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QUANTITATIVE PCR FOR THE DETECTION AND QUANTITATION OF ENVIRONMENTAL MICROORGANISMS: BASICS AND APPLICATIONS

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Introduction

Exposure to fungal and bacterial bioaerosols [such as spores, mycotoxins, endotoxins, bacterial cells, volatile organic compounds (VOC's) and (1-3)-ß-glucan] in the indoor environment has emerged as a significant health concern in residential environments as well as in occupational settings, including offices and industrial sites (such as facilities for composting, wastewater treatment, sludge, and recycling materials). Currently, measurements of fungal and bacterial exposures rely on air sampling for culturable fungi and bacteria or total fungal spore counts. Although sampling and testing for mycotoxins and fungal surrogates (such as ergosterol and glucan), endotoxins, or selected bacterial species are possible, the application has not been widely used.

In addition to air sampling, assessing indoor fungal contamination requires careful review of the building history and visual inspection by an experienced environmental professional. Suspected contamination should be collected by source sampling and confirmed to be fungal growth, bacterial growth, or both. This approach not only identifies the sources of contamination but also facilitates eventual removal and control of fungal and bacterial growth and their reservoirs. In addition, information should be gathered and samples may be taken and analyzed for endotoxins, allergens, and other bioaerosols to determine whether each individual bioaerosol may play a role in the health manifestation or not. A physician, whether a board certified occupational health physician, an immunologist, an allergist, or a pulmonary specialist, may be involved in the determination of the etiological agents responsible for human symptoms.

Although microorganisms (specifically fungi and bacteria) are a part of our environment, indoors or outdoors, exposure to some of the pathogenic microorganisms are known to cause a number of human diseases, such as anthrax, tuberculosis, legionellosis, aspergillosis, histoplasmosis, etc. Even exposure to normally innocuous microorganisms and their metabolites may lead to human disease, such an allergic rhinitis, hypersensitivity diseases, etc. The sampling and testing for the microorganisms using the conventional methods may take days to weeks. A rapid and reliable detection and identification of the microorganisms is essential for facilitating better assessment of microbial contamination and eventually the remediation process.

DNA or deoxyribonucleic acid is the genetic base for essentially all living things (except for some RNA viruses). Over the last fifty years, scientists have learned the biological, physical and chemical properties of DNA. Using these properties, scientists are now able to duplicate DNA *in vitro*. The duplication is exponential and millions of copies of DNA can be produced in one single process in the laboratory. This process of duplicating DNA in vitro is called Polymerase Chain Reaction (PCR). Several variations of PCR have been developed.

Organisms share a significant portion of their DNA. On the other hand, a portion of the DNA is unique to each species. By identifying the unique and specific DNA sequence(s) of individual species, a probe with fluorogenic dye can be designed to detect and quantify DNA products through the PCR. This PCR procedure is called quantitative PCR (QPCR) or real-time PCR. The term “real-time PCR” is because laboratorians can actually monitor and “visualize” the progress of PCR reactions through a computer.

Researchers at the U.S. EPA have utilized the QPCR principles, and have developed and patented a new DNA-based realtime quantitative PCR technique for the detection and quantitation of some indoor fungi and bacteria (for approximately 120 - 130 species). Laboratories (approximately 10 labs in the US) have licensed this technology from the U.S. EPA, and are currently offering this service for the rapid detection and quantitation of some indoor fungi and bacteria. The list of fungal and bacterial species can further be expended as primers and probes of additional species are identified and designed.

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Health Effects of Fungi

The adverse effects of fungal exposures on human health are not a new issue to human beings. Excessive moisture and water (dampness) indoors due to water intrusion or leaking are the key factors leading to fungal growth indoors. Fungi play a negative role to human health through three major ways: causing infections, trigger allergies, and producing mycotoxins and other secondary metabolites. The occurrences of diseases, such as aspergillosis, histoplasmosis, cryptococcosis, coccidioidomycosis, candidiasis are due to infections caused by fungi. *Aspergillus fumigatus* in particular has caused infections to patients who have a compromised immune system and is an important opportunistic pathogen in infection control in healthcare facilities.

Fungal spores and fragments are allergens, which can cause allergy and be asthma triggers, and the cause of rhinitis and sinusitis. Some fungi are linked to hypersensitive pneumonitis, and certain fungi have been associated to occupational respiratory diseases.

Fungi produce a wide variety of metabolites, which can have various adverse and beneficial effects to human health and well-being. They reportedly produce over 400 mycotoxins of varying potency. Approximately 46 genera of fungi are mycotoxin producers, including some common indoor fungal contaminants, such as *Aspergillus* spp., *Penicillium* spp., *Fusarium* spp., *Stachybotrys chartarum*, *Trichoderma* spp., etc. Mycotoxins are toxic to humans and animals mainly through ingestion. However, inhalation exposure cannot be overlooked. Mycotoxins may cause medical conditions to livers, kidneys, gastrointestinal tract, the heart, the central nerve system, and the immune systems. A number of mycotoxins are known carcinogens.

Indoor molds pose population health issues. Indoor mold and dampness were related to 50% increases in asthma and 60% increases in upper respiratory disease. Harvard and Canadian studies showed that 10% of the residential buildings had dampness problems to a degree, which could result in population health effects. Remediation should be taken following the determination of the presence of indoor molds and dampness. The Canadian Federal and Provincial Committee on Environmental and Occupational Health recommended that exposure to indoor mould be minimized, and recognized there is a relationship between the population health effects of mould and dampness and the existence of risk groups. It led to a 1990 National Building Code, which requires mechanical ventilation in residences and applies to most Canadians.

What Is PCR Technology?

Since Drs. Francis Crick and James Watson described the double helix DNA structure fifty years ago, many advances have occurred based on the biological, physical and chemical nature of DNA. The explosion in molecular biology technology has initiated a new era in fungal and bacterial detection. This new technology for fungal and bacterial detection is called polymerase chain reaction (PCR), a nucleic acid-based technology.

Every fungal and bacterial species has its own-unique, characteristic genetic signature, which varies among species and can serve as a genetic fingerprint for their identification. The power of PCR is to amplify the selected sequence of genetic fingerprint to the level that it can be easily detected and quantified.

At the forefront of PCR technology is called real time quantitative PCR (QPCR), which is currently used in our laboratory. In this system, a fluorogenic probe is added into the PCR amplification process. This probe is released, if the reactions occur, and emits fluorescence. The levels of accumulated fluorescent light can be detected and quantified in real time, during the amplification process. This fluorogenic probe is specially designed to bind only to the selected sequence of fingerprint, thus providing additional target specificity in detection.

With the progression of cutting-edge PCR technology, we are now able to rapidly, quantitatively, and accurately detect contaminant fungal and bacterial species in samples regardless of their culturability or viability.

Advantage of PCR Analysis

PCR technology not only overcomes the time-consuming process using conventional culture and microscopic analysis but also provides a sensitive, accurate and reliable analysis that you can count on. Furthermore, you can collect air

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samples in large air volume and for many hours. This will avoid the "snap shot" deficiency of most microbial air sampling of one to ten minutes. This also increases the quantitation sensitivity of the samples.

Following are the advantages of using QPCR analysis.

- Quick turnaround time (2-8 hours)
- Accurate fungal and bacterial identification
- Reproducible results
- Hours of air sampling
- Detection of fungal spores whether they are viable or not
- Quantitative and qualitative results
- Excellent detection and quantitation sensitivity

Users must be aware of the followings when considering using QPCR.

- Requests must include species
- QPCR detects only those species prescribed and will not detect those not on the prescribed list
- DNA does not cause infections if infectious agents are sought

Table 1. Comparisons of the spore counting method, the culturable method and the QPCR analysis.

	Spore Counting	*Culturable*	*QPCR*
Sample time (air samples)	Short duration – a few minutes.	Short duration – a few minutes.	Longer duration – hours to days.
Qualitative results: ID	Presumptive at best, varies depending on lab and its staff.	Good identification, lab dependent.	Precise, over 95% confidence.
Quantitative results	Subject to individual analyst and lab variations; wide statistical deviations.	Actual spore or bacterial counts always underestimated due to viability, dormancy and limitations of media used.	Accurate, based on calibrators/standards; low detection and quantitation limits.
Authority and protocol	No standardized protocol.	Conventional methods used in microbiology or mycology labs.	USEPA patented technology with specific protocol.
Human factors	Subject to analyst's educational background, training, human errors and bias.	Subject to analyst's educational background, training, human error and bias.	Less likely to be subject to human error and bias if analysts are properly trained and supervised.

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Table 2. Fungal spore identification based on spore morphology alone is presumptive. Many genera and species of fungi that produce spores are morphologically very difficult to distinguish or differentiate.

Fungal spore names	Some fungal groups, genera and species which may produce similar spore types
Alternaria	**Alternaria, Mystrospriella, Phoma glomerata, P. pomorum, Ulocladium**
Asp/Pen-like	*Aspergillus, Aureobasidium, Penicillium, Trichoderma, Absidia, Acremonium, Aphanocladium, Beauveria, Chromelosporium, Cladosporium* (young spores), *Phialophora, Gliocladium, Metarrhizium, Monocillium, Mortierella, Mucor, Paecilomyces, Thysanophora, Torulomyces, Verticillium,* amerospores, ascospores, basidiospores, yeasts
Aureobasidium	*Exophiala, Phialophora, Aspergillus, Penicillium*, many other fungi, and yeasts
Botrytis	*Botrytis*
Cercospora	*Cercospora, ascospores of Balansio, Cochliobolus,* and *Gaeumannomyces*
Chaetomium	*Chaetomium, Chaetomidium*
Cladosporium	*Cladosporium, Cladophialophora, Exophiala, Fulvia, Gonatobotryum, Hormoconis (Amorphotheca), Hyalodendron, Mycovellosiella, Periconiella, Phaeoramularia, Septonema, Stenella*
Curvularia	***Curvularia***
Drechslera/Bipolaris	*Drechslera/Bipolaris, Corynespora*
Epicoccum	***Epicoccum***
Fusarium	*Cylindrocarpon, Acremonium, Gliocladium, Microdontium, Monographella*
Ganoderma	***Ganoderma***
Nigrospora	*Nigrospora, Paathramaya*
Pithomyces	*Pithomyces, Ulocladium*
Rhizopus	*Rhizopus*
Scopulariopsis	***Scopulariopsis, Doratomyces, Trichurus***
Spegazzinia	*Spegazzinia*
Stachybotrys	*Stachybotrys, Memnoniella, Gliomastix, Periconia*
Stemphylium	*Stemphylium, Monodictys*
Torula herbarum	*Torula herbarum, Dendryphion, Dwayabeeja*
Ulocladium	*Ulocladium, Alternaria, Monodictys, Pithomyces*
amerospores	amerospores, single-celled spores (with less than 15:1 ratio), many spore types
ascospores	Spores of ascomycetes, amerospores, basidiospores
basidiospores	Spores of basidiomycetes, amerospores, ascospores

Comparing Results Derived From Spore Counting, Culturable, And QPCR Methods

Comparison of results derived from spore counting, culturable and QPCR methods is often requested because laboratories report different units for each method. It is important for the users of the laboartory services to have a basic understanding of how the units are derived.

Results derived from spore counting, culturable, and QPCR methods may be reported in different units. Spore count results may be presented as spores or structures, while culturable results are consistently reported in colony forming units (CFU's). QPCR results have been reported as templates (of DNA), spores, cells, spore-equilvalents or genome-equivalents. There has been no general concensus in reported units for either spore counting or QPCR results. Therefore, it may be different and difficult to compare or correlate results derived from the different methods.

Spore counting may include fungal structures other than spores. For exmaple, hyphal and mycelial fragments, fungal hairs, and conidiophores are important, and are usually counted and included in the results. Therefore, the unit "structures" is a better reflection of what are included. The culturable method counts colonies that are usually derived from spores (particularly for air samples) and occasionally from viable hyphal or mycelial fragments (from bulk or wipe samples of active fungal colonies). The standard unit of use is CFU's irregarless of the origin (whether spores, hyphal or mycelial fragments) of the colonies. Because QPCR is a relatively new method, the unit of use is not consistent. Because

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QPCR detects and quantifies DNA, templates of DNA or genome-equilvalents may be a better unit to use. However, these units have no direct relationship with fungal spores or structures at all. Fungal spores may be multicellular, multinuclear, or both. Spores within the same species may contain a different number of cells (example Alternaria alternata). An argument for using cells as the unit is also plausible since cell is the basic unit of spore, hyphal or mycelial fragments. Because spores are usually used as calibrators or standards in the QPCR method, it is reasonable to use spores as the unit, provided the spore calibrators are pure and without contamination of hyphal or mycelial fragments. In reports issued by EMLab P&K, spore-equilvalent is the unit of choice.

Although no direct comparison among results of spore counting, culturable, and QPCR methods is possible at this time, the pattern of the results should be similar or identical, if the laboratories are qualified and competent.

For bacteria, the same approach is used even though spore counting is not applicable to bacterial cells or spores. Individual bacterial cells and spores are usually too small and indiscernible under the compound microscope at the highest magnifications.

How To Select Your Species for QPCR Analysis

Some fungi are good to excellent indicators of water-damaged environments and materials (see PCR 01 below). In situations where fungal contaminants are well speciated and known, they are identified and selected for QPCR analysis. Occasionally, specific organisms are targeted because of available clinical and medical information (example, diagosis of Legionaire's disease or aspergillosis). Those who have limited experience with mould assessment may want to discuss this with the laboratory of their choice.

The following packages are specially designed to look for marker or signature fungi. Different combinations may be selected for specific purposes depending on the needs of each case.

PCR01

In this package is a **broad coverage of 23 fungal species that may be found in a water-damaged environment**.

Acremonium strictum
Alternaria alternata
Aspergillus flavus/oryzae
Aspergillus fumigatus
Aspergillus niger
Aspergillus ochraceus
Aspergillus sydowii
Aspergillus ustus
Aspergillus versicolor
*Eurotium (Asp.) amstelodami**
Chaetomium globosum
Cladosporium cladosporioides
Memnoniella echinata
Paecilomyces variotii
Penicillium aurantiogriseum
Penicillium brevicompactum
Penicillium chrysogenum
Pencillium purpurogeum
Pencillium variabile
Scopulariopsis brevicaulis/fusca
Stachybotrys chartarum
Trichoderma viride/koningii
Ulocladium botrytis

PCR02

This package covers **15 fungal species found in water-damaged environments**.

Acremonium strictum
Aspergillus fumigatus
Aspergillus niger
Aspergillus sydowii
Aspergillus ustus
Aspergillus versicolor
Chaetomium globosum
Memnoniella echinata
Penicillium aurantiogriseum
Penicillium brevicompactum
Penicillium chrysogenum
Pencillium purpurogeum
Pencillium variabile
Stachybotrys chartarum
Ulocladium botrytis

PCR03

This package is designed for detecting the signature fungal species that are associated with **water damage**.

Acremonium strictum
Aspergillus versicolor
Chaetomium globosum
Memnoniella echinata
Penicillium aurantiogriseum
Penicillium chrysogenum
Stachybotrys chartarum
Ulocladium botrytis

PCR04

This package is specially designed for detecting **important indoor *Aspergillus* species**.

Aspergillus flavus/oryzae
Aspergillus fumigatus
Aspergillus niger
Aspergillus ochraceus
Aspergillus sydowii
Aspergillus ustus
Aspergillus versicolor
*Eurotium (Asp.) amstelodami**

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PCR05

This package is specially designed for detecting important ***Aspergillus* species and *Penicillium* species** in the indoor environment.

Aspergillus ochraceus
Aspergillus sydowii
Aspergillus ustus
Aspergillus versicolor
Penicillium aurantiogriseum
Penicillium brevicompactum
Penicillium chrysogenum
Penicillium variabile

PCR06

Analysis for a single specific fungus or a combination from the list below can also be done.
The following fungi are currently available for real time PCR analysis.

Acremonium strictum
Alternaria alternata
Aspergillus flavus/oryzae
Aspergillus fumigatus
Aspergillus niger
Aspergillus ochraceus
Aspergillus penicillioides
Aspergillus restrictus/caesillus/conicus
Aspergillus sydowii
Aspergillus ustus
Aspergillus versicolor
*Eurotium (Asp.) amstelodami**
Chaetomium globosum
Cladosporium cladosporioides
Memnoniella echinata
Paecilomyces variotii
Penicillium aurantiogriseum
Penicillium brevicompactum
Penicillium chrysogenum
Penicillium citrinum
Pencillium corylophilum
Penicillium expansum
Penicillium purpurogeum
Penicillium roquefortii
Penicillium variabile
Scopulariopsis brevicaulis/fusca
Stachybotrys chartarum
Trichoderma viride/koningii
Ulocladium botrytis
Wallemia sebi

PCR07

Analysis for *Legionella pneumophila*, *L. micdadei* and *L. maceachernii* from environmental samples is available.

PCR10

Wood decay dry rot detection and speciation: *Poria (Meruliporia) incrassata*, *Serpula lacrimans*, and *Serpula himantioides*

* *Eurotium* (*Aspergillus*) group includes *Eurotium amstelodami*, *E. herbariorum*, *E. chevalieri*, *E. rubrum*, and *E. repens*.

<u>When And How To Use QPCR In A Microbiological Assessment In The Indoor Environment</u>

QPCR is a new tool in the microbiological assessment in the indoor environment. It may be used in conjunction with other sampling and testing methodology or independently. Because it is very specific and precise (not a screening tool), it is very important to have a thorough understanding of a project before deciding whether QPCR or other tools be selected.

Several of the combinations listed above cover 75 to 90% of common indicator fungi found in the water damaged environment. Special situations may select target organisms. For example, Aspergillus fumigatus, A. niger, A. ochraceus, A. flavus, and A. terreus may be selected if the sampling and testing is for hospitals and health care facilities where aspergillosis is of major concern. QPCR testing for Legionella penumophila and L. micdadei may be requested for water samples collected from cooling towers and hot water sources. Legionella penumophila is estimated to cause more than 80% of legionellosis. Between Legionella penumophila and L. micdadei the percentage is even higher.

QPCR has also been used forsenically or for quality assurance purposes. Air samples and fine dust samples have been sampled and tested with QPCR to determine if a mould remediation has been successfully completed. Fine dusts can be collected from personal items and contents from a mould contamination to determine the extend of contamination and whether cleaning has been effective. Chips of encapsulant on surfaces after a mould remediation have been sampled and tested with QPCR to determine whther an encapsulant is used to cover and mask mould spore contaminated dust or not.

Results of QPCR can also be used, as a quality assurance check, to test laboratory results of spore counts or culturable samples.

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QPCR can also be used to detect and identify fungi and bacteria that are not easily identifyable. For example, a primer-probe set has been designed for the detection of three wood decay fungi: *Poria (Meruliporia) incrassata*, *Serpula lacrimans*, and *Serpula himantioides*. These fungi do not frequently produce fruiting bodies for proper identification. It is very difficult or impossible to identify these wood decay fungi based on their vegetative growth. QPCR testing can solve the problem in a few hours.

Sampling Protocol for QPCR Analysis

A. Liquid Samples

a) Obtain sterile 15mL screw-cap tube for sampling. Obtain an extra tube as a field blank.
b) Keep the sampling tube closed until it is used. Fill up the tube with water sample.
c) Tightly cap the bottles. Make sure that water will not leak out during shipping and transporting.
d) Ship the samples on ice.

B. Bulk Dust Samples

a) Obtain 3-piece 37-mm cassettes, preloaded with 0.45 µm pore-size filters for sampling. Obtain an extra as a field blank.
b) A sufficient amount of dust is required for analysis, preferably 0.1 g or more.
c) Use clean masking tape to mark a surface area of your interest. A 12" x 12" area is suggested. However, you may increase the area or composite your samples from several 4" x 4" or 6" x 6" areas. Connect your filter cassette to the high volume pump with clean Tygon tubing of approximately 2-3 feet. Remove the top cover (open face) of the cassette and turn on the pump to sample by vacuuming. Sample within the marked area by vacuuming horizontally, vertically, and diagonally. When you finish sampling, turn the cassette up, cover it, and turn off the pump. Number your sample and record it on your chain of custody.
d) Other sampling devices, such as a vacuum cleaner interceptor bag, are also acceptable.

C. Air Samples

a) Obtain 3-piece 37-mm cassettes, preloaded with 0.45 µm pore-size filters, for sampling. Obtain an extra as a field blank.
b) Sufficient air volume is required for analysis, a minimum of 600 L (based on 4-6 L/min for 120-180 min) or more (up to 3,000 L) is suggested. Close face sampling is recommended.

D. Wipe/Swab Samples

a) Dip sterile cotton swab tip into sterile distilled water tube and moisten the cotton. Make sure the cotton is not too wet.
b) Use the wet cotton swab to wipe the suspected surface area. Label each sample clearly and record on COC.
c) Place the swab in a clean zip-lock bag or a sterile container.
d) Ship the samples on ice.

E. Bulk Samples

a) Place the selected sample in a clean zip-lock bag or into a sterile container. Please provide special instructions if needed on your Chain of custody. Clearly label each bag.
b) You can also wipe the suspected area of bulk samples with the use of the sterile wet cotton swab as described in D wipe/swab samples. Record the area on the COC. Send them as wipe/swab samples as described above
c) If the sample is wet, ship it on ice.

F. Chain of Custody (COC)

a) Write the sample number on the container/cassettes and on COC sheet. Use a short distinctive number for each sample.
b) Complete all sample information on COC sheet, such as sampling date(s), air volume, time, location, your project or job number, purchase order number(s) for the job, your name, company name, phone and fax number, and e-mail address. Keep your own record and send a copy with samples to the laboratory.

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G. Shipping Samples

a) For liquid samples, place containers in a clean plastic bag then put into an insulated box with blue ice or reusable ice packs to maintain the temperature between 2 to 8 °C. Do not use ice cubes or dry ice. Stuff the box with foam chips to provide sufficient cushion and seal the box securely for shipping.
b) Place air and dust samples in plastic bags and then in a cardboard box. Securely seal and tape the bag for shipping.
c) Ship liquid and wet samples on ice.
d) Send samples to the laboratory by overnight express carrier. Call and inform the laboratory. Take holidays into consideration.

References:

1. Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A., 1988, Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239: 487-491.
2. Haugland, R.A., Vesper, S.J., and Wymer, L.J., 1999, Quantitative measurement of *Stachybotrys chartarum* conidia using real time detection of PCR products with the TaqMan fluorogenic probe system. *Molecular and Cellular Porbes*, 13:329-340.
3. Edel, V., 2000, Polymerase Chain Reaction in Mycology: an Overview, in Applications of PCR in Mycology, edited by Bridge, P. D., Arora, D. K., Reddy, C. A., and Elander, R. P., *CAB International Publishing*.
4. Vesper, S., Dearborn, D. G., Yike, I., Allan, T., Sobolewski, J., Hinkley, S. F., Jarvis, B. B., and Haugland, R. A. (2000). Evaluation of *Stachybotrys chartarum* in the house of an infant with pulmonary hemorrhage: quantitative assessment before, during, and after remediation. *Journal of Urban Health* 77, 68-85.
5. Haugland, R. A., Brinkman, N. and Vesper, S. J. (2002). Evaluation of rapid DNA extraction methods for the quantitative detection of fungi using real-time PCR analysis. J. Microbiol. Methods. 50, 319-323.

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