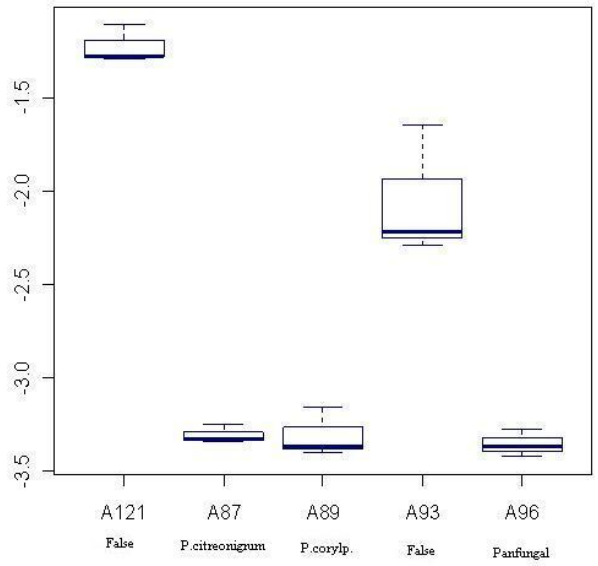
13


-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
A121
False
A48
False
A69
A.fumigatus
A77
A.terreus
A78
P.variotii
A89
P.corylp.
A93
False
A95
A.ustus

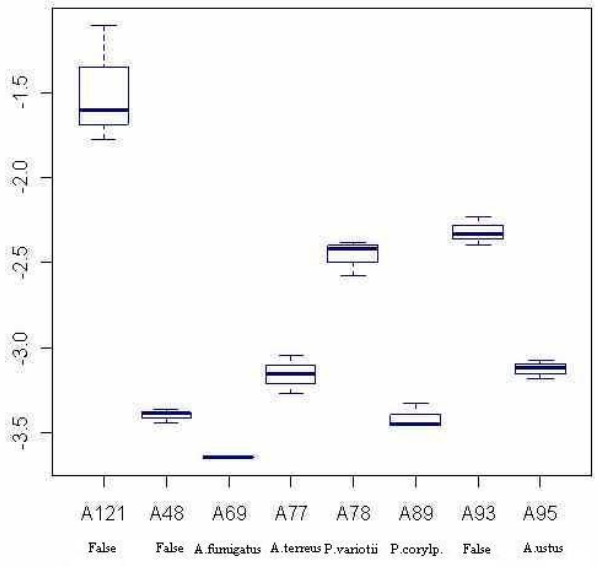
14


-2.5
-3.0
-3.5
A121
False
A48
False
A68
A.flavus
A69
A.fumigatus
A77
A.terreus
A78
A.ustus
A79
A.versicolor
A93
False

15


-1.5
-2.0
-2.5
-3.0
-3.5
A121
False
A87
P.citreonigrum
A89
P.corylp.
A93
False
A96
Panfungal

16


-1.5
-2.0
-2.5
-3.0
-3.5
A121
False
A48
False
A69
A.fumigatus
A77
A.terreus
A78
P.variotii
A89
P.corylp.
A93
False
A95
A.ustus