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# Improvement of phylum- and class-specific primers for real-time PCR quantification of bacterial taxa

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ABSTRACT

Mapping the distribution of phylogenetically distinct bacteria in natural environments is of primary importance to an understanding of ecological dynamics. Here we present a quantitative PCR (qPCR) assay for the analysis of higher taxa composition in natural communities that advances previously available methods by allowing quantification of several taxa during the same qPCR run. Existing primers targeting the 16S rRNA gene specific for Firmicutes, Actinobacteria, Bacteroidetes and for the α and γ subdivisions of the Proteobacteria were improved by largely increasing the coverage of the taxon they target without diminishing their specificity. The qPCR assay was validated *in vitro* testing artificial mixtures of 16S rRNA sequences and used to characterise the composition of natural communities developing in young marine biofilms. The possible contribution of the proposed technique in revealing ecological dynamics affecting higher bacterial taxa is discussed.



## 1. Introduction

The taxonomic level at which bacteria uniformly respond to environmental parameters can vary greatly, and coherent responses have been recorded from the sub-species (Koeppel et al., 2008; Ward et al., 2006) to the phylum level (Philippot et al., 2009). It can be argued that the number of adaptive features conserved in broad taxonomic divisions is limited; and genes, more than taxa, are responsible for bacterial distribution. However, the existence of genes that are unique to all members of high taxonomic groups such as phyla or classes (Gao et al., 2009; Gupta and Lorenzini, 2007; Gupta and Mok, 2007) suggests that ecological consistency may also be found in deep branches of the tree of life. Improving our capacity to characterise the distribution of higher bacterial taxa can contribute to reveal the factors underlying this coherence (Philippot et al., 2010).

A variety of methods are available to quantify microbial populations. Although ultra-high throughput sequencing methods now dwarf classical PCR-based approaches (Sogin et al., 2006), quantitative PCR (qPCR) provides a cheap alternative to determine the abundance of target DNA sequences. Using this technique it is possible to follow the kinetics of the reaction in progress, thus eliminating some of the bias which characterises end-point PCR (Sipos et al., 2007; v. Wintzingerode et al., 1997). Two alternative approaches exist to resolve gene expression from qPCR data; relative and absolute quantification. When implementing the first approach, the abundance of different genes is compared by calculating the efficiency of each primer pair used, and by normalising the quantification to a control gene (Bustin et al., 2005; Bustin and Nolan, 2004). Alternatively, absolute quantification requires the determination of a standard curve, constructed by amplifying known amounts of target DNA, which is then used to extrapolate the abundance of the target gene (Pfaffl and Hageleit, 2001; Rutledge and Côté, 2003). In order to develop the primers and qPCR assay described here, a relative quantification approach was undertaken.

qPCR has been used to quantify 16S rRNA genes for a decade (Suzuki et al., 2000) and its applicability to the field of microbial ecology has recently been reviewed (Smith and Osborn, 2009). Among the limitations of qPCR is the 16S rRNA gene offers limited scope to design primers (for SYBR green detection, as opposed to probes used in TaqMan assays) that are specific to broad bacterial taxa. Furthermore, the exponential increase of 16S rRNA sequences submitted to public databases makes it necessary to periodically re-evaluate existing primers. Nevertheless, the advantages of using qPCR, such as the utilisation of small amount of template, high sensitivity, large throughput processing and affordable cost, make it the technique of choice for many investigations aiming to characterise microbial communities.

Fierer et al. (2005) were the first to implement qPCR to determine higher-taxa composition in natural communities, and their assay has had a major role in revealing unexpected ecological dynamics among soil bacteria (Philippot et al., 2009; Philippot et al., 2011; Wessén et al., 2010). Although their approach represented an important improvement in respect to previously available techniques, the qPCR assay proposed by Fierer et al. is characterised by two limitations.

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Firstly, the method relies on the comparison of threshold data (Ct) from 9 separate qPCR runs, increasing both the time to generate data and the probability of introducing bias due to undetected temperature fluctuation in each thermocycle. Secondly, some of the primers used matched only a fraction of the target group (*e.g.* only 25% of deposited Firmicutes' 16S rRNA sequences possess the primer complementary sequence), posing a limit to the interpretation of qPCR data from a broad taxonomic perspective.

The aim of this study was to improve both the specificity (*i.e.* the ability to prevent amplification of un-targeted sequences) and the universality (*i.e.* the capacity of amplifying 'all' members of the desired taxon) of primers targeting the Bacteroidetes, Firmicutes, Actinobacteria and the α and γ subdivision of Proteobacteria. Furthermore, through a stepwise process of single nucleotide modification, primers were developed to maximise their specificity at the same annealing temperature, allowing the timely generation of data from a single qPCR assay.

## 2. Materials and methods

### 2.1. Primer design

In order to reveal phylum- and class-specific conserved regions, between 20 and 30 16S rRNA sequences for each taxonomic group were randomly downloaded from the Ribosomal Database Project II (Maidak et al., 2001) and grouped into fasta files. Sequences from each taxon were clustered using ClustalX (Thompson et al., 1997) and consensus sequences obtained using BioEdit (Hall, 1999). The alignments of these consensus sequences (Fig. S1) were visually inspected to improve existing primers and to design new ones. Primers were assessed in silico using the tool 'probe match' from the RDPII with data set options: strain 'both'; source 'both'; size>1200; quality 'good' (Maidak et al., 2001). The annealing temperature that maximised primer specificity *in vitro* was determined by using target and non-target DNA as a template in parallel gradient PCR reactions with annealing ranging from 55 °C to 68 ° C. The PCR thermocycle was composed of one initial denaturing step of 5 min at 95 °C, 30 cycles of 95 ° C for 15 s, gradient annealing for 15 s and 72 °C for 30 s, and a final elongation step at 72 °C for 5 min. Every PCR reaction contained 0.05 units/μl of Taq polymerase (Sigma Aldrich), 0.2 mM of each dNTP, 0.4 μM of each primer, 1× buffer, ~10 ng of DNA and water to 25 μl. Genomic DNA from 30 bacterial isolates that were genotyped by partial 16S rRNA sequencing (EMBL accession numbers from FN433050 to FN433079) was used as a template. A minimum of 3 target and 5 non-target gDNA was used to test each primer pair and *in vitro* specificity was claimed when only target DNA was amplified (as determined by standard gel electrophoresis). Primers were chosen and further modified to allow the use of the same annealing temperature for every pair designed. All primers tested are reported in Table S1.

### 2.2. Quantitative PCR optimization of thermocycle and reaction condition

To investigate the effect that the change of polymerase and reaction buffer had on the optimal annealing temperature, a first round of gradient qPCR (annealing at 58.9, 59.3, 59.8, 60.5, 60.9, 61.2, 61.5 and 62.5 °C) was performed for all taxon-specific pairs. Reactions were performed in sealed 96 well plates using a Chromo4 MJ-Research thermocycler (BioRad) and analysed with Opticon Monitor 3 software. All qPCR reactions contained 12.5 μl of 2× Faststart SYBR green (Roche Diagnostics Ltd), 10 μl of primers (final concentration 0.3 μM), 2.5 μl of the DNA template (equilibrated to 5 ng). Primer specificity was inferred from the shift of the threshold cycle (Ct), obtained by amplifying target compared to non-target sequences. It was found that 61.5 °C maximised specificity of all pairs and the ultimate qPCR protocol implemented is reported in Fig. 1. In order to rule out the formation of PCR artefacts, a melting curve was generated at this point by monitoring SYBR green fluorescence in the temperature ramp 60 to 95 °C with an increase of 0.5 ° C and a hold of 1″.

**TAXON-SPECIFIC PRIMER PAIRS USED**

| Target group | Name | Sequence |
|---|---|---|
| Universal | 926F | AAACTCAAAKGAATTGACGG |
| | 1062R | CTCACRRCACGAGCTGAC |
| α-Proteobacteria | α682F | CIAGTGTAGAGGTGAAATT |
| | 908αR | CCCCGTCAATTCCTTTGAGTT |
| γ-Proteobacteria | 1080γF | TCGTCAGCTCGTGTYGTGA |
| | γ1202R | CGTAAGGGCCATGATG |
| Bacteroidetes | 798cfbF | CRAACAGGATTAGATACCCT |
| | cfb967R | GGTAAGGTTCCTCGCGTAT |
| Firmicutes | 928F-Firm | TGAAACTYAAAGGAATTGACG |
| | 1040FirmR | ACCATGCACCACCTGTC |
| Actinobacteria | Act920F3 | TACGGCCGCAAGGCTA |
| | Act1200R | TCRTCCCCACCTTCCTCCG |

**PCR REACTION COMPONENTS**

2x SYBR green buffer containing taq polymerase
0.3 μM of each primer
DNA template
Water to 25 μl

**PCR THERMOCYCLE**

| | | |
|---|---|---|
| initial denaturing step | 95° C | for 5′ |
| 30 cycles | 95° C | for 15″ |
| | 61.5° C | for 15″ |
| | 72° C | for 20″ |
| final elongation step | 72° C | for 5′ |

**Fig. 1.** Taxon-specific qPCR protocol. The qPCR assay was optimised using Chromo4 MJ-Research thermocycler (BioRad) and Faststart SYBR green (Roche Diagnostics Ltd).

### 2.3. Primer efficiencies

The amplification efficiency of each primer pair was determined by standard procedure; making dilution series of target DNA, calculating a linear regression based on the Ct data points and inferring the efficiency from the slope of the line. Serial dilutions 1, 1:4, 1:16, 1:64 and 1:256 were utilised. Technical triplicates were tested for each dilution point and primer pair. A Non-Template Control (NTC) was included in each assay to confirm that the Ct value generated by the lowest concentrated DNA was not an artefact. Finally, Ct data were uploaded to an Excel spreadsheet generated by the authors and resulting efficiency graphs are given in Fig. S2.