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A rapid and extraction-free methodology for detection of SARS-CoV-2 using the 'QuantuMDx lyophilised SARS-CoV-2 RT-PCR detection Assay' for use in the screening of single/pooled saliva samples.

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INTRODUCTION

An accurate, rapid, non-invasive, and affordable diagnostic test is crucial in population control of COVID-19. Saliva is an advantageous sample type due to ease of collection, high viral content and accuracy in diagnosis. However, it is inhibitory to RT-PCR and usually undergoes extraction before molecular analysis.

The commercially available QuantuMDx SARS-CoV-2 RT-PCR Detection Assay (CE-IVD) was designed for use on extracted nasopharyngeal samples and has demonstrated excellent clinical performance in the diagnosis of COVID-19, however it had not been tested for use in direct PCR methodologies.

AIM

QuantuMDx set out to develop a methodology using the commercially available SARS-CoV-2 RT-PCR Detection Assay on saliva samples without the need for extraction of RNA. The assay master mix formulation cannot be changed however additives and other techniques could be used.

METHOD

In order to optimise a methodology a number of obstacles were overcome, including lysis of virus, RNA degradation and PCR inhibition. Optimisation and testing were performed using negative saliva samples spiked with inactivated (ATCC) or live (Newcastle University) SARS-CoV-2.

The main areas of development were:

- Dilution of sample
- Additives to prevent degradation of sample
- Additives to improve direct RT-PCR
- Thermal Conditioning
- Concentration of PCR mix
- Pooling of samples for rapid diagnostics

RESULTS

The optimised methodology for a non-pooled sample involves diluting the saliva with MSwab (Copan) which was spiked with additives in a preparatory step. Following a 15 minute incubation the sample(s) underwent a 2 minute heat-lysis step. The samples are added to master mix which has been rehydrated with a newly developed buffer, into a more concentrated form than the standard product presentation. The time to result from raw saliva is <125 minutes for up to 94 wells containing sample. Minimal pipetting steps reduced hands-on time to approximately 25 minutes, with 75 minutes for RT-PCR and 5 minutes for analysis. This is a marked reduction in time as using the PCR mix with a manual extraction step could take as long as 245 minutes, with 170 minutes of hands-on time.

The LOD for individual samples is $4x10^4$ virus/ml raw saliva, a value lower than what is typically shed from infectious individuals[1], and all without the need for a labour-intensive and time-consuming extraction step.

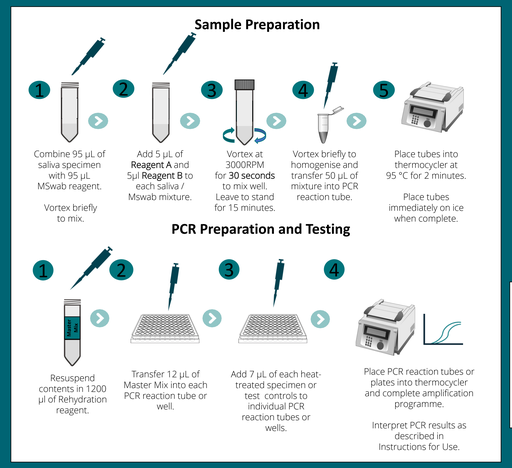


Figure 1 – User workflow for Saliva Direct methodology for use with QuantuMDx SARS-CoV-2 RT-PCR Detection Assay

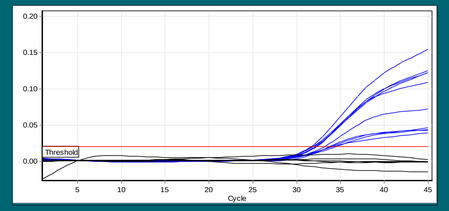


Figure 2- Demonstration of RT-PCR amplification of 5 sample (in duplicate at LOD, $4x10^4$ per ml raw saliva. Blue curves= Raw saliva samples spiked with inactivated virus (xxx) to a concentration of 4 x 10^4/ml before undergoing the workflow as shown in Figure 1. Black curves= Negative controls using the corresponding saliva samples .

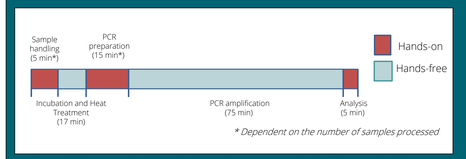
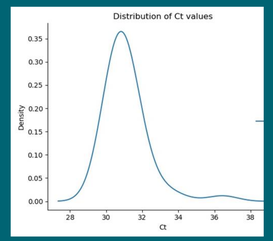


Figure 3- Pictogram depicting the time taken to perform Saliva Direct methodology

Combined with a pooling step, this protocol could facilitate mass-screening. 20 contrived saliva samples were pooled in multiple combinations. Pooling the 5 and 10 most inhibitory samples presented an LOD of $4x10^4$ virus/ml and $2x10^4$/ml respectively. This suggests that a sample with a $2x10^5$/ml viral load could be detected within a pool of 5 or 10, a viral load less than what is typically detected in a culture positive/infectious sample.

A.



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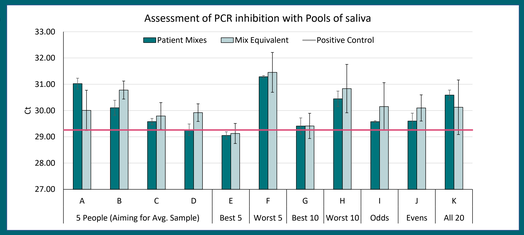

Figure 4A and 4B- Study looking at the distribution of inhibition in Saliva. In order to test the saliva assay with pools, a study was performed looking at the PCR inhibition apparent in individual samples and the difference when samples are pooled. Samples were spiked with 500 copies/rxn of inactivated SARS-CoV-2 and put through the saliva assay individually. The data were analysed (4A) and ranked in terms of inhibition (not shown). The samples were then pooled based on the previous information in pools of 5, 10, and one pool containing all samples. Figure 4B illustrates the variation of inhibition profiles. Samples within the '5 person mix' are the individual results from 5 saliva samples intended to be an equal spread of inhibition with no repeated samples across the 4 mixes. The 'best 5' are the results from the 5 least inhibited samples and the 'worst 5' are the 5 most inhibited. Similarly, the 'best 10' and 'worst 10' represent the least and most inhibited 10 saliva samples, respectively. 'Odds' and 'evens' are composed of samples which have been ranked 1-20 in terms of inhibition and then split into odds and evens to give 2 sets of data from 10 saliva samples with similar spread. Data are presented in the graph from the actual patient mix and from the 'mix equivalent' which is taken from the average Ct value of the same samples separately, on the same RT-PCR. The red line represents the positive control. Data appears to show the larger the pool, the less inhibition (other than when looking at 5 best v 10 best which is to be expected). This can be observed by looking at the average mixes of 5 and comparing it to the average mixes of 10 (odds and evens). It also occurs when pooling the worst 10 rather than worst 5. In almost every case the mix equivalent comes out with a high Ct than the actual pool result. This shows that some PCR inhibition is reduced by pooling samples.

Conc. (copies/ml raw saliva)	Theoretical single patient Conc. in a pool of 10 (copies/ml in 1 patient)	Sensitivity (%)		p-values (T-test)
		Least Inhibitory	Most Inhibitory	Least vs Most inhibitory
20,000	200,000	100%	100%	0.00
10,000	100,000	100%	70%	0.18
5,000	50,000	75%	40%	0.65

Figure 5- LOD study with the 10 least and most inhibitory pools. Samples were detected 100% of the time in the mix of the 10 most inhibitory samples at 20,000/ml. This is the equivalent of a single saliva sample within a 10 sample mix being at concentration 200,000/ml, a clinically relevant viral load for detection of infectious samples[1].

CONCLUSIONS

QuantuMDx have optimised a methodology for detecting SARS-CoV-2 in saliva specimens without the need for extraction/swabs using the commercially available QMDx SARS-CoV-2 RT-PCR detection assay product. The direct methodology cuts the time to result to <125 minutes with high sensitivity at clinically relevant concentrations of virus in single sample assays (LOD $4x10^4$/ml raw saliva).

A further study showed that the LOD is lowered when using pooled samples to $2x10^4$/ml which corresponds to a single sample viral load of $2x10^5$/ml, a number around the threshold for infectious individuals. With this layout 940 samples could be tested simultaneously on a single 96-well plate and used for high throughput testing for infectious individuals.

REFERENCES

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