Results

Primer design and selection

The genes kgp and hmuY were chosen as PCR targets for their specificity to *P. gingivalis* and their functional relevance as virulence factors. A region of the 16S rRNA encoding gene unique to *P. gingivalis* was also targeted; the 16S gene is the most common amplicon target for *P. gingivalis* in the literature. Each primer set was validated *in-silico* to have no off-target sites on the genomes of *P. gingivalis* or other species. Designed primers were validated by traditional PCR and gel electrophoresis, where the expected amplicon length for each target (insert numbers here) was observed in comparison to ladder. All amplicons agreed with their predicted size when compared to 100 base pair ladder, and hmuY1, kgp4, and 16S2 were selected for downstream use.

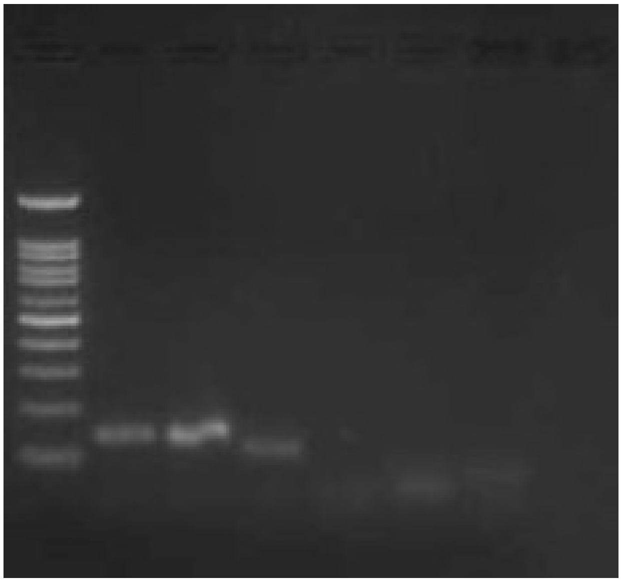

Figure #. Amplicons generated by three PCR primer sets visualized by gel electrophoresis using 100 bp ladder.

Probe validation by qPCR

TaqMan sequence specific fluorescent probes were designed for the three primer sets and qPCR was completed to produce standard curves, each with three replicates of template concentrations ranging from 0 – 395 000 genome copies. Each gene target showed excellent linearity ($R^2 > 0.98$), and an amplification efficiency above 80%. The curves also showed good reproducibility; the coefficient of variation (CV) of each curve did not exceed 2.6% and the inter-assay CV was under 2% for each target.

- Selection of kgp4, hmuY1, 16S2 and outlining of the assays
- Calibration curves (one for each singleplex)
- Amp eff. Linearity, precision (RSD range)

Multiplex validation by qPCR

The multiplex assay containing all three primer/probe sets was then validated on qPCR. All calibration curves showed strong linearity, with no curves having an R^2 below 0.97, and three of the nine below 0.98. The intra-assay precision does not vary from the singleplex curves for kgp ($t_{19.6} = 0.13$, $p = 0.90$), hmuY ($t_{18.3} = -0.94$, $p = 0.36$), nor 16S ($t_{23.3} = 0.22$, $p = 0.83$). For kgp and 16S, the amplification efficiency is comparable to the singleplex curves, however the hmuY target showed reduced efficiency in two curves, with the lowest at 78.6%. Since the other targets did not have reduced efficiency, it is unlikely that interference with other oligonucleotides in the multiplex caused hmuY amplification to be less efficient. The sensitivity of the multiplex was assessed using 95% lower limit of detection ($LOD_{95\%}$) determined by logistic regression. The kgp and 16S amplicons had an $LOD_{95\%}$ of 6.55 copies and 5.71 copies respectively, whereas the $LOD_{95\%}$ for hmuY was 57 copies. Notably there are four copies of the 16S amplicon in the *P. gingivalis* genome, which suggests increased sensitivity compared to kgp. While this was not observed in the LOD, the 16S cycle threshold values were consistently ~2 units lower than kgp for the four lowest copy number reactions.

***include or not? For all targets, there is a statistically significant increase in cycle threshold at each concentration from the singleplex and multiplex.

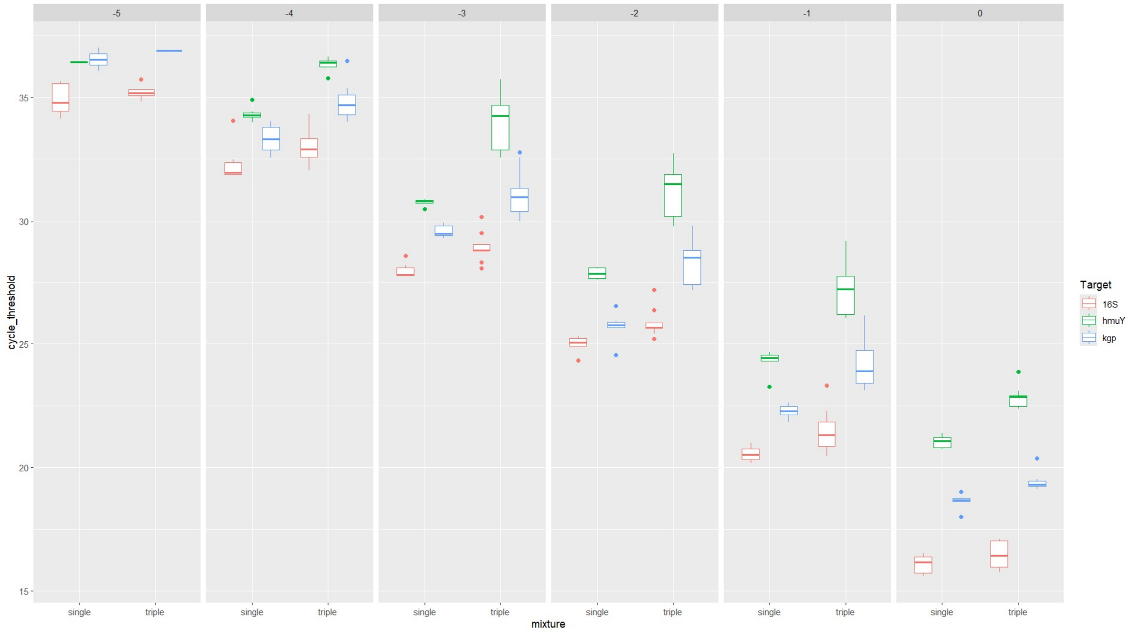

Table #. Quantitative PCR results of singplex and multiplex assays targeting three gene amplicons specific to *P. gingivalis*. Curves were generated with triplicate sets of standard solutions containing 0 to 395 000 copies of *P. gingivalis* genomic DNA.

Singleplex	kgp (FAM)			hmuY (HEX)			16S (ABY)		
	R^2	Amp. Eff.	Precision	R^2	Amp. Eff.	Precision	R^2	Amp. Eff.	Precision
Curve 1	0.997	90.9%	1.24%	0.999	103.9%	0.39%	0.997	81.1%	1.04%
Curve 2	0.992	84.0%	2.02%	0.992	97.7%	1.43%	0.986	74.8%	2.53%

Multiplex	kgp (FAM)			hmuY (HEX)			16S (ABY)		
	R^2	Amp. Eff.	Precision	R^2	Amp. Eff.	Precision	R^2	Amp. Eff.	Precision
Curve 1	0.996	86.9%	0.68%	0.996	96.5%	0.70%	0.997	79.8%	0.72%
Curve 2	0.988	82.0%	0.72%	0.980	88.9%	0.52%	0.989	76.2%	1.18%
Curve 3	0.970	83.0%	2.70%	0.973	78.6%	2.14%	0.979	74.6%	2.09%
Inter-assay Precision	1.73%			1.19%			1.66%		

Limits of detection:

(50%, 95%)

kgp: -4.91 (4.86 cp), -4.78 (6.55 cp)

hmuY: -3.99 (40.4 cp), -3.84 (57.1 cp)

16S: -4.99 (4.04 cp), -4.84 (5.71 cp)