

Evaluation of COVID-19 RT-qPCR test in multi-sample pools

Idan Yelin^{1*}, Noga Aharony^{1*}, Einat Shaer Tamar^{1*}, Amir Argoetti^{1*}, Esther Messer², Dina Berenbaum¹, Einat Shafran⁶, Areen Kuzli⁶, Nagham Gandali⁶, Omer Shkedi⁴, Tamar Hashimshony¹, Yael Mandel-Gutfreund^{1,7}, Michael Halberthal^{+3,4}, Yuval Geffen⁺⁵, Moran Szwarcwort-Cohen⁺⁶, Roy Kishony^{+1,7}

(1) Faculty of Biology, Technion - Israel Institute of Technology, Haifa, Israel. (2) Safety Unit, Technion - Israel Institute of Technology, Haifa, Israel. (3) Rambam Health Care Campus, Haifa, Israel. (4) Faculty of Medicine, Technion - Israel Institute of Technology, Haifa, Israel. (5) Bacteriology laboratory, Rambam Health Care Campus, Haifa, Israel. (6) Virology laboratory, Rambam Health Care Campus, Haifa, Israel. (7) Computer Science Department, Technion - Israel Institute of Technology, Haifa, Israel.

* These authors contributed equally to this manuscript

Corresponding author: Prof. Roy Kishony, Marilyn and Henry Taub Professor of Life Sciences, Faculty of Biology and Computer Science Department, Technion - Israel Institute of Technology, Haifa, Israel. +972-77-887-3737, rkishony@technion.ac.il

+ Correspondence regarding sample collection and medical interpretation should be set to M. H. (m_halberthal@rambam.health.gov.il), Y. G. (y_geffen@rambam.health.gov.il), or M. S-C (M_Szwarcwort@rambam.health.gov.il);

correspondence regarding the experimental procedure and data analysis should be sent to R. K. (rkishony@technion.ac.il).

Summary: A single positive sample can be detected in pools of up to 32 using the standard COVID-19 RT-qPCR test. Such pooling methodology, immediately applicable using current equipment and reagents, will allow routine population surveillance while conserving scarce resources.

Accepted Manuscript

Abstract

Background

The recent emergence of SARS-CoV-2 lead to a current pandemic of unprecedented scale. Though diagnostic tests are fundamental to the ability to detect and respond, overwhelmed healthcare systems are already experiencing shortages of reagents associated with this test, calling for a lean immediately-applicable protocol.

Methods

RNA extracts of positive samples were tested for the presence of SARS-CoV-2 using RT-qPCR, alone or in pools of different sizes (2-, 4-, 8-, 16-, 32- and 64-sample pools) with negative samples. Transport media of additional 3 positive samples were also tested when mixed with transport media of negative samples in pools of 8.

Results

A single positive sample can be detected in pools of up to 32 samples, using the standard kits and protocols, with an estimated false negative rate of 10%. Detection of positive samples diluted in even up to 64 samples may also be attainable, though may require additional amplification cycles. Single positive samples can be detected when pooling either after or prior to RNA extraction.

Conclusions

As it uses the standard protocols, reagents and equipment, this pooling method can be applied immediately in current clinical testing laboratories. We hope that such implementation of a pool test for COVID-19 would allow expanding current screening capacities thereby enabling the expansion of detection in the community, as well as in close organic groups, such as hospital departments, army units, or factory shifts.

Keywords: SARS-CoV-2, COVID-19, diagnostics, disease surveillance

Introduction

The ongoing pandemic of the recently-emerged SARS-CoV-2 is critically challenging health systems worldwide. The virus is characterized by fever and severe acute respiratory syndrome^{1,2}. As of March 17, the World Health Organization (WHO) has reported over 170,000 cases with over 10,000 new diagnoses added in 24 hours³.

Detecting carriers of the virus is fundamental to response efforts. It ensures the quarantine of COVID-19 patients to prevent local spread¹, and more broadly informs national response measures⁴. Nevertheless, as monitoring capacity is limited, testing in most countries is generally focused on acutely ill patients, while potentially infectious carriers at the community remain undiagnosed. As many countries are already experiencing shortages of diagnosis kits and factories struggling to keep with the demand⁵⁻⁷, it has become important to come up with new ways to conserve the reagents used for diagnostic tests. At the same time, as the disease is novel, it is of value to validate any modifications to the testing process before universal adoption^{8,9}.

Pooling diagnostic tests has been applied in other infectious diseases and is especially attractive as it requires no additional training, equipment, or materials. In this method, first suggested by Dorfman in 1943¹⁰ and perfected over the years¹¹⁻¹³, samples are mixed and tested at a single pool, and subsequent individual tests are made only if the pool tests positive. In addition to being used in the clinic for infectious disease diagnostics in previous epidemics^{14,15}, pooling has been proven to work for RT-qPCR^{16,17}, a time-consuming step for which the reagents are expected to be in short supply¹⁸. Nonetheless, as SARS-CoV-2 is a novel pathogen, it is unclear how diluting a sample containing its RNA would affect the sensitivity of this assay and the false-negative rate.

Here, we test the ability of the standard RT-qPCR test for detecting a single positive sample within a pool of negative samples. First, pooling RNA extracts of clinical samples, we tested previously-confirmed positive samples alone and combined with an increasing number of previously-confirmed negative samples and found that positive samples can still be well observed in pools of up to 32 samples, and possibly even 64 with additional PCR cycles. Second, pooling prior to RNA extraction was tested and successfully demonstrated for 8-sample pools.

Methods

Sample collection

Swabs from both nostrils and the throat were previously collected by healthcare providers and sent to the Virology laboratory at the Rambam Health Care Campus, Haifa, Israel. A volume of 130 microliter of the transport swab buffer was mixed with 270 microliter lysis buffer and RNA was extracted using magLEAD (Precision System Science). We obtained samples tested between March 4-15, 2020.

Individual RT-qPCR tests in the clinical laboratory

RT-qPCR was performed in the clinical laboratory to detect the presence of SARS-CoV-2 RNA with AgPath-ID™ One-Step RT-PCR Reagents (Thermo Fisher Scientific) in a Bio-Rad CFX 96 qPCR machine with WHO primers and probe (E_Sarbeco_R: ATATTGCAGCAGTACGCACACA, E_Sarbeco_F: ACAGGTACGTTAATAGTTAATAGCGT, E_Sarbeco_P: AACTAGCCATCCTTACTGCGCTTCG)¹⁹. Reactions were heated to 50°C for 30 minutes for reverse transcription, denatured in 95°C for 10 minutes and

then 46 cycles of amplification were carried in 95°C for 15 seconds and 55°C for 32 seconds. Fluorescence was measured using the FAM parameters.

Pooling prior to RT-qPCR

We arbitrarily chose 5 positive samples and 67 negative samples. 66 of the negative samples were mixed into pools of different sizes containing equal volumes of 1, 2, 4, 8, 16 and 32 unique samples. Negative pools of size 1 and 2 were prepared in duplicates made of different samples to determine whether different negative-sample composition in the pool affected the detection of positive samples. The final 67th sample was mixed with the pool of negative samples as control for the positive samples. The negative pools were distributed in 6 rows of a 96-well plate, 5ul per well, and 10ul of the positive samples and the 67th negative sample were distributed in the 7th row. 5ul of the positive samples were then diluted into the “pool” of 1 negative sample to make a ½ dilution, then the ½ dilution was diluted in the 2 samples pool to make a ¼ dilution etc., up to 1/64. Finally, 20ul of the RT-qPCR reagent mix were added to each well.

Pooled-samples RT-qPCR in the research laboratory

Laboratory RT-qPCR procedure was performed according to the procedure for individual samples in the clinical laboratory, on an identical qPCR machine and program and with reagents used at the Rambam Health Care Campus. To conserve resources and allow multiple pooling and duplicates of the same sample, each sample was diluted by X0.4 prior to mixing with reagents.

Pooling prior to RNA extraction

To test the ability of pooling prior to RNA extraction, transport swab buffers were taken from the collection tubes of 3 previously-confirmed positive samples and mixed at equal volumes with the sample transport buffer from the collection tubes of 7 previously-determined negative samples. A volume of 500 microliter from the pooled tube was mixed with 2ml lysis buffer for inactivation and RNA was extracted using NUCLISENS easyMAG (biomerieux) and eluted in 50 microliter elution buffer. RT-qPCR was performed in the clinical laboratory to detect 3 SARS-CoV-2 genes: N, E, and RdRP. RNaseP was used as an internal control. RT-qPCR was done using Seegene - Allplex 2019-nCov Assay, in a Bio-Rad CFX 96 qPCR machine. Reactions were heated to 50°C for 20 minutes for reverse transcription, denatured in 95°C for 15 minutes and then 45 cycles of amplification were carried in 94°C for 15 seconds and 58°C for 30 seconds. Fluorescence was measured using four fluorescence channels: FAM (E gene), HEX (internal control), Cal Red 610 (RdRp gene), Quasar 670 (N gene).

Ethical approval

This study was granted exemption from IRB approval for use of deidentified discarded RNA samples of COVID-19 tests by The Rambam Health Care Campus IRB committee.

Results

Pooling Prior to RT-qPCR Test

The original diagnostic run at the Rambam Health Care Campus was robust. Positive samples had on average 135 ± 32 -fold stronger fluorescence relative to negatives, and the positive samples reached the threshold, which we set at a fluorescence of 300 according to CDC guidelines⁸, at the 25.5 ± 6.1 cycle (Figure 1A). The five positive samples selected similarly averaged at Ct of 24.5 ± 3.1 and maximum fluorescence of 5164 ± 912 (Figure 1B).

As the number of negative pooled samples increases, the amplified RNA reaches the threshold later, as expected from a diluted sample (Figure 2). Except for a single replicate (POS #2), all samples reached the threshold in 32-sample pools. For most samples we observed a linear correlation between when the threshold is reached and the doubling of the pool size. On average, for each dilution by a factor of 2, we observed an increase of 1.24 in Ct, corresponding with the expectation that an RNA sample that is diluted twice as much will ideally require one more cycle to double in amount and reach the same fluorescence (Figure 3). The observed linearity indicates that in most cases there is no RNA interference with the reverse transcriptase or DNA polymerase enzyme. From the average slope of 1.24, the expected Ct following pooling was extrapolated for all positive samples and sensitivity was calculated for different pool sizes and different cycle cutoffs (Supplementary Figure 1). Even for a relatively restrictive cutoff of 40 cycles, pooling would reach sensitivity of 96% for a pool size of 16 samples.

Of the ten tested replicates, only duplicate B of sample #2 did not cross the threshold in pools of 32. Moreover, with the exception of this specific duplicate, the fluorescence of all 64-sample pools increased in a sigmoidal manner. In contrast, negative samples in duplicate B in the 64-, 32-, and 2-sample pool also began to increase but none maintained a sigmoidal pattern or crossed the threshold (Figure 2).

Pooling Prior to RNA Extraction

To test whether the pooling approach can be applied even before RNA extraction, the transfer media of three single positive samples were pooled each with the transfer media of 7 negative samples. Following RNA extraction of the pooled samples, extracts were subjected to amplification of genes N, RdRp and E (each measured in a separate channel). All samples, even a relatively weak sample with Ct values between 29.0-31.8, were detected in the 8-sample pools in all three channels. Compared to unpooled tests, the Ct of pools increased, on average, by 3.6 ± 2.1 , 2.9 ± 0.45 and 2.4 ± 0.11 for genes E, RdRp and N, respectively (Fig. 4, Supplementary Fig. 2). The average increase in Ct was 2.9, which is in accordance with an 8 fold dilution of the initial samples.

Discussion

We found that a single clinical sample with SARS-CoV-2 RNA can be consistently detected in a pool of up to 16 to 32 samples. Pooling this way leads to only a linear increase in the threshold cycle (Ct). Even for a relatively restrictive cutoff, pooling would reach sensitivity of 96% for a pool size of 16 samples. For high-fold pooling, our data shows an estimated false negative rate of 10% (1 out of 10), which is relatively small compared to the inherent clinical sensitivity of the standard assay²⁰. We further demonstrate that pooling works well also when applied prior to RNA extraction.

RT-qPCR could be further optimized for the detection of low-concentration RNA. For instance, additional amplification cycles could lower detection limit allowing better detection for pools of more than 32 samples, which based on extrapolation of the data we expect would allow the 64-sample pools of positive sample #5 to cross the threshold. In addition, some abnormalities as with duplicate B in positive samples #5 and #2 could have been due to interference from contamination in one or more of the samples. The unusual peaks for positive sample #2 could be due to a changing salt concentration that disturbed the TaqMan probe. Since both these issues could be solved by

further diluting the RNA samples with water, it is worthwhile to explore whether diluting samples with different ratios of water could improve the integrity of the signal in pooled results. While we tested samples with a range of different signal strengths, the detection of samples with even lower signals may warrant the use of smaller pools. Alternatively, adding a few additional PCR cycles could be considered as a means to increase detection rate of such low viral load samples. In general, as RT-qPCR kits and protocols vary internationally, use of suggested pooling may require validation for each specific setting.

These results can be used not only for pooling, but also in multiplexing and any other signal compression techniques where samples are mixed to reduce the number of tests. We hope that this proof-of-concept will encourage others to develop mathematical and computational tools tailored for the pooling of SARS-CoV-2 tests.

Pooling is especially useful for routine community survey and for monitoring of cohesive groups. Local and global epidemic response critically depend on determining carriage frequency in the population, which is greatly enabled by pooling techniques. Furthermore, pooling techniques can be used for routine monitoring of essential work groups, such as hospital staff, military units, and factory workers. While the frequency of infection in these groups may be low, diagnosing even a single positive person typically requires quarantine of the entire group to prevent further spread in the community. In these surveillance applications, pooling may allow more routine monitoring and detection of low frequency of carriage thereby informing policy makers, reducing transmission, and alleviating the strain on healthcare services.

Funding

This work was supported by Yad Hanadiv - The Rothschild Foundation in Israel.

Conflict of Interest

The authors declare no conflict of interest regarding the publication of this article.

Accepted Manuscript

References

1. Huang, G. *et al.* Triaging patients in the outbreak of the 2019 novel coronavirus. *medRxiv* 2020.03.13.20035212 (2020).
2. Zhou, P. *et al.* Discovery of a novel coronavirus associated with the recent pneumonia outbreak in humans and its potential bat origin. *bioRxiv* 2020.01.22.914952 (2020)
doi:10.1101/2020.01.22.914952.
3. *Coronavirus disease 2019 (COVID-19) Situation Report*. https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200317-sitrep-57-covid-19.pdf?sfvrsn=a26922f2_4 (2020).
4. CDC. Coronavirus Disease 2019 (COVID-19) Situation Summary. *Centers for Disease Control and Prevention* <https://www.cdc.gov/coronavirus/2019-ncov/cases-updates/summary.html> (2020).
5. Esfandiari, S. Wuhan, China, faces such a shortage of coronavirus test-kits that people say getting one is like 'winning the lottery'. *Business Insider* (2020).
6. Ramsey, L. & Woodward, A. The US is struggling to test more people for the coronavirus. Now it's facing a shortage of the materials used to run those tests. *Business Insider* (2020).
7. Knaus, C. & Doherty, B. Global shortage of Covid-19 test kits hits Australia as other nations limit exports. *The Guardian* (2020).

8. CDC. Research Use Only Real-Time RT-PCR Protocol for Identification of 2019-nCoV. *Centers for Disease Control and Prevention* <https://www.cdc.gov/coronavirus/2019-nCoV/about/testing.html> (2020).
9. Li, C. *et al.* High sensitivity detection of SARS-CoV-2 using multiplex PCR and a multiplex-PCR-based metagenomic method. *bioRxiv* 2020.03.12.988246 (2020)
doi:10.1101/2020.03.12.988246.
10. Dorfman, R. The Detection of Defective Members of Large Populations. *Ann. Math. Stat.* **14**, 436–440 (1943).
11. Liu, A., Liu, C., Zhang, Z. & Albert, P. S. Optimality of group testing in the presence of misclassification. *Biometrika* **99**, 245–251 (2012).
12. Nguyen, N. T., Bish, E. K. & Aprahamian, H. Sequential prevalence estimation with pooling and continuous test outcomes. *Stat. Med.* **37**, 2391–2426 (2018).
13. Bilder, C. R. & Tebbs, J. M. Pooled-testing procedures for screening high volume clinical specimens in heterogeneous populations. *Stat. Med.* **31**, 3261–3268 (2012).
14. Nguyen, N. T., Aprahamian, H., Bish, E. K. & Bish, D. R. A methodology for deriving the sensitivity of pooled testing, based on viral load progression and pooling dilution. *J. Transl. Med.* **17**, 252 (2019).
15. Litvak, E., Tu, X. M. & Pagano, M. Screening for the Presence of a Disease by Pooling Sera Samples. *J. Am. Stat. Assoc.* **89**, 424–434 (1994).
16. Arnold, M. E. *et al.* Evaluation of the pooling of swabs for real-time PCR detection of low titre shedding of low pathogenicity avian influenza in turkeys. *Epidemiol. Infect.* **141**, 1286–1297 (2013).
17. Taylor, S. M. *et al.* High-throughput pooling and real-time PCR-based strategy for malaria

detection. *J. Clin. Microbiol.* **48**, 512–519 (2010).

18. Herper, M., Begley, S., Boodman, E., Robbins, R. & Facher, L. Thermo Fisher to produce millions of coronavirus diagnostic tests - STAT. *STAT* <https://www.statnews.com/2020/03/14/thermo-fisher-to-produce-millions-of-coronavirus-diagnostic-tests/> (2020).
19. *Diagnostic detection of 2019-nCoV by real-time RT-PCR.* https://www.who.int/docs/default-source/coronaviruse/protocol-v2-1.pdf?sfvrsn=a9ef618c_2.
20. Wang, W. *et al.* Detection of SARS-CoV-2 in Different Types of Clinical Specimens. *JAMA* (2020) doi:10.1001/jama.2020.3786.
21. *Guidance and standard operating procedure: COVID-19 virus testing in NHS laboratories.* <https://www.england.nhs.uk/coronavirus/wp-content/uploads/sites/52/2020/03/guidance-and-sop-covid-19-virus-testing-in-nhs-laboratories-v1.pdf> (2020).

Accepted Manuscript

Figure 1: Selected Positive Samples are Representative of their Population. A) Fluorescence of all samples as collected and tested at Rambam Health Care Campus between March 4-15. Selected positive samples are coloured (#1:red, #2:blue, #3:green, #4:gold, #5:purple) and are representative of all other positive-testing samples (gray). B) Threshold cycle (Ct) of Positive Samples from Rambam Health Care Campus showing 26 positive samples (out of 388 samples tested) had an average Ct of 25.5+/- 6.1 (standard deviation). Out of these samples, we selected 5 representative positive samples marked POS #1-5 (colored circles).

Figure 2: Fluorescence of pooled samples increases over RT-qPCR cycles.

Fluorescence over cycle during RT-qPCR for all positive samples (#1-#5 and negative control from top to bottom) diluted in different numbers of negative samples (dark red - no dilution, light orange - dilution in 63 negative samples) over duplication A and B (left to right). Almost all pooled positive samples amplify in a sigmoidal curve that crosses the threshold.

Dots represent the cross point of the fluorescence threshold (threshold = 300, gray dashed line).

Figure 3: Positive samples are consistently detected when diluted with up to 31 negative samples.

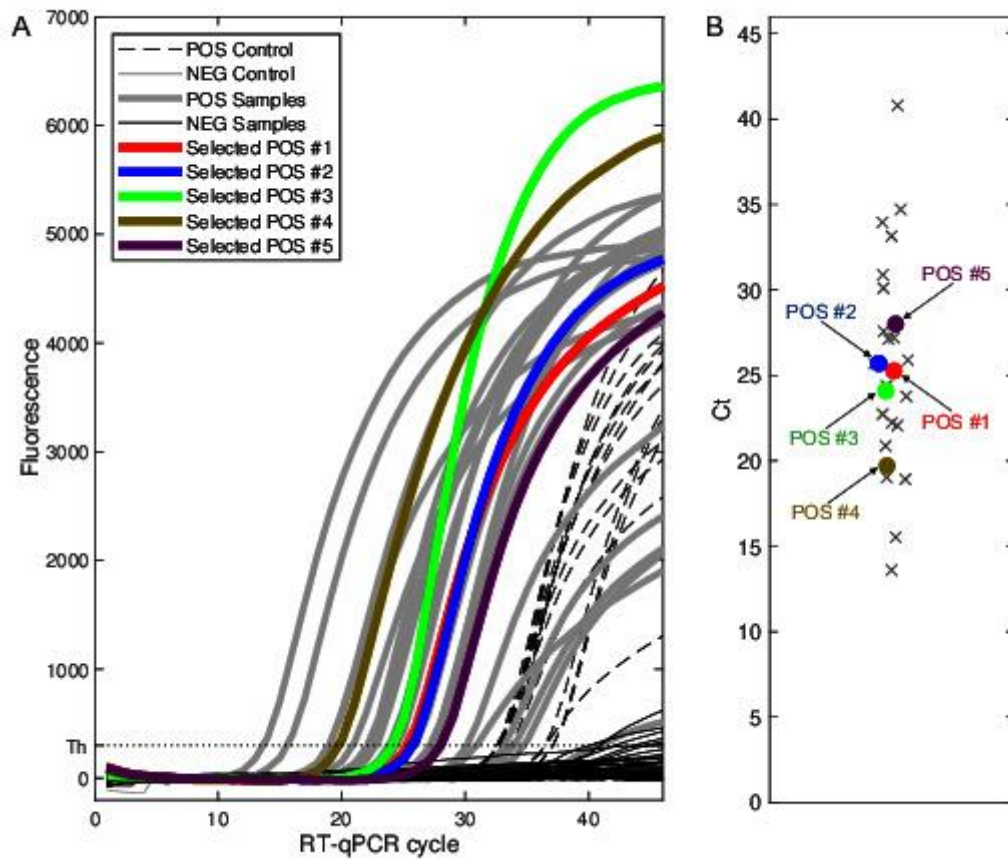
Pool size containing a single positive sample over the RT-qPCR cycle where it crosses the threshold (Filled line - duplicate A, dashed line - duplicate B). Most positive samples reach the threshold at a later Ct as they are more diluted. Samples #2 and #5, which reached the threshold later than others, grew nonlinearly relative to other samples.

Figure 4: Detection of positive samples pooled with negative samples prior to RNA extraction.

Automatically detected Ct of positive samples A, B and C measured individually (Single, blue) or when pooled with 7 negative samples (Pool, red) for all 3 viral genes (E, RdRp, and N). All three genes were identified in all three samples when measured individually or in pools. Measured Ct of pools was on average higher by 2.9, consistent with an effective dilution of 8 fold.

Accepted Manuscript

Figure 1



Accepted

Figure 2

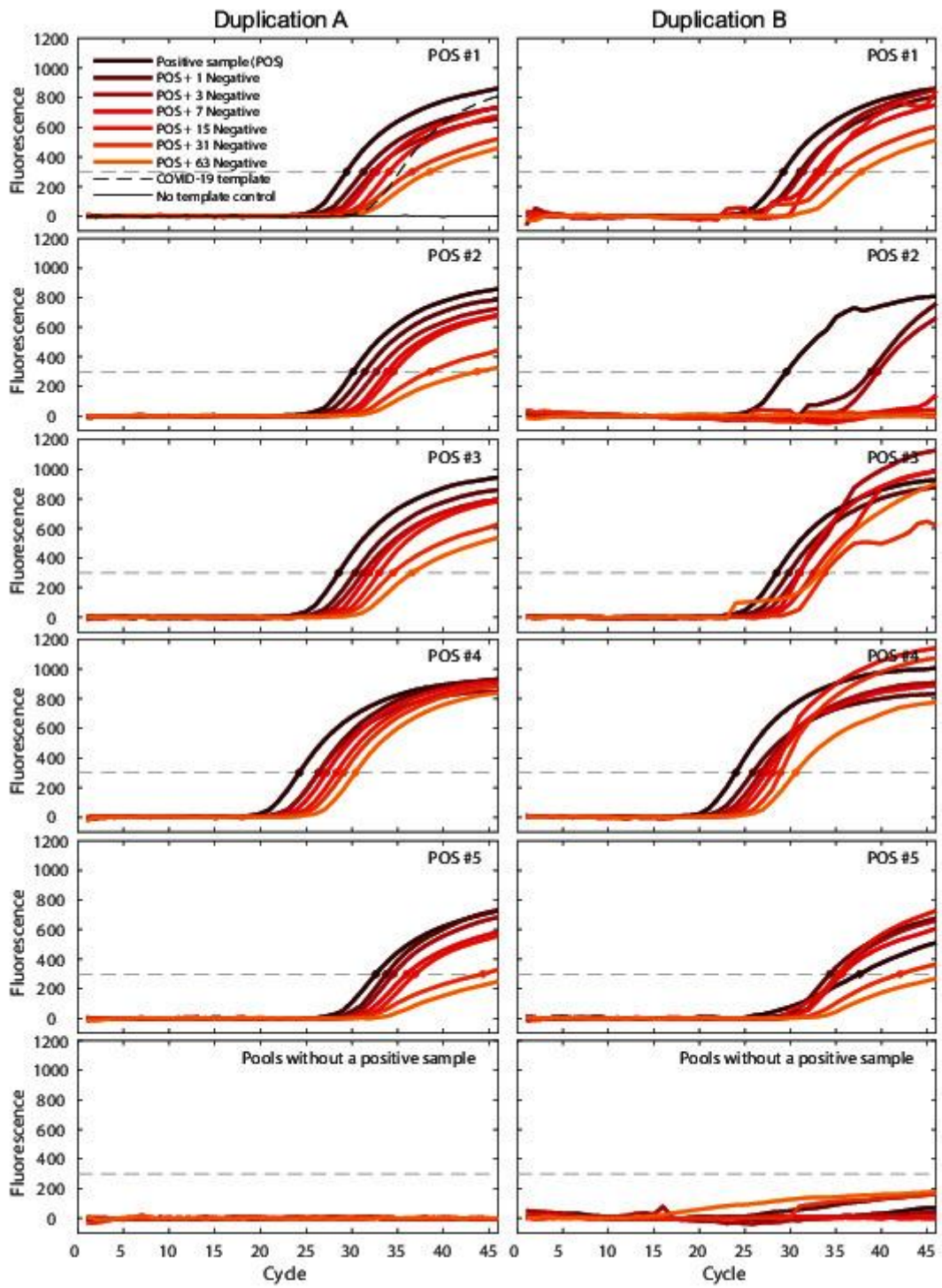
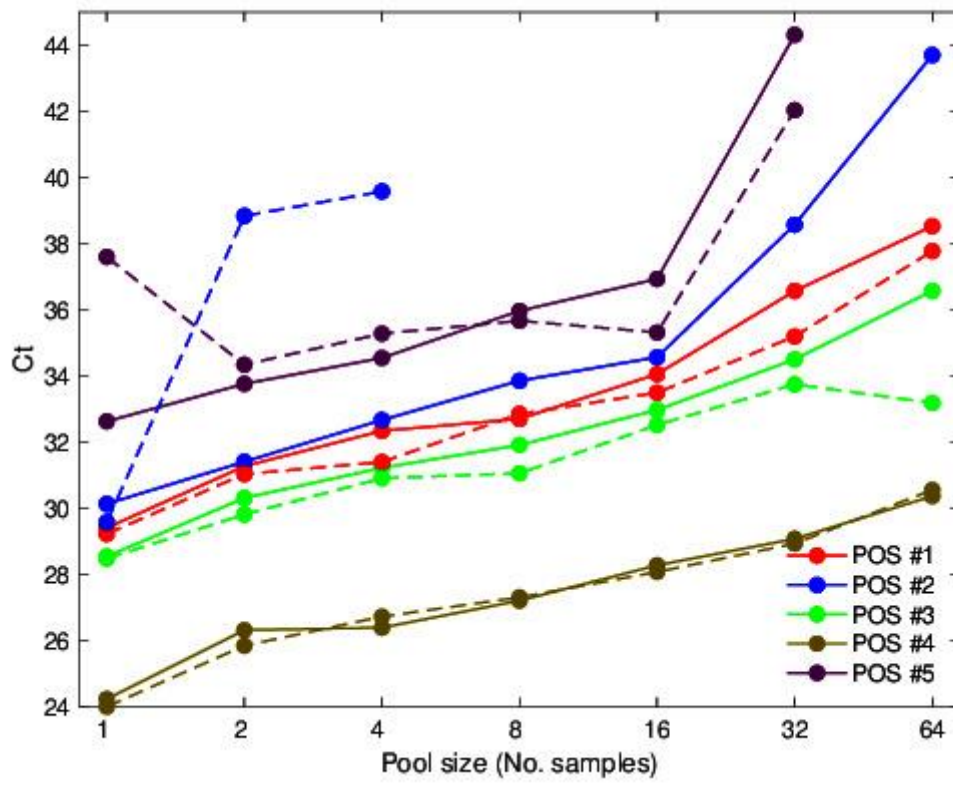
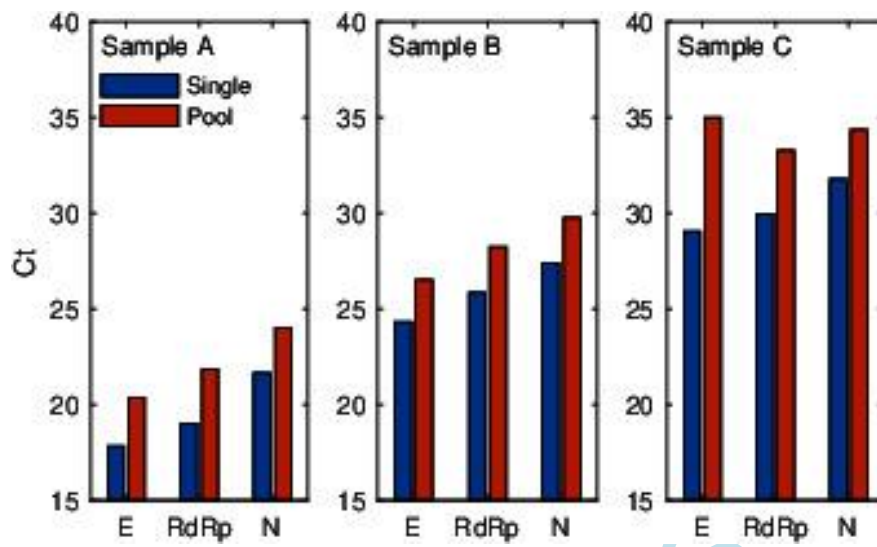


Figure 3



Accepted

Figure 4



Accepted Manuscript