# A duplex qPCR-application for determining Gram-positive and Gram-negative bacteria from environmental samples

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## Introduction

Microbes are present everywhere in the environment and humans continuosly interact with them. These interactions can affect the human health in many ways; microbes can cause infectious diseases and induce the development of inflammatory and autoimmune diseases, such as farmer's lung (4), however, in last years it has been postulated that exposure to microbes in early life can prevent the development of allergy (1). Nowadays people spend most of their lives in indoor environments, and are exposed to microbes present there. Therefore, it is important to study the microbes in indoor environments and develop accurate methods for the exposure assessment. At present, endotoxin activity and amount of muramic acid is used for assessment of Gram– negative and –positive bacteria in epidemiological studies (2, 5). These methods, however, are somewhat unspecific.

Aim of this research is to develop a new quantitative multiplex real-time PCR –method to detect and quantify Gram-positive and Gram-negative bacteria in samples collected from house dust, indoor air and building materials.

## Materials and methods

Primers and dual-labeled TaqMan probes were designed for 16S rRNA-gene location based on sequences retrieved from NCBI Gene Bank. Multiple sequences from 20 Gram-positive and 19 Gram-negative bacterial genera were aligned using the Vector NTI Align X software. One set of primers and two probes were designed so that both probes matched within the same 144 bp amplification product (Table 1.). Specificity of the primers and probes, also their secondary structures were checked.

| Oligonucleotide | bp | GC % | Tm °C | 5' modifi-cation | 3' modifi-cation |
|---|---|---|---|---|---|
| Gram-F | 19 | 58 | 59.5 | - | - |
| Gram-R | 22 | 55 | 59.2 | - | - |
| Grampos-P | 29 | 45 | 68.3 | 6-FAM | BHQ1 |
| Gramneg-P | 27 | 52 | 69.3 | Cy5 | BHQ3 |

**Table 1.** Physicochemical properties of primers and probes.

The function and specificity of the qPCR -assay was tested with single and duplex probes and different bacterial and fungal strains listed in Table 2. DNA-extractions were made from 100 µl of microbial suspensions with Sigma-Aldrich's Gen-Elute™ Plant Genomic DNA Miniprep Kit including 1 minute of beat beating. Finnzymes's Dynamo Probe qPCR-kit with 400 nM of primers, 100 nM of probes and 2 µl of template in a 25µl volume was used. 35 cycles of 94°C 15 sec and 64°C 60 sec were run. All reactions were pipetted with Corbett CAS-1200 pipetting robot and run with Corbett Rotor-Gene 3000.

| Genera – Species | Strains | Gram stain | qPCR Gram+ | qPCR Gram- |
|---|---|---|---|---|
| *Bacillus cereus* | ATCC 11778 | + | ++ | – |
| *Staphylococcus aureus* | ATCC 25923 | + | ++ | – |
| *Mycobacterium mucogenicum* | DSM 44625 | + | – | – |
| *Streptomyces californicus* | Strain A4 | + | – | + |
| *Streptomyces spp.* | 10 strains | + | + | ++ |
| *Arthrobacter globiformis* | 106.028 | + | ++ | – |
| *Promicromonospora citrea* | 106.031 | + | + | – |
| *Micromonospora aurantiace* | 106.029 | + | + | – |
| *Amycolatopsis sacchari* | 106.030 | + | + | – |
| *Nocardia carnea* | 106.036 | + | + | + |
| *Pseudonocardia sp.* | 106.035 | + | + | + |
| *Rhodococcus rhodochromes* | 106.033 | + | + | ++ |
| *Escherichia coli* | ATCC 25922 | – | – | ++ |
| *Pseudomonas aeruginosa* | Strain 2179 | – | – | ++ |
| *Enterobacter aerogenes* | ATCC 13048 | – | – | ++ |
| *Fungal strains* | 15 strains | n.d. | – | – |

**Table 2.** Bacterial and fungal strains, that were used for testing the method.

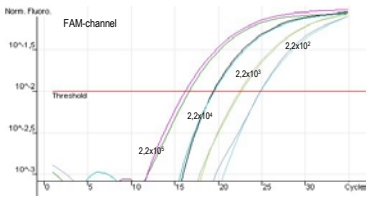


**Figure 1.** *Bacillus cereus* (ATCC 11778) standards in FAM-channel, where Gram-positive probe is detected.

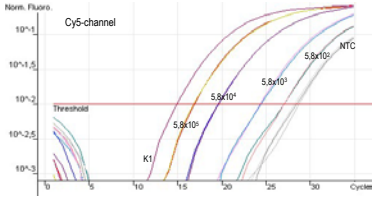


**Figure 3.** *Pseudomonas aeruginosa* (strain 2179) standards in Cy5-channel, where Gram-negative probe is detected.

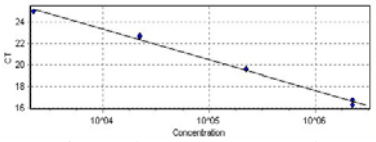


**Figure 2.** Standard curve of *Bacillus cereus* standards, controls and NTC's. R = 0.99709, $R^2$ = 0.99418, efficiency 1.24.

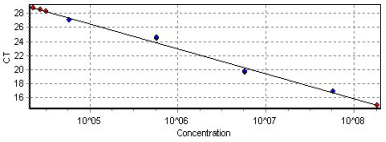


**Figure 4.** Standard curve of *Pseudomonas aeruginosa* standards, controls and NTC's. R = 0.99290, $R^2$ = 0.98584, efficiency 0.92.

## Results and discussion

Sensitivity of the assay was not equal for both probes, due to competition between the probes since they bind in same gene location in 16S rRNA. Reaction efficiency was slightly better for the Gram-positive probe than the Gram-negative (100 % vs. 92 %). Gram-negative probe detected all *Streptomyces* species and some *Actinobacteria* better than Gram-positive probe, because of 1-2 bp difference in 5' end of the probe. *Mycobacterium mucogenicum* remained totally undetected due to a 1 bp difference in the probe. All Gram-negative bacteria were determined successfully (Table 3). Also all different fungal strains gave negative results.

The $C_T$ values for non-template control reactions in the Gram-negative assay were consistently between 26.38 and 29.45 (about 130-180 copies/reaction), whereas in the Gram-positive assay they remained undetected. This is most probably due to background coming from the mastermix, since the used DNA-polymerase is produced in *E. coli*. TaqMan chemistry is so sensitive method that both recombinant and native *Taq DNA*-polymerases, regardless of the source, give false-positive results with similar $C_T$ values (6).The background could be avoided by using *DNase* I –treatment for DNA-polymerase before run, so that all minor amounts of contaminants would be degraded (6).

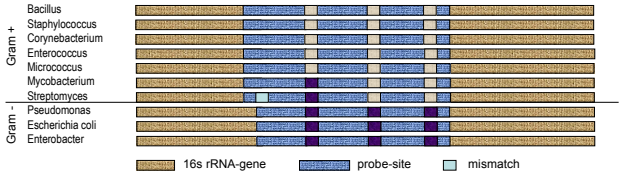


**Table 3.** Illustration of sequence alignment of the area where probes have been designed .

## Conclusions

- The developed assay detects efficiently and specifically both Gram-negative and –positive bacteria, with the exception of some actinobacteria
- Master-mix (recombinant DNA-polymerase produced in *E.coli*) gives a considerable amount of background in the Gram-negative assay, increasing the detection limit of Gram-negative bacteria up to 200 copies / reaction

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## References

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