Quantitative Analysis of Multi-Species Oral Biofilms by TaqMan Real-Time PCR

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Oral infectious diseases, including dental caries, various forms of periodontitis and oral malodor, are not caused by a single pathogen. The etiology of these diseases is known to be associated with bacterial accumulation and plaque composition on the hard and soft tissues of the oral cavity. Therefore, the quantitative, as well as qualitative, analysis of the microorganisms present in oral biofilms, namely dental plaque, subgingival plaque and tongue debris, is important for diagnosis and rational treatment decisions. The quantitative microbial analysis of oral multi-species biofilms also provides useful information for establishing the etiology of oral infectious diseases. Recently, a 5′ fluorogenic, nuclease-based, real-time polymerase chain reaction (PCR) technique has been increasingly employed for the quantitative microbial assessment of the human oral cavity. We review the development and use of TaqMan real-time PCR for quantifying oral bacteria, its role in the diagnosis of oral infectious diseases and their microbial etiology.

Keywords: TaqMan real-time PCR; Quantification; Oral bacteria; Biofilm

The human oral cavity contains more than 500 bacterial species that interact with each other and with their host tissues.1,2 These complex interactions result in the formation of microbial biofilms as dental plaque, subgingival plaque and tongue surface debris leading to dental caries, periodontal disease and oral malodor. Recent studies have suggested that several oral bacteria are opportunistic pathogens that cause systemic disease, such as bacterial endocarditis,3-5 aspiration pneumonia,6 pre-term low birth weight7 and cardiovascular disease.8 Consequently, it is important to know the microbial composition of the oral cavity for the diagnosis and rational treatment of these diseases. Among the various procedures used to detect oral bacteria, such as microbial culture, immunological assays, enzymatic methods and molecular biology, a polymerase chain reaction (PCR)-based diagnostic technique has become a powerful and increasingly popular tool due to its rapidity, sensitivity and specificity.9 However, it is difficult to quantify accurately the number of bacteria using conventional PCR because the reactions are evaluated after gene amplification is completed.10 In addition, the quantification of PCR products can be affected by contamination, interfering substances and unequal amounts of collected samples.11 PCR is difficult to use for routine diagnosis due to the time required for sample handling and post-PCR analysis.

Recently, a real-time PCR assay using a TaqMan probe (a fluorescent DNA probe based on the 5′ to 3′ exonuclease activity of Taq polymerase) has been developed for quantitative DNA analysis.12 The oligonucleotide probe, with a reporter fluorescent dye attached to its 5′ end and a quencher dye attached to its 3′ end, hybridizes to the target gene. During PCR amplification, the quencher dye is cleaved by the 5′ nuclease activity of Taq polymerase resulting in the accumulation of reporter fluorescence. The release of the fluorescent dye during amplification allows for rapid detection and quantification of DNA.13 Compared with other methods for quantifying oral pathogens, including flow cytometry14 and DNA-DNA hybridization,15 the TaqMan real-time PCR assay has advantages in terms of sensitivity and rapidity. Furthermore, by using universal primers and probes, the total number of bacteria and the proportion of target microorganisms in single clinical specimens can be calculated. The SYBR Green I assay can also be used with another type of real-time PCR. SYBR Green I dye binds specifically to double-stranded DNA and
enables the detection of products accumulating during the PCR.16 This assay has the advantage of being simple and less expensive but does not use a probe specific for the nucleotide sequence of the target microorganism. On the other hand, the high specificity of the primers and probes in TaqMan PCR enable it to distinguish target pathogens from the numerous closely related species in the oral cavity. Quantitative analysis is essential for monitoring therapeutic efficacy in clinical trials, gaining insight into oral biofilms and diagnosis of oral infectious diseases. This review describes the development and application of TaqMan PCR for determining the quantitative microbial composition of multi-species biofilms in the human oral cavity.

Development of TaqMan Real-Time PCR for the Detection of Oral Bacteria

The use of TaqMan technology for the quantitative analysis of oral bacteria is a recent phenomenon; its development is outlined in Table 1. Many oligonucleotide primers and probes of oral bacteria is a recent phenomenon; its development is outlined in Table 1. Many oligonucleotide primers and probes for target microorganisms are derived from 16S ribosomal RNA (rRNA) gene sequences, although some are based on species-specific gene sequences associated with virulence factors.17-19 As each bacterium may contain a variable number of 16S rRNA sequences, 16S rRNA-derived primers and probes may overestimate the number of target microorganisms. Primers and probes based on a single copy gene sequence would provide a more accurate measure of the absolute number of bacteria. The amplification and detection are generally carried out with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The oligonucleotide primers and probe sequences designed for this system can be used with other systems, such as the LightCycler (Roche Diagnostics GmbH, Penzberg, Germany) (see Authors’ Reply),20 but a new standard curve should be generated from the amplification plot for each system employed for diagnosis and monitoring.

Specimens are taken from the regions associated with infectious disease: supragingival dental plaque with dental caries,18 subgingival plaque with periodontitis,17,19,21,22 carious dentine with infected pulp or chronic caries23 and tongue surface debris with oral malodor.24,25 In combination with such specimens, we normally use saliva as a PCR template that reflects the overall condition of the oral cavity. In fact, bacteria causing dental caries and periodontitis are more frequently detected in saliva than in the dental plaque.17,18,26

Detection of the Relative Amount of the Target DNA in Clinical Specimens

Porphyromonas gingivalis, a black-pigmented, gram-negative, anaerobic rod, is considered to be the most important pathogen in adult-onset periodontitis27,28 and oral malodor.29 Lyons et al.30 developed a nested, two-step, real-time PCR for comparing the number of P. gingivalis cells with that of the total bacterial cells in subgingival dental plaque. The first amplification targeted conserved sequences in the bacterial 16S and 23S rRNA genes. In the second stage, nested PCRs were used to quantify the number of P. gingivalis cells or the total number of bacterial cells. With previous methods it has been especially difficult to determine the total number of bacteria. This study showed that real-time PCR offers the prospect of determining the number of P. gingivalis and the total number of bacteria in plaque samples directly without culturing. To measure the relative amount of P. gingivalis and Actinobacillus actinomycetemcomitans in subgingival dental plaque and saliva samples, we normalized the number of target DNA copies to that of the 16S rRNA gene using a simplification of the comparative threshold cycle (ΔΔCt) method that was provided in User Bulletin #2 of ABI PRISM 7700 Sequence Detection System by Applied Biosystems.17 The critical threshold cycle (Ct) is defined as the first cycle in which fluorescence is detectable above the background and is inversely proportional to the logarithm of the initial number of template molecules. The fold-difference (N) in the number of the target organism-specific gene copies relative to the number of 16S rRNA gene copies was determined as follows:

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N = 2^{\Delta \Delta Ct} = 2^{(Ct_{\text{target DNA}} - Ct_{16S\text{-rRNA}})}
\]

The ΔΔCt values for the sample and the 16S rRNA were determined by subtracting the average Ct value for the target gene from that of the 16S rRNA gene. The simplified ΔΔCt method provides the required simplicity and rapidity for clinical diagnosis.

Using TaqMan real-time PCR and the simplified ΔΔCt method, we performed quantitative analysis of P. gingivalis to clarify the relationship between the bacterial numbers and the periodontal status.31 Before periodontal treatment, a significant positive correlation between the log-translated number of P. gingivalis and pocket depth was found (y=1.14x – 2.01, r²=0.37, p<0.0001). This indicated that the number of P. gingivalis cells increased approximately 10-fold for each 1 mm increase in pocket depth. Simple regression analysis of the ratio of P. gingivalis bacteria in relation to pocket depth also showed a positive correlation (y=4.69x – 13.4, r²=0.40, p<0.0001), which indicated that the percentage of P. gingivalis cells in the total bacterial cells in subgingival plaque specimens increased 4.69% for each 1 mm increase in pocket depth. Subsequently, changes in pocket depth and the number of P. gingivalis bacteria before and after treatment were monitored. There were significant reductions in both the absolute number (Wilcoxon’s signed-rank test, p<0.01) and the percentage (p<0.001) of P. gingivalis cells before and after periodontal treatment. As there was a significant difference (paired t-test, p<0.0001) in pocket depth before and after treatment, these results suggest that the absolute and relative numbers of P. gingivalis bacteria are closely related to periodontal status and that quantitative analysis of this bacterium is important for evaluating periodontal therapy.
Development of TaqMan Assay for Quantifying Oral Treponemes

In addition to *P. gingivalis*, oral treponemes have been implicated in periodontitis and oral malodor. These helically shaped spirochetes are present in significantly elevated numbers in plaque samples from deep-pocket sites of patients with severe periodontitis. Most oral spirochetes either cannot yet be grown or are extremely difficult to grow in culture because they have lost the ability to synthesize many essential molecules that they normally obtain from their host. Real-time PCR is a valuable tool for detection of such organisms that are difficult to grow in culture. This assay is able to detect and quantify the target organisms directly in oral specimens.

Using TaqMan real-time PCR, we evaluated the absolute and relative numbers of *Treponema denticola* bacteria in 53 subgingival dental plaque samples from nine patients prior to periodontal treatment. Although *T. denticola* is the most-studied spirochete due to its virulence in periodontal disease and is one of the predominant oral treponemes found in the gingival crevice, it is difficult to grow in culture. Simple regression analysis showed a significant positive correlation between the log-transformed number of *T. denticola* cells and pocket depth (y=0.61x + 1.51, r²=0.23, p<0.0001). A significant positive correlation was also observed between the percentage of *T. denticola* bacteria and pocket depth (y=4.90x – 12.60, r²=0.28, p<0.0001). Asai et al. developed a TaqMan real-time PCR assay to detect the absolute number of three oral treponemes, *T. denticola*, *Treponema vincentii* and *Treponema medium* in subgingival plaque. They found that *T. denticola* and *T. medium* were detected in patients with serious periodontal diseases (pocket depths 4 mm to 8 mm), whereas *T. vincentii* was detected mainly in shallow pockets of 4 mm to 5 mm depth.

Previously, dark-field microscopy was used to establish the relationship between number of subgingival spirochetes and severity of periodontitis. Dark-field microscopy is suitable for determining the overall number of spirochetes but is unsuitable for identifying specific spirochete species in clinical specimens due to similarities in shape. These reports demonstrate that TaqMan real-time PCR is suitable for detection and quantification of oral treponemes in cases where identification using culture and visual observation is difficult. In addition, TaqMan PCR is able to differentiate between spirochete species and, therefore, may be useful in studying the etiological role of different oral spirochetes at each stage of periodontitis.

Quantitative Studies Showing the Relationship Between Periodontal Bacteria

Periodontitis is thought to arise from complex microflora consisting of putative periodontopathic bacteria, such as *P. gingivalis*, *T. denticola*, *Tannerella forsythia* (formerly *Bacteroides forsythus*) or *Tannerella forsythensis* and *Fusobacterium* spp. These bacteria have been co-isolated from affected periodontal sites and it has been suggested that periodontitis is initiated by cooperation between the different species. We established a TaqMan real-time PCR assay to quantify the absolute and relative numbers of *T. forsythia* and *Fusobacterium* spp. in clinical specimens and to demonstrate their co-localization in periodontal pockets. Spearman’s rank correlation showed a significant positive correlation between the percentage of each bacterium and the pocket depth of each sample (r=0.48 for *T. forsythia*, and r=0.50 for *Fusobacterium* spp., p<0.001). There was a significant correlation between the proportions of both bacteria in each pocket (r=0.75, p<0.001). Between-subject analysis was performed to avoid cross-contamination of sites; the correlation between the mean proportions of these two bacteria for each subject was likewise very strong (r=0.88, p<0.001). The significant relationship between the two bacteria and pocket depth suggests that both species grow well in deeper periodontal pockets. Furthermore, when *Fusobacterium* spp. and *T. forsythia* were quantified in samples from periodontal pockets of different depths, both bacteria were detected more frequently in samples from periodontal pockets ≥4 mm (χ² test, p<0.001). *T. forsythia* was not detected in subgingival plaque, from where *Fusobacterium* spp. were absent (Fisher’s exact probability test, p<0.001) or in samples from shallow pockets ≤3 mm depth. In fact, *T. forsythia* was not detected at any *Fusobacterium*-negative site (p=0.03) suggesting co-localization between *T. forsythia* and *Fusobacterium* spp. in deep periodontal pockets. These preliminary tests show that real-time PCR may be used to clarify the relationship between oral periodontopathic bacteria in periodontal pockets.

TaqMan Assay for Quantification of Periodontal Bacteria Associated With Oral Malodor

Oral malodor is mostly caused by oral pathology, mainly from malodorous compounds produced by oral bacteria. Major compounds responsible for oral malodor are volatile sulfur compounds (VSCs), including hydrogen sulfide (H₂S), methyl mercaptan (CH₃SH) and dimethyl sulfide (CH₃SCH₃). About 90% of the VSCs in the oral cavity are H₂S and CH₃SH and some periodontal pathogens such as *P. gingivalis*, *Prevotella intermedia*, *T. denticola* and *Fusobacterium nucleatum* generate significant amounts of H₂S and CH₃SH from L-cysteine and L-methionine. Consequently, there has been renewed interest in the relationship between oral malodor and periodontal disease. On the other hand, it has been reported that the main source of odor is usually the tongue microbiota. Tanaka et al. employed TaqMan real-time PCR to measure the number of anaerobes in tongue biofilm samples and examined the association between oral malodor and tongue periodontal pathogens including *P. gingivalis*, *P. intermedia*, *Prevotella nigrescens*, *T. denticola* and *T. forsythia*. Species-specific primer and probe sets were designed from the variable regions of the 16S rRNA gene sequences and a universal primer and probe set was used for quantifying the total proportion of bacterial cells in specimens. Among the VSCs, H₂S

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178 TaqMan PCR for oral biofilms CM&R 2005 : 3 (August)
concentration was strongly correlated with the amount of P. intermedia and P. nigrescens bacteria, whereas CH$_3$SH concentration was strongly correlated with P. gingivalis and P. nigrescens (Spearman’s rank correlation coefficient, $p<0.0001$). The total proportion of these five anaerobes on the tongue dorsa was strongly correlated with total VSC level ($r=0.88$), but only weakly correlated with the degree of organolepsis ($r=0.29$) suggesting that these periodontal pathogens on the tongue dorsa contribute greatly to VSC production. Furthermore, the proportions of P. gingivalis, P. nigrescens and T. forsythia and the total of the five anaerobes on the tongue dorsa were higher in patients with periodontitis than in those without periodontitis. Recently, the importance of oral malodor has been recognized as it carries considerable social stigma in our modern society. Quantification of VSC-producing bacteria is important for diagnosis and therapeutic assessment of oral malodor. Conventional tests, gas chromatography, organoleptic tests and portable sulfide monitors are essential tools for evaluating oral malodor, however, these tests cannot measure any direct correlation between the disease and pathogenic bacteria. In addition to these conventional tools, real-time PCR will support diagnosis of oral malodor pathogens and contribute to control of VSC production.

Quantification of Cariogenic Pathogens and Early Microbial Colonizers in Dental Biofilms

Streptococcus mutans (serotype c, e and f mutants streptococci) and Streptococcus sobrinus (serotype d and g mutants streptococci) are directly involved in formation of human dental caries.$^{50,51}$ The water-insoluble forms of glucan produced from dietary sucrose by S. mutans and S. sobrinus are closely associated with pathogenicity. These glucose polymers provide a structure for aggregation of mutants and other oral streptococci and modify dental biofilm porosity, thus increasing availability of nutrients for continued bacterial metabolism. Consequently, the levels of these cariogenic pathogens in the human oral cavity are predictive of caries activity and the transmission risk of mutants streptococci. Although various methods have been developed to identify mutants streptococci, a quantitative assay as an index of caries activity has not yet been established. We have described a TaqMan real-time PCR assay for quantitative detection of S. mutans and S. sobrinus in saliva and dental plaque.$^{18}$

The S. mutans- and S. sobrinus-specific primers and probes were derived from the gtfB$^{52}$ and gtfT$^{53}$ genes, which encode glucosyltransferases involved in the synthesis of insoluble forms of glucan. The limits of detection and quantification by real-time PCR were linear over the range $1.7 \times 10^3$ to $1.7 \times 10^7$ colony forming units (CFU) for S. mutans and $1.1 \times 10^3$ to $1.1 \times 10^6$ CFU per reaction mixture for S. sobrinus. Using this assay, the numbers of S. mutans and S. sobrinus in saliva and dental plaque were examined in 10 patients. The absolute and relative numbers of these bacteria have been shown to vary between saliva and dental plaque. Both bacteria were detected more frequently in saliva: 10 in saliva and 8 in dental plaque for S. mutans and 7 in saliva and 5 in dental plaque for S. sobrinus. Our results suggest that saliva may be suitable for estimating individual caries risk, but the localized caries risk may be closely dependent on the microbial composition of dental plaque. Furthermore, based on the notion that dental plaque is a complex microbial community growing as a biofilm on enamel surfaces, the most recent evidence suggests that dental caries have a multi-bacterial etiology and, therefore, it is important to gain insight into the total bacterial composition of dental plaque.

During dental biofilm formation, Actinomyces spp. and several Streptococcus spp., including Streptococcus mitis, Streptococcus oralis, Streptococcus sanguinis and Streptococcus gordonii, adhere to the acquired pellicle on the tooth surface and are the predominant colonizers in the very early stages.$^{54}$ Different specific intra- and inter-generic co-aggregations between these primary colonizers and secondary colonizers have been demonstrated,$^{55-57}$ and it appears that early colonizers of dental plaque play an important role in progressive biofilm formation and the characterization of mature dental plaque. However, there have been no reports of any specific relationship between mutants streptococci and other oral bacteria. Quantitative analysis is required not only for accurate diagnosis of caries risk but also for studying biofilm formation and etiology. We developed a real-time PCR technique for quantitative detection of four early colonizer microorganisms in dental biofilms: Actinomyces naeslundii, Actinomyces viscosus, S. mitis and S. gordonii.$^{58}$ Using TaqMan PCR, we calculated the numbers of these four early colonizers and two cariogenic bacteria, S. mutans and S. sobrinus, in dental plaque. Specimens were obtained from the buccal side of the upper first molar in 5 individuals who had refrained from brushing for 12 hours. Large numbers ($\geq 10^3$ copies) of S. mitis were detected in all samples, while S. gordonii and A. viscosus were minor species in terms of prevalence (2 for S. gordonii and 1 for A. viscosus) and number (theoretical data below the detection limits except for 1 specimen). The frequency and number of S. mutans, S. sobrinus and A. naeslundii differed among individuals. As TaqMan PCR can monitor bacterial numbers in biofilms, it can be used to clarify the behavior of oral bacteria in dental biofilm formation. Thus, it may play a role in future biofilm research.

Designing Species-Specific Primers and TaqMan Probes Using Genomic Subtractive Hybridization

The key to the success of the TaqMan method is designing specific primers and probes based on the nucleotide sequence of the target microorganism. Genes encoding species-specific proteins or 16S rRNA are generally used as targets for real-time PCR.$^{18,23}$ However, it is difficult to isolate specific nucleotide sequences from the genes of closely related species. The classification of the viridans group streptococci, including S. mitis, S. oralis and S. gordonii, has long been considered difficult. Indeed, on the basis of 16S rRNA sequence, S. mitis and S. oralis share $>99\%$ sequence identity with each other and $\sim 97\%$ sequence identity with S. gordonii.$^{59}$ Although the
DNA–DNA hybridization between strains belonging to the mitis group showed that the DNA–DNA similarity values for the entire chromosome are estimated to be <60%. It is difficult to identify species-specific DNA sequences because much of the genome project for these bacteria has not yet been finished. *Actinomyces* spp. also shows extensive phenotypic and serologic variations. Most of the former *A. viscosus* have been classified as *A. naeslundii* genospecies 2. We employed genomic subtractive hybridization to identify genomic differences between the two closely related strains of *Streptococcus* or *Actinomyces* spp. and designed species-specific primers and probes for TaqMan real-time PCR. Subtractive hybridization was initially developed to identify differences in cDNA pools, but it has also been successfully used to identify genomic differences between closely related strains of *Helicobacter pylori*, *Mycobacterium tuberculosis* and *Neisseria meningitidis*. Chromosomal DNA of *S. oralis* ATCC 10557 was used as driver DNA for *S. gordonii* DL1 and *S. mitis* 903, and chromosomal DNA of *A. naeslundii* ATCC 51655 and *A. viscosus* ATCC 43146 were used as driver DNAs for each other. The oligonucleotide adaptors used in the first subtractive hybridization were 5′-GATCCCTCGGTGA-3′ (RBam12) and 5′-AGCACCTCCAGCCTCACCAG-3′ (RBam24), and the second adaptors were 5′-GATCCGTTCATG-3′ (JBam12) and 5′-ACCGACGTCTACATCTCAAGC-3′ (JBam24). The amplification products of the second-round PCR, which appeared as a smear of products <700 bp upon electrophoresis with ethidium bromide, were digested with Sau3AI cloned into BamHI-digested pBluescript II SK+ (Stratagene, La Jolla, CA) and used to transform *Escherichia coli* DH5α (TAKARA Bio Company, Shiga, Japan). Eight colonies were selected randomly from each cDNA bank containing 200 colonies and the nucleotide sequences were determined using an ABI PRISM 310 genenic analyzer (Applied Biosystems, Foster City, CA). A total of 32 fragments of 150 bp to 550 bp contained diverse sequences. Sequence homology between the subtracted fragments and those in the GenBank database was determined using BLAST by means of the WWW server at the National Center for Biotechnology Information (Bethesda, MD: http://www.ncbi.nlm.nih.gov/BLAST). A nucleotide database search (blastn) revealed that 21 insertions have no significant sequence homology to any known DNA sequences in GenBank, and the nucleotide sequences of 7 fragments were detected in the driver bacterial DNA. Three insertions are homologous to 16S rRNA genes of oral bacteria and another 8 insertions share homology with genes encoding unknown proteins. The *S. gordonii*, *S. mitis*, *A. naeslundii* and *A. viscosus*-specific primers and TaqMan probes derived from the subtractive DNA fragments are listed in table 1. The blastx similarities of the protein-encoding region in the fragments showed expected values of >0.1, except for the *S. gordonii* sequence (E=7e−37). This technique is useful for designing species-specific primers and probes for real-time PCR, especially for organisms for which whole genome sequence information is not yet available.

**Conclusion**

TaqMan real-time PCR has the advantage of providing rapid, accurate, sensitive and quantitative detection. It allows the continuous monitoring of DNA amplification eliminating the need for gel electrophoresis. Its rapidity and ease of handling are very important for the diagnosis of infections requiring timely identification of bacterial pathogens, such as bacterial meningitis, septicemia and communicable respiratory diseases. Oral infectious diseases are not caused by a single critical pathogen. The etiologies of dental caries, periodontal diseases and oral malodor are associated with bacterial accumulation and plaque composition. Therefore, the key for diagnosis of oral infections is to establish the microbial composition and the number of opportunistic pathogens present in oral biofilms rather than the rapid identification of the microorganisms concerned. TaqMan real-time PCR is an excellent method for the quantitative detection of oral bacteria and for the determination of relative numbers of different bacteria. Using genomic subtractive hybridization, it is easy to design primers and TaqMan probes for target microorganisms by directly subtracting the chromosomal DNA of a related strain from that of the target bacterium.

The human oral cavity contains more than 500 species of bacteria that colonize host tissues and co-aggregate with one another and other oral microorganisms. Following the proposal that the specific adhesion between different bacterial species contributes to the development of dental plaque, the microbial composition of dental plaque and the co-aggregation of the dominant organisms in biofilm formation have been extensively studied. Various procedures have been used to study these bacterial interactions, including laser confocal microscopy, spectrophotometry, co-aggregation assays, bacterial culture, molecular biology and microcalorimetry. These *in vitro* studies have shed light on many aspects of specific interactions between oral bacteria in dental biofilm. TaqMan real-time PCR can be used for direct target quantification without culturing and is useful for detecting species that are extremely difficult to grow in culture. This technique allows the visualization of the microbial ecosystem in the oral cavity and will likely be increasingly employed in future biofilm research.

**References**

Table 1. Development of TaqMan real-time PCR for the detection of human oral bacteria.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Sequence of primer or probe (5′–3′)</th>
<th>Target genes</th>
<th>Clinical specimens</th>
<th>Equipment</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td><strong>Actinobacillus actinomycetemcomitans</strong></td>
<td>Forward, CAG CAT CGA TCG TCT TA Reverse, TCA GCC CCT TCT TCT TCG TAG GT Probe, TCA AGG ATT CCT CAC GCA CTC TCC GCA G</td>
<td>lktA</td>
<td>Subgingival plaque saliva</td>
<td>ABI PRISM 7700 Sequence Detection System</td>
<td>17</td>
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<td>Forward, CCC ATC GCT GGT TGG TTA Reverse, GGC AGC TAG GGC GAC C Probe, CTC CTG TAT AGC CCA TTG TAG CAC GTG TGT</td>
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<td>Subgingival plaque</td>
<td>LightCycler system</td>
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<td>Forward, ACG CAG AGC ATT GAC TGA ATT TAA Reverse, GAT CTC CAC AGC TAT ATG GCA GCT A Probe, TCA CCC TTC TAC CTT TG CAT GGG</td>
<td>lktC</td>
<td>Subgingival plaque</td>
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<td><strong>Porphyromonas gingivalis</strong></td>
<td>Initial forward, GGA TTA GAT ACC CTC GTA GTC Initial reverse, GGA GTA TTG AGC CTT Second forward, CCG ACC TCT ACA GTA TTG TTC G Second reverse, CCG ACC TCT ACA GTA TTG AAC GCA GCT A Probe, ATG AGC TCA AAT CAG CAC GGC CCT TAC AT</td>
<td>16S rRNA gene and 23S rRNA gene</td>
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<td>16S rRNA gene</td>
<td>Carious dentine</td>
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<td>Forward, TAC CCA TCG TCG CCT TGG T Reverse, CGG ACT AAA ACC GCA TAC ACT TG Probe, GCT AAT GGG AGC CAT TAT CTT ACA GCT</td>
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<td><strong>Porphyromonas endodontalis</strong></td>
<td>Forward, GCT GCA GTC CAA CGT TAG TCT TG Reverse, TCA GTG TCA GAC GGA GCC TCG TAG TAC Probe, CAT TCC GCA TAC CCT CGG CCT TCT CTA GC</td>
<td>16S rRNA gene</td>
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<td><strong>Micromonas micros</strong></td>
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<td><strong>Treponema denticola</strong></td>
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<td><strong>Treponema vincentii</strong></td>
<td>Forward, GTC TCA ATG CAT AGA AA Reverse, CAA GCC TTA TCT CTA AGA CT Probe, GAC GGG GCC CGC CAC AGG C</td>
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<td><strong>Treponema medium</strong></td>
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<td>Subgingival plaque</td>
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### Table 1 continued from page 181.

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<th>Clinical</th>
<th>Equipment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tannerella forsythia</strong></td>
<td>Forward, GGG TGA GTA ACG CGT ATG TAA CCT Reverse, ACC CAT CCG CAA CCA ATA AA Probe, CCC GCA ACA GAG GGA TAA CCC CGG</td>
<td>16S rRNA gene</td>
<td>Subgingival plaque</td>
<td>ABI PRISM 7700 Sequence Detection System</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Forward, ATC CTG GCT CAG GAT GAA CG Reverse, TAC GCA TAC CCA TCC GCA A Probe, ATG TAA CCT GGC CCG AAC AGA GGA ATA AC</td>
<td>16S rRNA gene</td>
<td>Subgingival plaque</td>
<td>ABI PRISM 7700 Sequence Detection System</td>
<td>22</td>
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<tr>
<td></td>
<td>Forward, AGC GAT GGT AGC AAT ACC TGT C Reverse, TTC GGC GGG TTA TCC CTC Probe, TGA GTA ACG CGT ATG TAA CCT GCC CGC</td>
<td>16S rRNA gene</td>
<td>Subgingival plaque</td>
<td>LightCycler system</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Forward, TCC CAA AQA CGC GGA TAT CA Reverse, AGC GTG GGC ATG TCA TGG T Probes, CCG CGA CTT GAA ATG GTA TTA TTC CTC</td>
<td>BspA anigen gene</td>
<td>Subgingival plaque</td>
<td>ABI PRISM 7700 Sequence Detection System</td>
<td>19</td>
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<td><strong>Fusobacterium spp.</strong></td>
<td>Forward, CGC AGA AGG TGA AAG TCC TGT AT Reverse, TGG TCC TCA CTG ATT ACC ACA GA Probe, ACT TTG CTG CCA AGT AAC ATG GAA CAG</td>
<td>16S rRNA gene</td>
<td>Subgingival plaque</td>
<td>ABI PRISM 7700 Sequence Detection System</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Forward, AAG CGC GTG GTC TAG GTG ATG T Reverse, TGG ATG TCC GCT TAC TCT TGC AG Probe, CAA CGC AAT ACA GAG TTG AGC TAC GCA CCA CCA</td>
<td>16S rRNA gene</td>
<td>Carious dentine</td>
<td>ABI PRISM 7700 Sequence Detection System</td>
<td>23</td>
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<tr>
<td><strong>Prevotella spp.</strong></td>
<td>Forward, CGC GCC AAG TAG CGT GCA Reverse, TGG ACC TTC CGT ATT ACC GC Probe, ATG AAG GAC GCG CGT ATT CCA ATG CAG</td>
<td>16S rRNA gene</td>
<td>Carious dentine</td>
<td>ABI PRISM 7700 Sequence Detection System</td>
<td>23</td>
</tr>
<tr>
<td><strong>Prevotella melaninogenica</strong></td>
<td>Forward, GTG GGA TAA CCT GCC GAA AG Reverse, CCA ATC CAT TCA CGA TAA ATC TTT A1 Probe, CAA ATC TGA TGC CGT CAT CGA AGA CTA TGC</td>
<td>16S rRNA gene</td>
<td>Carious dentine</td>
<td>ABI PRISM 7700 Sequence Detection System</td>
<td>23</td>
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<tr>
<td><strong>Prevotella intermedia</strong></td>
<td>Forward, TCC ACC GAT GAA TCT TGT GTC Reverse, ATC CCA CCT TCC CTC CAC TC Probe, CGT CAG ATG CCA ACT TAT GTG GAC AAC ATC G</td>
<td>16S rRNA gene</td>
<td>Subgingival plaque</td>
<td>LightCycler system</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Forward, CGG TGG AAA GAC GGC CTA A Reverse, CCA ATC CCT TAC CGG RA Probe, CCC GAT GTG TTT CAT TGA CCG CAT C</td>
<td>16S rRNA gene</td>
<td>Subgingival plaque</td>
<td>LightCycler system</td>
<td>24</td>
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<tr>
<td><strong>Cariogenic pathogen</strong></td>
<td><strong>Streptococcus mutans</strong> Forward, GCC TAC AGC TCA GAA GATG CTA TTC T Reverse, GCC ATA CAC TCA TCA TGA ATT GA Probe, TGG AAA GGA CCG TCG TCG TTA TGA A</td>
<td>gtfB</td>
<td>Dental plaque Saliva</td>
<td>ABI PRISM 7700 Sequence Detection System</td>
<td>18</td>
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<td></td>
<td><strong>Streptococcus sobrinus</strong> Forward, TCC AAA GGC AAG ACC AAG CTA GT Reverse, CCA GCC GGA GAT TCA GTA CTG TGT Probe, CCT GCT CCA CCG ACA AAG GCA GC</td>
<td>gtfT</td>
<td>Dental plaque</td>
<td>ABI PRISM 7700 Sequence Detection System</td>
<td>18</td>
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<tr>
<td><strong>Early microbial colonizer in human dental biofilm</strong></td>
<td><strong>Streptococcus gordonii</strong> Forward, GGT GTT GTT TGA CCC GCC GTT CAG Reverse, AGT CCA GCC CAC GAC CAG Probe, AAC CTT GAC CCG CTC ATT ACC AGC TAG TAT G</td>
<td>The gene encoding putative type I site-specific DNase</td>
<td>Dental plaque</td>
<td>ABI PRISM 7700 Sequence Detection System</td>
<td>58</td>
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<tr>
<td></td>
<td><strong>Streptococcus mitis</strong> Forward, GAG TCC TGC ATC AGC CAA GAG Reverse, GGA TCC ACC TTT TCT GCT TGC TGA C Probe, TGG TCC CAA GTA GAG CCA ACC AAA CT</td>
<td>The gene encoding putative Glycine-rich protein</td>
<td>Dental plaque</td>
<td>ABI PRISM 7700 Sequence Detection System</td>
<td>58</td>
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Table 1 continued from page 182.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Sequence of primer or probe (5’-3’)</th>
<th>Target genes</th>
<th>Clinical specimens</th>
<th>Equipment</th>
<th>Reference</th>
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<tbody>
<tr>
<td><em>Actinomyces naeslundi</em></td>
<td>Forward, TCG AAA CTC AGC AAG TAG CGG Reverse, AGA GGA GGG CCA CAA AAG AAA Probe, GGG TAC TCT AGT CCA AAC TGG CGG ATA GCG</td>
<td>The gene encoding unknown protein</td>
<td>Dental plaque</td>
<td>ABI PRISM 7700 Sequence Detection System</td>
<td>58</td>
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<tr>
<td><em>Actinomyces viscosus</em></td>
<td>Forward, ATG TGG GTC TGA CCT GCT GC Reverse, CAA AGT CGT TCA CGG TCC G Probe, ACG GAG GTG AAC GGT GGA AG</td>
<td>The gene encoding unknown protein</td>
<td>Dental plaque</td>
<td>ABI PRISM 7700 Sequence Detection System</td>
<td>58</td>
</tr>
<tr>
<td><strong>Universal</strong></td>
<td>Forward, TCC TAC GGG AAG CAG CAG T Reverse, GGA CTA CCA CCA GGG TAT CTA ATC TGT TT Probe, CTT ACC CCC GGG AAG GTT ATT CCC CG</td>
<td>16S rRNA gene</td>
<td>1</td>
<td>ABI PRISM 7700 Sequence Detection System</td>
<td>21</td>
</tr>
<tr>
<td><strong>Universal</strong></td>
<td>Forward, CCA TGA AGT CCG AAT CGC TAC TAG Reverse, GCT TGA CGC GGG GTG T</td>
<td>16S rRNA gene</td>
<td>1</td>
<td>ABI PRISM 7700 Sequence Detection System</td>
<td>21</td>
</tr>
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</table>

* Applied Biosystems, Foster City, CA
* Bio-Rad Laboratories Inc., Hercules, CA


65. Perrin A, Nassif X, Tinsley C. Identification of regions of the chromosome of *Neisseria meningitidis* and *Neisseria gonorrhoeae* which are specific to the pathogenic *Neisseria* species. Infect Immun 1999;67:6119-6129.


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