2.6 Primer and Probe Design

The genes encoding a lysine specific gingipain (kgp), a heme acquiring hemophore (hmuY), and 16S rRNA were selected as targets for development of the multiplex assay. Potential amplicon regions were chosen on the target genes for high specificity to the target gene and inclusivity of *P. gingivalis* strains. Target regions were also chosen for a consistent number of copies in the genomes of multiple strains. Primers were designed on the target amplicon regions according to criteria provided by the Primer Express software user guide. These criteria included a melting temperature (T_m) of 58-60℃, a GC content close to 50%, length close to 20 residues, and an amplicon length between 50 and 150 base pairs. Probes were designed to use TaqMan chemistry and be 3'-NFQ MGB modified. Their design criteria included a T_m between 68-70℃, GC content close to 50%, and length close to 16 residues. More stringent and specific probe design considerations can be found in the Primer Express software user guide. Primer Express was used to validate the criteria and check for secondary structures in the primers and probes.

kgp
Twenty-seven kgp sequences (supp. Table 1) from varying strains of *P. gingivalis* were aligned in Geneious Prime and a consensus sequence was obtained. Candidate amplicon regions were chosen based on specificity to the kgp gene. Notably, a region not conserved between kgp and other gingipains (i.e. RgpA/RgpB) was used. Three primer/probe sets were then designed by the criteria listed above. Refseq genomes of *P. gingivalis* strains W83, TDC60, W50, ATCC 33277, and A7A1-28 were imported to Geneious prime from NCBI and each primer/probe set was verified to hit only the intended target. Finally, the candidate primer/probe sets (Table 2) were checked for off target amplicons using the NCBI Primer Blast tool and ordered from Alpha ADN for experimental validation.

hmuY
Twenty-nine HmuY (supp. Table 1) sequences from varying strains of *P. gingivalis* were aligned in Geneious Prime and a consensus sequence was obtained. Three primer/probe sets were then designed by the criteria listed above. Refseq genomes of *P. gingivalis* strains W83, TDC60, W50, ATCC 33277, HG66, and A7A1-28 were imported to Geneious prime from NCBI and each primer/probe set was verified to hit only the intended target. Finally, the candidate primer/probe sets (Table 2) were checked for off target amplicons using the NCBI Primer Blast tool and ordered from Alpha ADN for experimental validation.

16S rRNA
Twenty-four 16S rRNA (supp. Table 1) sequences from varying strains of *P. gingivalis* were aligned in Geneious Prime and a consensus sequence was obtained. Two candidate primer/probe sets were designed in species specific regions of the 16S rRNA gene. Refseq genomes of *P. gingivalis* strains W83, TDC60, W50, ATCC 33277, HG66, and A7A1-28 were imported to Geneious prime from NCBI and each primer/probe set was verified to hit only the intended target. Notably the 16SrRNA gene is present four times in *P. gingivalis*, which suggests potential higher sensitivity as a qPCR target. Finally, the candidate primer/probe sets (Table 2) were checked for off target amplicons using the NCBI Primer Blast tool and ordered from Alpha ADN for experimental validation.

Table 2. Polymerase chain reaction (PCR) primer pairs selected for testing on *P. gingivalis* genomic DNA template.

Target name	Primer Pair Sequences (5' – 3')	T_m (℃)	%GC	Length (bp)	Amplicon length
kgp3	715F AATACGCCGGTTCGTATGCT	58.2	50	20	89 bp
	803R AAGCCCTTTTGAGCCTTCCA	59.8	50	20	
kgp4	769F AAGCCTTGGCTCACTTGGAA	58.5	50	20	142 bp
	910R CAGCACTAGCTGCCAATCCA	58.6	55	20	
kgp5	836F AAGTAGGAACGACAAACGCCTC 941R GTGTCACCAACCAAAGCCAAGA	58.4	50	22	106 bp
		59.9	50	22	
hmuY1	380F CTTGCCACTTTCGCCACAA	59.4	53	19	137 bp
	516R CGAAATACGAAACGTGGCAGT	58.8	48	21	
hmuY2	93F CCATCAGCACCACGAACG	58.2	61	18	101 bp
	193R CAGGGATTTGCTTCAGGTGG	59.1	55	20	
hmuY3	145F CGGCAGGACCGTGAGAGA	59.5	67	18	56 bp
	200R GAACGCACAGGGATTTGCTT	58.8	50	20	
16S2	168F CGGACTAAAACCGCATACACTTG 275R TGAGCCGTTACCTCACCAACA	59.6	48	23	108 bp
		60.1	52	21	
16S1	448F TACGGGAATAACGGGCGATA	58.9	50	20	110 bp
	557R CGGATAACGCTCGCATCCT	59.8	58	19	

2.7 Primer Pair Validation

All nine primer pairs were resuspended according to the manufacturer's instructions and 10 µM working solutions were prepared. Positive and negative control PCR reactions using pure *P. gingivalis* stock genomic DNA and water as templates were used to check the amplification capability of the primer pairs. The total reaction volume was 20 µL with 1 µL of 10µM forward primer, 1µL of 10 µM reverse primer, 6 µL PCR grade water, 10 µL of GoTaq Green Mastermix and 2 µL of template DNA. The thermal cycler conditions were as follows: 10 minutes at 95℃ for initial denaturation, 40 cycles of 15 seconds at 95℃ (denaturation) and 60 seconds at 60℃ (combined annealing/extension), and final elongation at 72℃ for 5 minutes. The PCR product was visualized on a 2% agarose gel run for 80 minutes at 80 volts.

2.8 Verification of amplicons by Sanger Sequencing

PCR product obtained from primer pairs kgp4, hmuY1, and 16S2 were cleaned using HighPrep PCR product purification beads according to the protocol provided by the manufacturer. Purified DNA was eluted in 20 µL of PCR grade water.

2.9 Probe Validation by qPCR

Probe design was finalized for one primer set targeting each gene which appeared to have the most promising results. These were kgp4, hmuY1, and 16S2.

Table 3. TaqMan probes designed to work with associated primer pairs for quantitative polymerase chain reaction (qPCR).

Target name	Probe Name and Sequence	T_m (℃)	%GC	Length (bp)
kgp4	843P 5'-FAM AACGACAAACGCCTCTA MGB- 3'	68.0	47	17
hmuY1	413P 5'-HEX ATAGCGGTGAAGAGC MGB-3'	68.0	53	15
16S2	239P 5'-ABY ATGCGTCCCATTAGC MGB-3'	70.0	53	15

The probes were resuspended according to the manufacturer's instructions and 10 µM and 20 µM working solutions were prepared. All qPCR reactions were carried out on the Applied Biosystems QuantStudio 3 RT-PCR instrument in a MicroAmp Fast 96 well reaction plate. Serial tenfold dilutions of *P. gingivalis* stock genomic DNA ranging from 1 ng/µL to 1E-06 ng/µL (template copy numbers: ~400 000 to ~0.4) were used as the template for preparation of a calibration curve. PCR grade water was used as the negative control. Each reaction had a final primer concentration of 900 nM and a final probe concentration of 250 nM. Each reaction also contained 5 µL of Fast Advanced qPCR mastermix and 1 µL of template DNA solution. The total reaction volume was 10 µL and the remaining volume was made up with PCR grade water. The thermal cycling conditions were chosen according to the manufacturer's instructions, and are as follows: 2 minutes at 50℃, 20 seconds at 95℃, and 40 cycles of 1 second at 95℃ and 20 seconds at 60℃, with fluorescence measurements taken every cycle.

Each primer/probe set was tested by running standards as outlined above in at least 3 replicates. Calibration curves were generated by plotting the $\log_{10}$ of template concentration against cycle threshold. Amplification efficiency was determined from the slope of the fitted linear trendline and linearity was assessed by R^2. Sensitivity was determined as the lowest concentration of template that was detected in all replicates. Reproducibility was assessed by relative standard deviation at each template concentration.