# Sensitive detection of *Porphyromonas gingivalis* in oral samples by digital PCR.

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## Background

The oral microbiome is a complex community of microbes that has key contributions to oral and overall health (Sharma et al. 2018). *Porphyromonas gingivalis* is an anaerobic, Gram-negative bacterium that is considered a 'keystone pathogen' of severe gum disease (periodontitis; How et al. 2016). It promotes imbalance of the oral microbiome and expresses virulence factors including a class of cysteine proteinases called gingipains (Freeze 2022). *P. gingivalis* can also enter the bloodstream and promote cardiovascular disease, cancer, and Alzheimer's disease (Freeze 2022). Given its clinical significance, many molecular methods of *P. gingivalis* detection have been established including quantitative PCR (qPCR; Clais et al, 2015; Marin et al. 2018), most commonly targeting the 16S rRNA gene. However, targeting of virulence genes specifically (especially for reverse-transcription experiments), and the use of the newer, highly sensitive digital PCR technique is more limited. Quantitative and digital PCR both rely on fluorescent probes to detect PCR product as it is produced, however dPCR does not require a standard curve. In dPCR, a PCR reaction is partitioned into thousands of individual reactions, ideally with either 0 or 1 template molecule. Counting the number of partitions that produce fluorescence after thermal cycling gives the absolute number of template molecules in the original sample.

## Objective

This research aims to design & validate A three-target multiplex PCR assay that:

- Uses sequence specific TaqMan fluorescent probes
- Works on quantitative and digital PCR instruments
- Works with genomic DNA and reverse-transcribed RNA (cDNA) samples.

Targets:

16S rRNA – common species marker
kgp – virulence gene: lysine gingipain
hmuY – virulence gene: heme binder

## Results

Table 1. Designed primer/probe sets used for quantitative and digital PCR experiments.

| Target | Oligo Sequence | $T_m$ (°C) | %GC | Length (bp) | Amplicon Length (bp) |
|---|---|---|---|---|---|
| kgp | **769F** 5'-AAGCCTTGGCTCACTTGGAA-3' | 58.2 | 50 | 20 | 142 bp |
| | **910R** 5'-CAGCACTAGCTGCCAATCCA-3' | 59.8 | 50 | 20 | |
| | **843P** 5'-FAM AACGACAAACGCCTCTA MGB-3' | 68.0 | 47 | 17 | |
| hmuY | **380F** 5'-CTTGCCACTTTCGCCACAA-3' | 59.4 | 53 | 19 | 137 bp |
| | **516R** 5'-CGAAATACGAAACGTGGCAGT-3' | 58.8 | 48 | 21 | |
| | **413P** 5'-HEX ATAGCGGTGAAGAGC MGB-3' | 68.0 | 53 | 15 | |
| 16S rRNA | **168F** 5'-CGGACTAAAACCGCATACACTTG-3' | 59.6 | 48 | 23 | 108 bp |
| | **275R** 5'-TGAGCCGTTACCTCACCAACA-3' | 60.1 | 52 | 21 | |
| | **239P** 5'-ABY ATGCGTCCCATTAGC MGB-3' | 70.0 | 53 | 15 | |

Gel electrophoresis (Figure 4) shows that all primers produced only 1 band in traditional PCR, and the band was of the predicted size. Next, quantitative PCR standard curves were prepared (Figure 5) to verify the TaqMan fluorescent probes and ensure no dye interference in the multiplex. Standard curves showed strong linearity.

## Methods

### Primer & Probe design

Target amplicon region

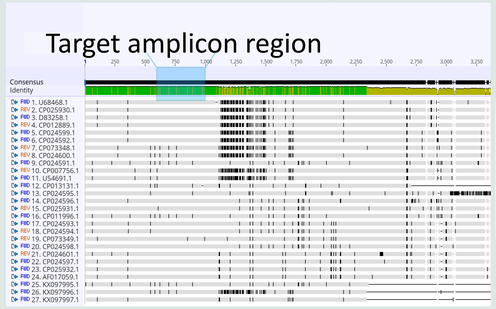


Figure 1. Multiple sequence alignment of 27 *kgp* sequences downloaded from NCBI on the Geneious Prime software. Primer pairs were designed from the consensus sequence within the target amplicon region.

Designed potential primer and probe sets for the three gene targets (kgp, hmuY, 16S rRNA) using NCBI DNA sequences and the visualization software *Geneious Prime*.

Primer Criteria
- ✓Melting Temp: 58°C – 60°C
- ✓GC Content: 40% - 60%
- ✓Length: 15 bp – 21 bp
- ✓Amplicon length ~150 bp

TaqMan MGB Probe Criteria
- ✓Include Unique 5' Fluorophore
- ✓Melting Temp: 68°C – 70°C
- ✓GC Content: 40% - 60%
- ✓Length: 15 bp – 17 bp
- ✓No G residues on 5' or 3' end
- ✓Only 1 CC dinucleotide allowed

Check primers/probes for species and gene specificity *in silico*.

Test primers by endpoint PCR and gel electrophoresis on *P. gingivalis* genomic DNA.

### Quantitative PCR

Validation of the primer/probe combos in standard curves using *P. gingivalis* genomic DNA.

Figure 2. QuantStudio 3 qPCR instrument.

Reaction Mixture
- 5 µL Fast Advanced qPCR MasterMix (2x)
- 900 nM Forward + Reverse Primer
- 250 nM Fluorescent Probe
- 0 – 1 ng Genomic DNA Template
- Water to 10 µL

Thermal Cycling Conditions
- 20 seconds @ 95 °C
- 40 cycles:
  - 1 second @ 95 °C
  - 20 seconds @ 60 °C

### Digital PCR

Running extracted genomic DNA, and RNA derived cDNA from real oral samples.

Figure 3. AbsolutQ dPCR instrument.

Reaction Mixture
- 2 µL DNA Digital PCR MasterMix (5x)
- 900 nM Forward + Reverse Primer
- 250 nM Fluorescent Probe
- 1 µL DNA Template
- Water to 10 µL

Thermal Cycling Conditions
- 10 minutes @ 96 °C
- 40 cycles:
  - 5 seconds @ 96 °C
  - 15 seconds @ 60 °C

## Results cont.

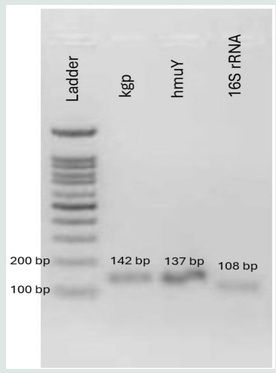


Figure 4. PCR amplicons from *P. gingivalis* gDNA visualized by gel electrophoresis.

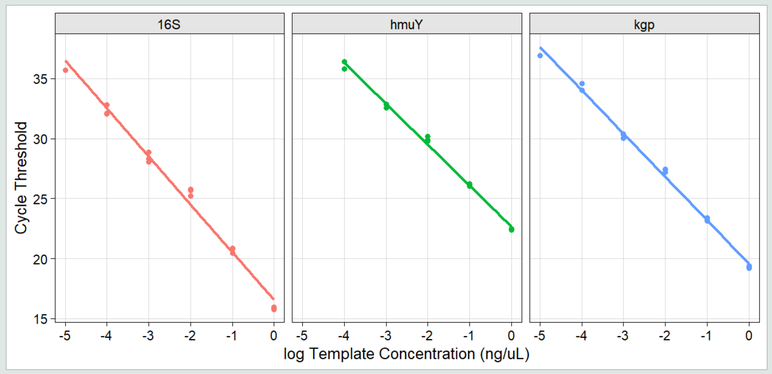


Figure 5. Quantitative PCR standard curves generated from the triple target assay using *P. gingivalis* genomic DNA as the template. The template concentrations correspond to a range of ~4 to ~400 000 *P. gingivalis* genome copies.

Each probe has a unique fluorophore:
FAM – kgp (515 nm)
HEX – hmuY (555 nm)
ABY – 16S (570 nm)

The qPCR results show that these do not interfere significantly, and the instruments can differentiate their emission. Reductions in amplification efficiency between single and multiplex reactions were minimal.

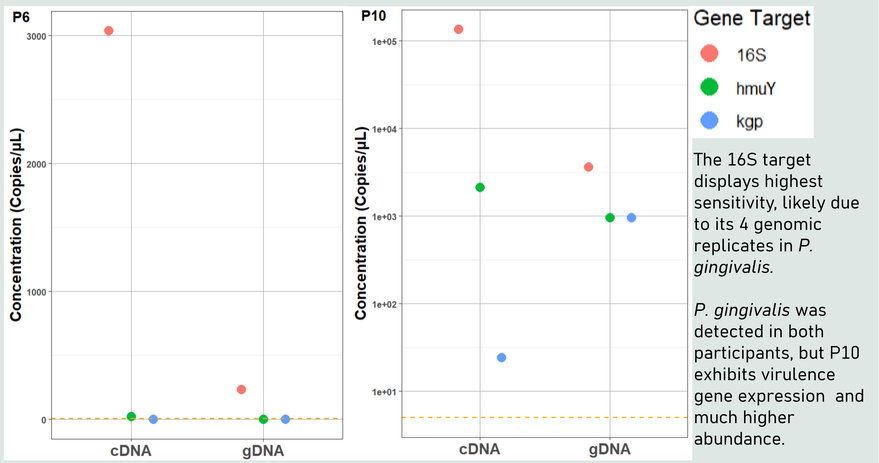


Figure 6. Digital PCR results from oral microbiome gDNA and cDNA samples of two individuals (P6 & P10; sample collection: Freeze 2022). Values above the orange line (5 copies) are considered detections. gDNA indicates *P. gingivalis* abundance, and cDNA indicates gene expression.

The 16S target displays highest sensitivity, likely due to its 4 genomic replicates in *P. gingivalis*.

*P. gingivalis* was detected in both participants, but P10 exhibits virulence gene expression and much higher abundance.

## Conclusion

A sensitive dPCR assay was successfully developed to determine *P. gingivalis* abundance and assess expression of two virulence genes in oral samples.

## Future Work

- Collect more gDNA and cDNA samples from participants to run on dPCR and further establish the precision and accuracy of the assay.
- Use the assay to assess a potential supplement or treatment against *P. gingivalis:*
  - Freeze (2022) collected samples from participants before and after nitrate supplementation, to investigate its effect on Porphyromonas abundance.

References: 1. Sharma, N. et al. (2018). Oral microbiome and health. AIMS Microbiology, 4(1), 42–66. doi: 10.3934/microbiol.2018.1.42 2. How, K. Y. et al. (2016). Porphyromonas gingivalis: An Overview of Periodontopathic Pathogen below the Gum Line. Frontiers in Microbiology, 7, 53. doi: 10.3389/fmicb.2016.00053 3. Freeze, G. (2022). The Effect of Nitrate Supplementation on Porphyromonas gingivalis Abundance in the Oral Microbial Community [Honours]. Thompson Rivers University. 4. Clais, S. et al. (2015). Comparison of viable plate count, turbidity measurement and real-time PCR for quantification of Porphyromonas gingivalis. Letters in Applied Microbiology, 60(1), 79–84. doi: 10.1111/lam.12341 5. Marin, M. J. et al. (2017). Detection and quantification of Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis and Streptococcus oralis in blood samples with different microbiological identification methods: An in vitro study. Archives of Oral Biology, 74, 55–62. doi: 10.1016/j.archoralbio.2016.11.007