Implementation of an efficient SARS-CoV-2 specimen pooling strategy for high throughput diagnostic testing

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Abstract

The rapid identification and isolation of infected individuals remains a key strategy for controlling the spread of SARS-CoV-2. Frequent testing of populations to detect infection early in asymptomatic or presymptomatic individuals can be a powerful tool for intercepting transmission, especially when the viral prevalence is low. However, RT-PCR testing – the gold standard of SARS-CoV-2 diagnosis – is expensive, making regular testing of every individual unfeasible. Sample pooling is one approach to lowering costs. By combining samples and testing them in groups the number of tests required is reduced, substantially lowering costs. Here we report on the implementation of pooling strategies using 3-d and 4-d hypercubes to test a professional sports team in South Africa. We have shown that infected samples can be reliably detected in groups of 27 and 81, with minimal loss of assay sensitivity for samples with individual Ct values up to 32. We report on automation of sample pooling, using a liquid-handling robot and an automated web interface to identify positive samples. We conclude that hypercube pooling allows for the reliable RT-PCR detection of SARS-CoV-2 infection, at significantly lower cost than lateral flow antigen (LFA) tests.

Introduction

A novel coronavirus, SARS-CoV-2, emerged at the end of 2019 in the city of Wuhan, China. The highly transmissible nature of SARS-CoV-2[1] has resulted in a pandemic which continues to persist. South Africa (SA) and other African countries are currently facing a resurgence or “second wave” of infections, which in some countries is more severe than the first.[2–4] Although there are approved vaccines against SARS-CoV-2, these are not yet available in amounts sufficient to control the pandemic. To make matters worse, there are new SARS-CoV-2 variants that have been identified in SA,[5] which may be associated with higher transmissibility and hence a more rapid spread of the virus. Therefore, there is an urgent need for more efficient population screening and isolation of infected individuals to reduce the transmission of SARS-CoV-2.

Pre-symptomatic or asymptomatic individuals, who are infectious viral carriers,[6] are the hidden drivers of the pandemic. They represent an estimated proportion ranging from 18–81% of infections,[7] thereby posing a major challenge to the containment of SARS-CoV-2. If such individuals can be efficiently detected through frequent, repeated population testing at scale and thereby enabled to isolate before they infect others, the spread of the virus can be prevented.[8] Therefore, efficient and affordable, high throughput SARS-CoV-2 testing is highly desirable as a means to control the pandemic.

Reverse-transcription real-time polymerase chain reaction (RT-PCR) testing is the gold-standard technology used for SARS-CoV-2 diagnosis. This test can cost up to US$ 56 (ZAR 850) per test, making high-throughput RT-PCR testing of every individual impractical. Sample pooling offers an attractive solution. By combining samples and testing them together, instead of performing individual tests, one can significantly reduce the number of tests and the associated labour and consumable costs. This method was first proposed by Dorfman[9] in 1943. Hypercube pooling, developed by Mutesa *et al* (2020),[8]

requires even fewer tests and yields greater cost savings. For example, at viral prevalences $p < 0.05\%$, hypercube pooling yields a 100-fold cost-reduction versus 22-fold for Dorfman's algorithm. Hypercube pooling therefore offers a highly affordable means of testing large numbers of samples. We also describe a quantitative cost comparison with lateral flow antigen (LFA) tests, finding hypercube pooling to have a significant cost advantage at low viral prevalence (Supplementary Information - Cost comparison of hypercube-based pooled tests vs. lateral flow antigen tests).

Hypercube pooling has been elegantly explained in the literature.[8] Briefly, the samples to be tested are divided into equally sized subsamples which are pooled together according to a mathematical algorithm. Here we report on pooled testing methods which can uniquely identify infected samples among groups of 27 and 81 samples (using 3- and 4-dimensional hypercubes, respectively), in far fewer than 27 or 81 tests. If the group is negative, then all individual samples in that group are deemed negative. If a group is positive, then its sub-samples are recombined in the form of slices within the hypercube, corresponding to different overlapping sub-pools. Groups of 27 or 81 samples are sub-pooled into 9 and 12 slices, respectively, each consisting of 9 or 27 subsamples, respectively. Each individual sample is then represented in 3 or 4 different slices, respectively, and the test results for the slices can be used to infer which individual sample is positive, based on its consistent detection within each of the hypercube slices, without an individual test ever being required. The slicing patterns for these groups are shown in the Supplementary Information Tables 1 and 2 and the complete workflows are presented in Fig. 1.

Although the pooling strategy described above offers considerable benefits over individual testing, it does involve tedious and repetitive pipetting steps, which can be cumbersome when performed manually and can raise the risk of human error. For example, a 3-d hypercube involves 108 precise pipetting steps in different combinations, *i.e.*, a group pool of 27 sub-samples plus 9 slice sub-pools of 9 sub-samples each. The 4-d hypercube involves 405 pipetting steps, *i.e.*, the first, group pool of 81 sub-samples plus 12 slice sub-pools of 27 sub-samples each. To reduce these intensive demands on laboratory staff as well as the risk of human error, we have implemented an automated pipeline using a liquid-handling robot (Opentrons OT-2). This open source robotic platform lends itself to scalability and rapid deployment. The OT2 is modularly constructed and utilises widely available (non-proprietary) electronic components that are neither difficult nor expensive to repair or replace. Flexibility is offered through the Python Application Programming Interface (API) which simplifies custom protocol development. Additional laboratory automation we describe here is the automatic determination of the individual positive result(s) and the de-bundling (or ungrouping) of pooled results into individual results using an automated web interface. Advantages of automated sample pooling include increased human-resource time (which can be better allocated for data analysis, experimental setup, etc.), elimination of the possibility of human error (due to fatigue or distraction, for example), reducing costs associated with manual labour and the flexibility to easily replicate or modify protocols based on the starting number of samples and desired pooling strategy.

Results

4-d 81-sample hypercube experiments:

The results of the 4-d hypercube experiments, in which a single positive sample is diluted up to 81-fold, are shown in Fig. 2. The plots are based on the Ct scores obtained for each of the three target genes (experiments A – H, Supplementary Information Table 4).

The operational sensitivity of the pooled test procedure is decreased for samples with an initial (undiluted) Ct value exceeding 32 as indicated by experiments D and H, where amplification in the 81-pool samples was not successful (less than three of the target genes were detected).

Efficiency – number of tests saved using the pooling method:

We received approximately 121 samples per week, over an 8 week period for testing. By testing 3 groups of 27 or 81 pools, an approximate 40-fold reduction in the number of tests used was achieved (24 tests versus 968 tests for pooled testing and individual testing, respectively).

Laboratory automation:

We created an automated and easy to use web interface to assist in de-bundling of pooled testing results and inference of the positive sample(s) in a pool.

The de-bundling module requires as input the analysis file generated from the pooled results and an individual sample identification file to generate the report. The de-bundling report can then be easily integrated into a laboratory information management system (LIMS) such as LabWare 7 in the case of our laboratory, for individual result reporting.

To infer the positive sample, the user specifies the pool size, i.e. 27 or 81, the number of positive slices and their respective slice numbers. The application then uses this information to determine and output individual positive test results. Both applications (freely available at http://krisp2.ukzn.ac.za:8080/rega-pooling/pooling; Supplementary Information Fig. 4) were used for all the analysis and reporting of results described in this paper.

Discussion

Early identification and isolation of SARS-CoV-2 infected individuals remains a key strategy to interrupt community transmission. In this pilot study, we successfully implemented hypercube pooling for frequent testing of a professional sports team. We show that this procedure reliably detects a single positive sample in a group of 81, provided the starting sample has a Ct $\leq$ 32. Whilst there is a need for further evaluation, particularly with samples with a wider range of Ct values and pools with multiple positive samples (for different viral prevalence scenarios), these results suggest that the pooling strategy is indeed a promising approach for cost-efficient RT-PCR testing.

Other studies have demonstrated successful implementation of pooled testing using groups of up to 5 and 8 samples each.[6] To our knowledge, this is the first study that has implemented the SARS-CoV-2

hypercube-based pooled testing strategy, using group sizes as large as 81.

We selected for analysis samples with Ct values that are typical for the population under study. For comparison, we also selected samples with Ct values that are one standard deviation away from the mean Ct value. We used historical data from the population of samples tested routinely in KZN to determine the distribution of Ct values present at various percentile ranges (Supplementary Information Fig. 1). The asymmetrical (left-skewed) distribution of the data may be attributed to the population of samples that was used to generate the curve i.e. symptomatic people with active disease (and therefore lower Ct scores) presenting at health facilities.

The retention of the sensitivity of the pooled test procedure as the hypercube dimension size (and consequently the dilution) increases is an obvious concern.

Our results demonstrated no loss of assay sensitivity for samples with an initial (undiluted) Ct value ≤ 32 (Fig. 2). Samples with higher Ct values would typically have a lower viral load, which may be deemed as clinically and / or epidemiologically insignificant, since the infection is not likely to be contagious (a cut-off Ct > 30 can be associated with non-infectious samples).[10]

Propagating SARS-CoV-2 from clinical samples can also be used as a valuable proxy for infectiousness. There are, however, conflicting reports on the cut-off Ct at which the virus is not cultivable which can range anywhere from 30 to > 35.[11,12]

In any event, high Ct values are expected to be associated with low infectivity;[13] the maximum viral load occurs during the onset of symptoms and presents the highest risk of transmission.[14] In respiratory samples, the viral load is highest during the initial stage of infection (patients in the early stages of infection usually have Ct values of 20–30 or less[12]), and reaches a peak in the second week followed by lower viral loads.[15] The pooling strategy we have implemented is therefore most beneficial when applied to low-prevalence, asymptomatic or pre-symptomatic people who are on the viral load incline. In addition, the effects of the loss of assay sensitivity can be mitigated by implementing more frequent testing – a solution which could be more easily afforded given the cost savings of the pooled testing strategy.

There have also been reports of inherent inaccuracy due to higher false negative rates associated with the pooled testing method.[16] Our method, however, includes many consistency checks. For example, detection of a single positive slice pool with the other slice pools negative is indicative of a testing mistake.[8] This is especially beneficial when compared to methods such as binary testing methods[17] which rely on repeated testing of the positive sample in subsequent rounds of testing, which can incorrectly terminate the identification of the positive where there are false negative results. Another cause of false negative results is sequence variation at primer or probe binding sites on the viral RNA.[18] In South Africa, a new SARS-CoV-2 lineage (501Y.V2) characterised by various mutations in the spike (S) gene has been reported.[3] However, there was no apparent loss of sensitivity for the S-gene in the RT-PCR assay used in this study.

We have developed and successfully implemented a pilot SARS-CoV-2 pooled testing strategy for a prominent South African rugby team. The successful implementation of pooled testing requires that 3 important criteria are met *viz*. (i) efficiency, (ii) sensitivity and (ii) operational feasibility[6]. Our study was successful in satisfying each of these criteria by (i) achieving a 40-fold reduction in test usage (and therefore cost) compared to individual testing. The cost reduction is most impressive when all individuals are negative, because 27 or 81 negative results can be achieved using just one qPCR reaction, in a 3-d or 4-d hypercube, respectively. This was the case with the screening of the South African Rugby team at the KRISP laboratory, where, during the first 8 weeks, all of the samples were tested using pooled tests of 27 or 81 samples, and only in the 9th week of consecutive tests were an additional round of slice testing required as the prevalence rose; (ii) no significant loss of sensitivity for samples with appreciable viral loads (Ct $\leq$ 32) was demonstrated and (iii) the practical nature of our pooling workflow, *i.e.*, the implementation of liquid-handling robots for automated pooling and software applications for the automated identification of the positive sample.

There are more conventional methods for high-throughput diagnostic testing which are available. For example, SARS-CoV-2 lateral flow antigen (LFA) tests are point of care rapid tests that can be used for large-scale screening. Although LFAs may be appealing because they are cheap, do not require a laboratory with specialized equipment or personnel and can provide results in 15–30 minutes, they do present some limitations; the sensitivity of this type of technology can have considerable variation (average sensitivity of 56.2% (95% CI 29.5 to 79.8%),[20] thereby decreasing its utility in screening certain populations (such as health-care workers and other front-line personnel). We have performed a detailed cost comparison of the hypercube-based pooled testing strategy compared to the LFAs. We estimate that, at prevalences below 0.43%, it costs over 6 times more to achieve reliable detection of SARS-CoV-2 infectious individuals by using LFAs compared to hypercube testing (assuming a detection probability of 99.9%) (Supplementary Information - Cost comparison of hypercube-based pooled tests vs. lateral flow antigen tests).

The pooled testing strategy described in this study, together with the downstream automation has led to a significant reduction in cost, kit usage and turnaround time at our laboratory. Our application of hypercube pooling to frequent testing of a professional sports team can potentially be extended to other low prevalence, asymptomatic population groups. Further evaluation of this approach in different epidemic situations (with variable prevalence) is required.

Online Methods

SARS-CoV-2 clinical diagnostics:

The standard protocol used for SARS-CoV-2 detection at the KRISP Laboratory (University of KwaZulu-Natal) is to extract viral ribonucleic acid (RNA) from nasopharyngeal and / or oropharyngeal swab samples. This is followed by RT-PCR (TaqPath COVID-19 CE-IVD RT-PCR kit, ThermoFisher Scientific, MA, USA) for the detection of three SARS-CoV-2 target genes (viz. S-gene, N-gene and ORF1ab). A sample is

considered “positive” if at least 2 out of the 3 target genes are amplified with an above-background fluorescence signal (cycle threshold, Ct) £ 40 PCR cycles. A sample is considered “inconclusive” if 1 out of the 3 target genes are positive (Ct £ 40) and should be repeat tested. A sample is considered “negative” if none of the 3 target genes are amplified. Swab samples collected in viral transport medium (VTM) for routine SARS-CoV-2 surveillance in KwaZulu-Natal, South Africa were used in this study. All experiments were conducted in accordance with relevant local guidelines and regulations.

Historical data from a population of (approximately 1200) samples tested routinely in KZN was used to determine the distribution of Ct values present at various percentile ranges (Supplementary Information Fig. 1). Positive samples used for each of the experiments were chosen based on their respective Ct scores to represent the range of different Ct values.

Pooling experiments were conducted to verify: (1) the successful detection of the positive sample using a 27-sample pool 3-d hypercube; and (2) the successful detection of the positive sample using an 81-sample pool 4-d hypercube (i.e. at an increased dilution).

27-sample, 3-d hypercube experiments

Three positive samples were used in three independent pooling tests to evaluate proof-of-concept and determine whether these samples would still result in successful PCR amplification when diluted 9- and 27-fold using 3-d hypercubes.

Each positive sample was combined with 26 negative swab samples as follows: sample tubes were labelled from 1 to 27. Samples were added to each of the labelled tubes, with the positive sample inserted blindly (i.e. in the position of the hypercube not known to the person performing the pooling experiments or the analysis of results). Each sample was vortexed briefly and 200 ml was manually pipetted into a separate collection tube containing the pooled sample. The collection tube (total volume of 5400 ml) was then briefly vortexed and 200 ml was used for paramagnetic bead-based RNA extraction using the Chemagic360 automated extraction system (PerkinElmer Inc., Cat.# CMG-1049, MA, USA). The purified nucleic acid was eluted in 100 ml of elution buffer. 10 ml of RNA was then used in the RT-PCR reaction (TaqPath™ COVID-19 CE-IVD RT-PCR Kit, Thermo Fisher Scientific).

Pools that were positive were sub-pooled into 9 slices, containing 9 samples each (Supplementary Information - Table 1). For each of the experiments, every individual sample was represented in 3 different slices.

81-sample, 4-d hypercube experiments

Positive samples were chosen based on their respective Ct values to ensure that they were within the range of those typically present in the distribution (18 – 34) and used in independent pooling experiments.

One positive swab sample was combined with 80 negative swab samples in each of eight experiments. For each experiment, sample tubes were labelled from 1 to 81, and the positive sample was inserted blindly. Each sample was vortexed briefly and 200 ml was added to a separate collection tube containing the pooled sample. The collection tube (total volume of 16 200 ml) was then briefly vortexed and 200 ml was used for RNA extraction and subsequent RT-PCR, as described above. The pool was further sub-pooled into 12 slices containing 27 samples each (Supplementary Information - Table 2). Each individual sample was represented in 4 different slices.

Based on the consistent amplification of the positive sample across the respective slices in each of the 3-d and 4-d hypercube experiments, the location of the positive sample can be deduced.

Laboratory robotics:

The robotics work flow is summarised through steps 1-4 (Supplementary Information Fig. 3):

1. A new 200 µl tip is collected by the single-channel 300 µl electronic pipette.
2. The electronic pipette draws 200 µl from the sample rack for the current slice iteration.
3. The electronic pipette dispenses the sample in the relevant sample sub-pool.
4. The tip is discarded into the robot's trash.

The programming codes for the 3-d and 4-d pooling experiments using the OT-2 liquid handling robot is available at https://github.com/krisp-kwazulu-natal/efficient-SARS-CoV-2-pooled-testing-strategy-code

Software for de-bundling and inference of positive results

Custom scripts were implemented in Python for de-bundling and inference of positive results from a pool of 27 or 81 samples. When inferring positive results, our scripts take into account degenerates and can infer more than one positive per pool at low prevalence. For ease of use, we developed a web-based front end using the Java Web Toolkit (Jwt) an open-source, robust Java web front end (GUI; http://webtoolkit.eu/jwt) library. Minimal user interaction is required as user interaction is limited to uploading the files to be processed or providing pool details in the case of inference and initiating the analysis. A report detailing the analysis results is provided to the user once the processing is complete. Our de-bundling and inference software is freely available and can be accessed at the URL: http://krisp2.ukzn.ac.za:8080/rega-pooling/pooling. The programming scripts for the software we developed is available at https://github.com/krisp-kwazulu-natal/efficient-SARS-CoV-2-pooled-testing-strategy-code

Declarations

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LS, UJA, WN and NT designed the study; LS, UJA and SAM co-ordinated the experiments, performed validation of the pooling experiments, data analysis and interpretation; LS and UJA wrote the manuscript;

WN, NT and RL contributed to editing the manuscript; WN and NT contributed to the theory; IS, SB, TL contributed to the development and implementation of the robotics automation workflow; HT and EJS developed and implemented the scripts used for the automated web applications; FP and TdO supervised the project; all authors reviewed and approved the final manuscript.

Ethics declarations

We used de-identified remnant nasopharyngeal and oropharyngeal swab samples from patients testing positive for SARS-CoV-2 by RT–qPCR from public health laboratories in South Africa. The project was approved by University of KwaZulu-Natal Biomedical Research Ethics Committee (protocol reference no. BREC/00001195/2020; project title: COVID-19 transmission and natural history in KwaZulu-Natal, South Africa: epidemiological investigation to guide prevention and clinical care).

Competing interests

The authors declare no competing interests.

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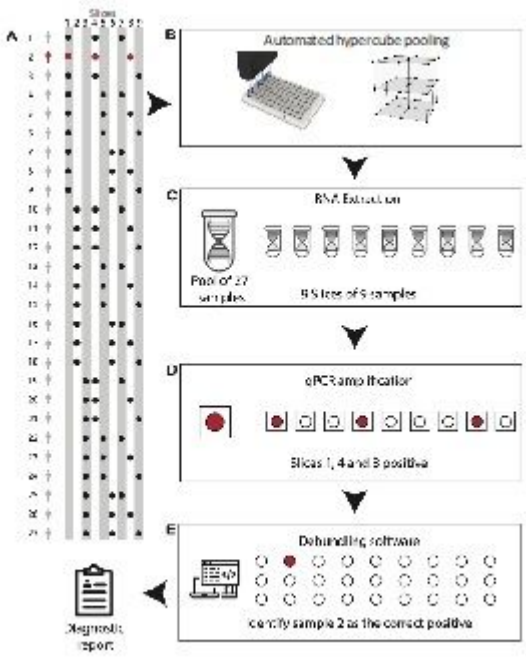
Figures



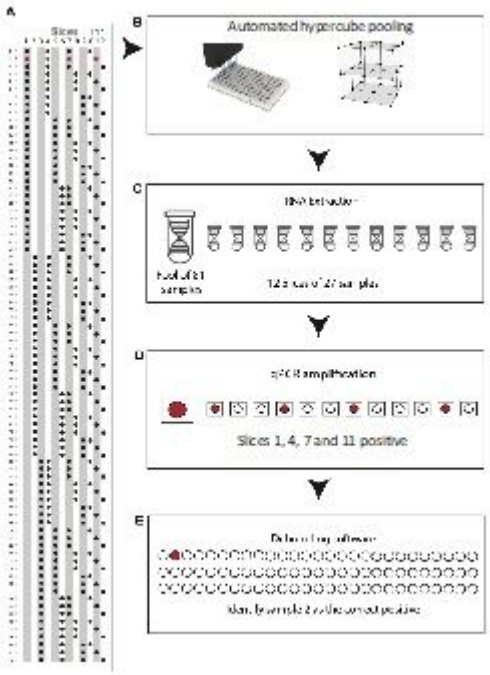


Figure 1

Examples of 3-dimensional (left) and 4-dimensional (right) hypercube workflows; (A) one positive sample (red, sample 2) is contained in a group of 27 samples or, respectively, 81 samples; (B) 9 (resp. 12) slice pools, each consisting of 9 (resp. 27) sub-samples, are created using a liquid handling robot; (C) RNA extraction is done on the group pool of 27 (resp. 81) samples (1 reaction) and 9 (resp. 12) slice pools (9 or 12 reactions); (D) qPCR results are positive for the group of 27 (resp. 81) samples and the respective slices 1, 4 and 8 (resp. 1, 4, 7, and 11); (E) De-bundling software is used for the automatic determination of the positive sample in the hypercube and for generating individual diagnostic reports.

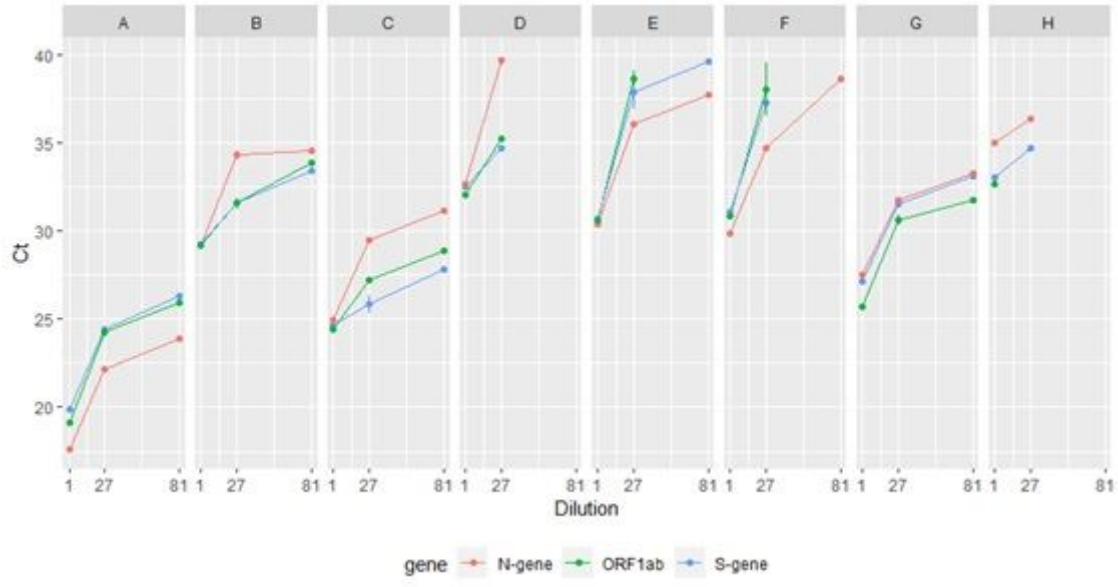


Figure 2

Detection of a single positive sample out of 81 in a 4-d hypercube. Ct scores are shown for: a) the undiluted sample, b) the mean and standard errors for the 4 slice pools in which the positive sample is diluted 27-fold, and c) the hypercube pool in which the single positive is diluted 81-fold.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryinformation.pdf