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Experimental and Mathematical Optimization of a Pooling Test for Detection of SARS-CoV-2 in a Population with Low Viral Load

Hyeongseok Jeong, Jooyeon Lee, Shinhye Cheon, Kyung Mok Sohn, Jungok Kim, Sungmin Kym, and Yeon-Sook Kim

Division of Infectious Disease, Department of Internal Medicine, Chungnam National University School of Medicine, Daejeon, Korea

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Corresponding Author:
Yeon-Sook Kim, MD, PhD
Division of Infectious Diseases, Department of Internal Medicine, Chungnam National University School of Medicine, 282 Munhwa-ro, Jung-gu, Daejeon 35015, Korea.
Tel: +82-42-280-6791
Fax: +82-42-280-6970
E-mail: alice@cnuh.co.kr

ORCID iDs
Hyeongseok Jeong
https://orcid.org/0000-0002-4539-079X
Jooyeon Lee
https://orcid.org/0000-0002-0898-4433
Shinhye Cheon
https://orcid.org/0000-0002-1783-121X
Kyung Mok Sohn
https://orcid.org/0000-0002-3237-044X
Jungok Kim
https://orcid.org/0000-0002-0694-1579
Sungmin Kym
https://orcid.org/0000-0003-3518-966X
Yeon-Sook Kim
https://orcid.org/0000-0003-1142-5488

ABSTRACT

Background: A pooling test is a useful tool for mass screening of coronavirus disease 2019 (COVID-19) in the pandemic era. We aimed to optimize a simple two-step pooling test by estimating the optimal pool size using experimental and mathematical validation.
Materials and Methods: Experimental pools were created by mixing one positive respiratory sample with various numbers of negative samples. We selected positive samples with cycle threshold (Ct) values greater than 32 to validate the efficiency of the pooling test assuming a high likelihood of false-negative results due to low viral loads. The positivities of the experimental pools were investigated with a single reverse-transcription polymerase chain reaction (RT-PCR) using the U-TOP™ COVID-19 Detection Kit Plus (Seasun Biomaterials, Daejeon, Korea). We used the Dorfman equation to calculate the optimal size of a pooling test mathematically.
Results: Viral RNA could be detected in a pool with a size up to 11, even if the Ct value of a positive sample was about 35. The Dorfman equation showed that the optimal number of samples in a pool was 11 when the prevalence was assumed to be 0.66% based on the test positivity in Daejeon, Korea from April 1, 2020 to November 10, 2020. The efficiency of the pooling test was 6.2, which can save 83.9 of 100 individual tests.
Conclusion: Eleven samples in a pool were validated optimal experimentally assuming a prevalence of 0.66%. The pool size needs modification as the pandemic progresses; thus, the prevalence should be carefully estimated before pooling tests are conducted.

Keywords: Pooling test; Optimization; Pool size; SARS-CoV-2

INTRODUCTION

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has become a pandemic disease despite global efforts. As of November 9, 2020, 50,232,068 confirmed cases including 1,254,567 deaths have been reported to the World Health Organization, and around 504,752 new cases per day were reported [1]. The ongoing COVID-19 pandemic has become a nationwide challenge for healthcare systems and their infrastructure. Transmission during the presymptomatic period characterizes COVID-19 and the majority of patients with COVID-19 are mild or asymptomatic

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Conflict of Interest
No conflicts of interest.

Author Contributions
Conceptualization: YSK, HJ. Data curation: HJ. Formal analysis: HJ. Methodology: YSK, HJ, SK. Validation: JL, SC, KMS, JK. Visualization: HJ. Writing - original draft: HJ. Writing - review & editing: YSK.

[2-4]; therefore early detection and containment of infected cases are crucial to prevent the virus from spreading. Of particular importance is the need to survey large asymptomatic populations to trace asymptomatic carriers who are difficult to identify and isolate, to assure healthcare personnel are not infected through the regular screening process [5], to routinely screen large groups of patients without known exposure prior to hospitalization [6, 7], and to estimate the regional point prevalence of COVID-19 [8].

The standard laboratory diagnosis of COVID-19 is based primarily on nucleic acid amplification tests using the real-time reverse-transcription polymerase chain reaction (RT-PCR) assay [9]. Real-time RT-PCR is a well-known test method for detecting viral genes with high sensitivity and specificity. It usually takes one hour for RNA extraction and 30 minutes to 3 hours for RT-PCR depending on the automated commercial instruments. In this pandemic situation, to increase capacity and preserve chemical reagents used in PCR, group testing with the technique of pooling samples has been adopted in many countries [10, 11]. In this method, instead of testing patients individually, the extracted RNA from respiratory specimens are mixed equally before amplification and then tested together running a single RT-PCR. A negative result implies that all samples in the pool are negative, while a positive result implies that at least one sample in the pool is positive. If the pooled sample is positive, then each sample needs to be re-tested individually to find out which samples were positive. Because samples are pooled together and fewer tests are run overall, fewer chemical reagents are needed, running time is reduced, and results can be reported to patients quickly. Previously, the grouped pooling strategy has been used in the screening process for the detection of the human immunodeficiency virus and hepatitis B and C viruses in blood products, and for detecting various infectious diseases [12-14]; thus, its efficiency has been established.

An important consideration before implementing the pooling test is the likelihood of false-negative results. Samples are diluted by mixing them with negative samples resulting in less viral genetic material available to amplify. Previous studies have shown that there is no decrease in test sensitivity when sample sizes were 3 to 8 [6, 7, 15], and the pooling of up to 30 samples can be possible, while borderline-positive single samples resulted in the escape of detection in large pools [16]. In some clinical settings, the false-negative result could be more worrisome; therefore, the pooling strategy should be validated properly. Asymptomatic cases of COVID-19 have a lower SARS-CoV-2 viral load than those of symptomatic cases [17]. However, the likelihood of false-negatives in a pooling test used to screen a population with low viral loads has not been reported yet. Another issue is the optimization of the strategy. The efficiency of pooling tests depends on the limit-of-detection, sensitivity, specificity of the assay, and the prevalence of the disease in the population [10, 15, 18]. The prevalence of COVID-19 in a population is a key parameter of efficiency. Lower prevalence enables the use of large optimal pool size. As the prevalence of COVID-19 increases, the cost savings of the test decreases because more pooled tests will return positive results and those specimens will need to be retested individually [10].

Therefore, we aim to investigate the effect of pool size on the sensitivity of detecting SARS-CoV-2, focusing on positive samples with low viral loads. Also, we proposed an efficient pooling strategy by estimating the optimal pool size using the prevalence of Daejeon, Korea as a variable.

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MATERIALS AND METHODS

1. Sample collection and design of experimental pools

We performed a series of experiments to evaluate the effect of the pool size on the detection sensitivity of SARS-CoV-2 using real-time RT-PCR. We created experimental pools of various sizes. One experimental pool contained one positive sample diluted with a variable number of negative samples. For example, to make a pool with a size of five, one positive sample was mixed with four negative samples. A total of 11 experimental pools with sizes ranging from 5 to 15 were created using the same positive sample. Viral RNA was extracted from the experimental pool and real-time RT-PCR was performed. We checked the cycle threshold (Ct) value of each RT-PCR to determine the positivity of the experimental samples. The schematic diagram of the experiments is shown in **Figure 1**. In the present study, the maximum pool size was defined as the maximum number of samples mixed in a pool, where the result preserves the positivity.

Positive and negative SARS-CoV-2 samples used in the experimental pools were combined nasopharyngeal and oropharyngeal swabs collected at a community hospital with 1,300 beds located in Daejeon, Korea from March 1, 2020, to May 31, 2020. Swabs were collected by healthcare workers according to the recommended protocol and swab samples were

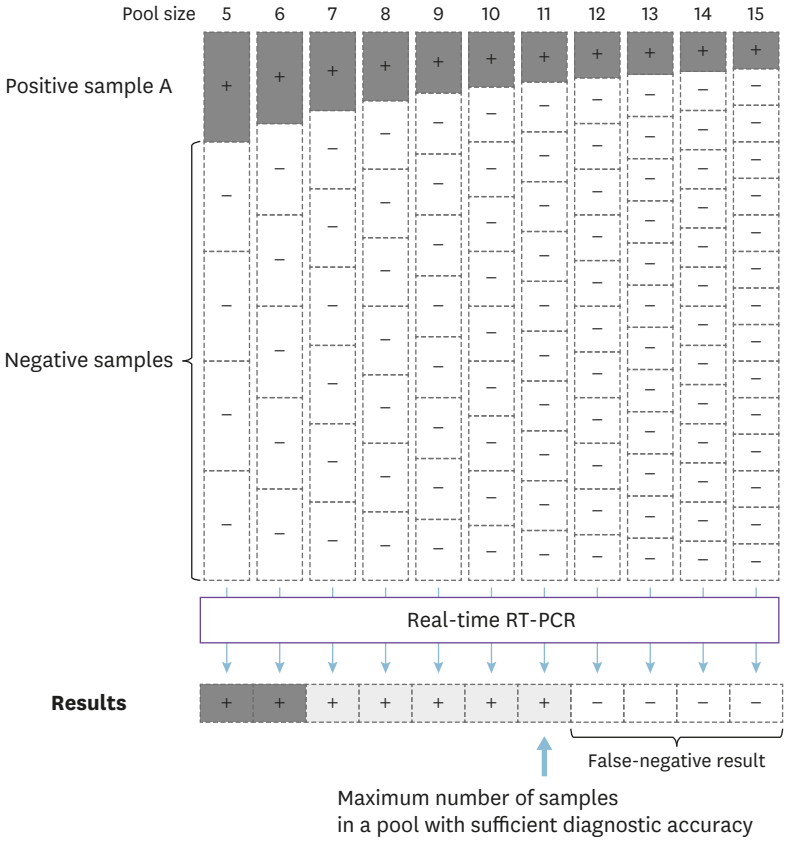

Figure 1. A schematic diagram of the experiments. Eleven experimental pools were generated with one positive sample A. An experimental pool was tested to detect SARS-CoV-2 with real-time RT-PCR and the maximum number of samples in a pool with sufficient sensitivity was determined.
SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; RT-PCR, reverse-transcription polymerase chain reaction.

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placed in a vial containing viral transport media under the procedures of the hospital. As we focused our investigation on the high likelihood of false-negative from pooled samples, we selected a positive sample with Ct values ranging from 33 to 35. To increase the reliability of the study, we repeated the same experiments using the other two positive samples. Overall the number of positive samples was three (annotated as A, B, and C), and the total number of experimental pools was 33.

2. RNA extraction and real-time RT-PCR assay

Viral RNA was extracted from 200 μL of a swab sample using the manual QIAamp DSP virus kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The extracted RNA was amplified using commercial RT-PCR kit, the U-TOP™ COVID-19 Detection Kit Plus (Seasun Biomaterials, Daejeon, Korea) on the detection system, CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The kit used in the study were targets two distint regions in open reading frames 1ab (*Orf1ab*), one region in nucleocapsid protein gene (*N*), envelope protein gene (*E*), and spike protein gene (*S*) of SARS-CoV-2. Real-time RT-PCR reactions were performed in 30 μL reactions containing 15 μL of 2 x reaction buffer, 1 μL Enzyme Mix, 4 μL of reaction mix, and 10 μL virus RNA template. The reaction conditions for amplification were 50°C for 30 minutes, 95°C for 15 minutes, 42 cycles at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. According to the manufacturer's guideline, a sample was defined as positive if the viral genome was detected at a Ct value of ≤38 for the fluorescein amidites (FAM), hexachloro-fluorescein (HEX), Quasar 705, and Cy5 signals, reporter dyes of detection probes for *Orf1ab*, *N*, *E* and *S* genes, respectively.

3. Simulation of the efficiency of the pooling the test

The pooling strategy used in the present study was a simple two-stage testing algorithm known as Dorfman pooling [19]. Assuming that the sensitivity and specificity of the gene amplification tests were 1.0, the efficiency of Dorfman pooling was expressed with the pool size (n) and prevalence of disease (P) as described below [18, 19]. Efficiency in the equation refers to the average maximum number of single tests that can be replaced with one pooling test [18].

$$\text{Efficiency} = \left\{1 + \frac{1}{n} - (1 - \text{P})^n\right\}^{-1}$$

We simulated the efficiency using n and P as variables. We ranged n from 5 to 50. In determining the prevalence, we used an average of test positivity (positive tests/total number of tests concluded) as recommended by the Center for Disease Control and Prevention (CDC) [10]. In the present study, we used the positivity data in Daejeon, Korea from April 1, 2020 to November 10, 2020 as an example.

We used R version 3.6.1 (Foundation for Statistical Computing, Vienna, Austria) [20] in the calculation and graphical illustration of the mathematical equation.

4. Ethics statement

The institutional review board (IRB) of Chungnam National University Hospital approved the study (IRB No.2020-06-079). Informed consent was obtained from the patients and the research was performed on anonymized, de-identified RNA samples.

Table 1. The Ct values of positive samples (A, B, and C) and experimental pools with various sizes

Target genes	Positive Samples	Experimental Pools[a] Number of samples in a pool (n)										
		5	6	7	8	9	10	11	12	13	14	15
Sample A												
Orf1ab gene	33.7	35.2	35.3	35.2	35.4	35.5	35.7	35.8	35.8	36.3	36.6	37.4
N gene	32.7	34.3	34.7	34.8	35.1	35.4	35.2	36.0	36.0	35.5	36.1	36.2
E gene	33.4	34.4	35.0	35.6	35.2	35.5	35.1	35.9	35.6	36.0	36.0	36.0
S gene	33.2	34.5	34.8	35.1	35.4	35.1	35.5	35.9	36.2	36.3	36.0	36.3
Sample B												
Orf1ab gene	34.9	35.9	36.4	37.1	36.7	37.0	36.4	37.3	37.1	37.4	37.4	37.3
N gene	33.9	35.3	36.3	36.3	36.6	35.9	36.3	37.8	37.8	38.2	38.7	38.9
E gene	34.4	35.1	35.7	35.9	36.3	36.5	35.5	36.4	36.3	37.2	37.0	36.9
S gene	34.5	35.5	36.0	36.7	36.3	36.6	36.1	37.0	36.7	37.1	37.1	37.0
Sample C												
Orf1ab gene	35.9	36.4	37.1	37.3	37.8	37.5	37.6	37.8	37.7	38.8	38.4	38.2
N gene	34.9	36.6	37.0	37.6	37.7	37.4	37.9	37.7	37.8	38.2	38.8	38.9
E gene	34.8	36.2	36.8	37.0	37.2	37.6	37.1	37.2	38.0	37.8	38.1	39.0
S gene	35.3	37.0	37.3	37.5	37.7	37.3	37.7	37.8	38.3	38.7	39.8	39.4

Orf1ab, open reading frames 1 ab; *N*, nucleocapsid protein; *E*, envelope protein; *S*, spike protein.
[a]Gray cells in the table indicate positive results and white cells indicate negative results.

RESULTS

1. Maximum number of samples in a pool in the experimental pools

Table 1 shows the Ct values of the experimental pools according to pool size and target genes. The average Ct values of four target genes in samples A, B, and C were 33.3 (range 32.7 - 33.7), 34.4 (33.9 - 34.9), and 35.2 (34.8 - 35.9), respectively. The pooled samples generally showed higher Ct values than those obtained from a single sample. All experimental pools still preserved positivity until the pool size increased up to 11. Among the experimental pools, the maximum number of samples in a pool that showed positive results were different from each other. Experimental pools generated using sample A showed positive results until the pool size increased up to 15 for all target genes, while experimental pools generated using sample B showed negative results in pools with sizes larger than 12 when targeting the N gene. The experimental samples generated with sample C that showed higher initial Ct values than samples A and B, showed negative results for all target genes when pool sizes were larger than 11. Also, there were differences in Ct values among the target genes. For example, the maximum number of samples in a pool were 12, 12, 13, and 11 for *Orf1ab*, *N*, *E*, and *S* genes, respectively, among experimental pools generated with sample C. Overall, it was concluded that the pool size should not be larger than 11 for sufficient sensitivity.

Figure 2 shows the differences in Ct values between the positive samples and the pooled samples ($Ct_{\text{pooled sample}} - Ct_{\text{positive sample}}$) according to the pool size. When the pool size was 8, the median difference in Ct values was 2.15 with an interquartile range (IQR) of 1.8 - 2.4. The maximum difference was shown when the pool size was 15, with a median value of 3.3 and an IQR of 2.50 - 4.02. As the pool size increased, the differences in Ct values increased, with a linear regression model of: Ct difference = 0.187 × pool size (n) + 0.498 (R^2 =0.53).

2. Estimation of optimal sample size using the Dorfman equation

Figure 3 shows the efficiency of the simple two-step pooling test with pool size (n) and prevalence (P) as variables. The contour plot shows the average maximum number of individual tests which can be replaced with one pooling test. For example, if the prevalence is assumed to be 0.1% and we use 10 as the sample number in a pool, then the efficiency of the

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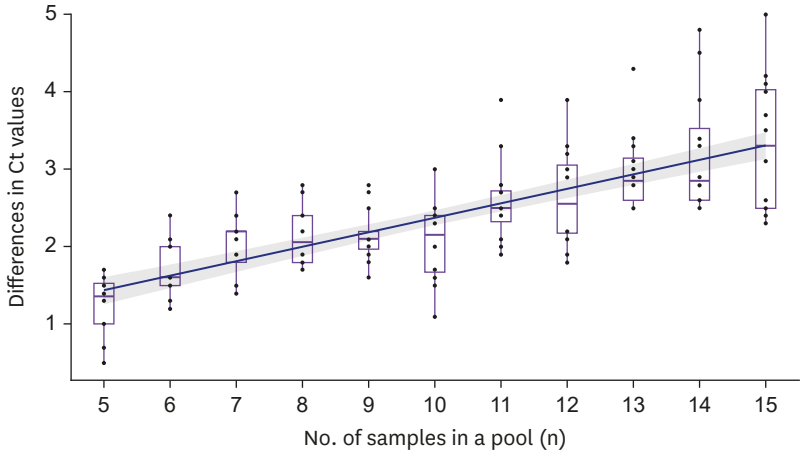

Figure 2. Differences in Ct values between positive samples and experimental pools according to pool size. The box plot shows the median and the upper and lower quartiles of differences. Linear regression with a grey zone expressing a 96% confidence interval was used.
Ct, cycle threshold.

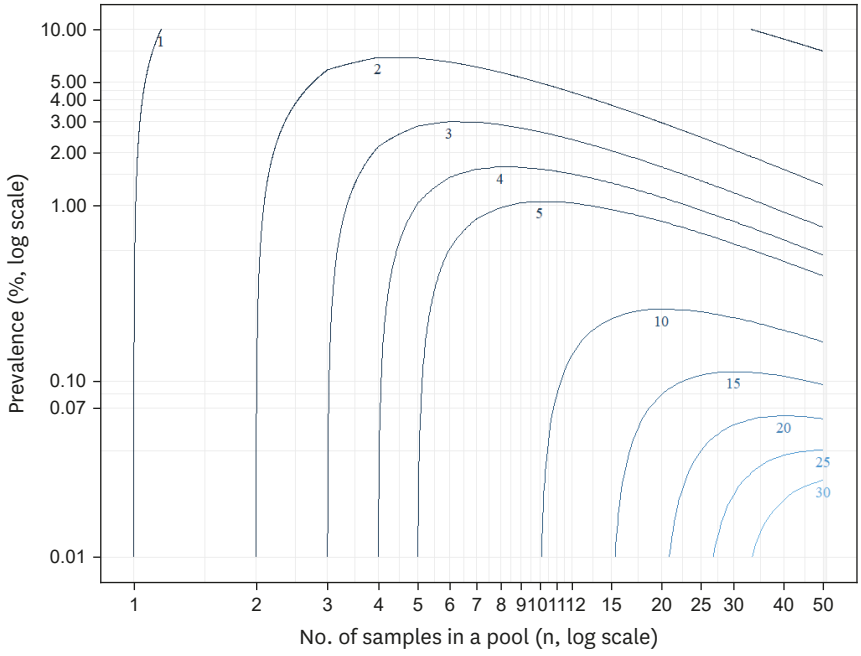

Figure 3. A contour plot of the average maximum number of single tests equivalent to one pooling test. The results shown are the mathematical simulation of the Dorfman equation.

test is determined to be 9.1. This means that when we use 10 samples in a pool, one pooling test can replace a maximum of 9.1 individual tests. Given the prevalence, the maximum efficiency can be calculated to be 15.9 when the pool size is 32.

The average test positivity of samples from Daejeon, Korea from April 1, 2020 to November 10, 2020 was 0.66% (410 confirmed cases over a total of 61,721 tests). When we use 0.66% as the prevalence, the optimal size of a pool is 11 and the efficiency is 6.2, which can save 83.9 of 100 tests as shown in **Table 2**.

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Table 2. Optimal pool size and efficiency of a simple two-step pooling test given various prevalences

Prevalence (%)	Optimal pool size (n)	Efficiency (average number of individual tests that can be replaced by one pooling test)	Number of tests saved (compared for every 100 individual tests)
0.01	101 → 11[a]	20.1 → 10.9[b]	95.0 → 90.1[c]
0.1	32 → 11[a]	15.9 → 9.8[b]	93.7 → 89.8[c]
0.5	15 → 11[a]	7.2 → 6.9[b]	86.1 → 85.5[c]
0.66	13 → 11[a]	6.3 → 6.2[b]	84.1 → 83.9[c]
1	11	5.1	80.4
3	6	3.0	66.7
10	4	1.7	41.2

[a]The theoretically calculated optimal pool sizes were changed to 11 based on the experimental results.
[b]The efficiencies of Dorfman pooling were corrected for a pool size of 11.
[c]The numbers of tests saved were corrected for a pool size of 11.

3. Efficiency gaining

The efficiencies of the simple two-step pooling test under the optimal sample size are addressed in **Table 2**. When the prevalence is assumed to be 3%, then the optimal size of a pool is determined to be 6, and the maximum efficiency of the Dorfman equation is 3.0. This suggests that the number of tests can be saved by 66.6 compared to single tests when we need to test 100 samples individually. Our experiments showed that the pool size should be 11 or less to preserve the test sensitivity when screening a population with low viral loads. So, in the case where the optimal pool size is calculated to be 12 or higher, then the optimal pool size should be changed to 11. For example, assuming the prevalence is 0.1%, the optimal size is calculated to be 32, which should be changed to 11.

DISCUSSION

As COVID-19 has become a pandemic disease, the healthcare infrastructure is facing a need for mass screening. A pooling strategy was pursued to increase test throughput and to limit the use of chemical reagents. The present study examined whether the pooling assay was feasible to test large numbers of individuals efficiently, especially for screening a population with low viral loads. A key requirement of the pooling test is to retain sufficient diagnostic accuracy. In this study, we investigated the effect of pool size on the detection of SARS-CoV-2 by conducting a series of experiments, focusing on the likelihood of false-negative results. To do this, we used only one positive sample containing a small amount of viral RNA in the experimental pools.

In previous studies, pools of sizes between 3 and 10 were usually used in the detection of SARS-CoV-2 in pooled samples with various Ct values [6, 8, 15, 21]. When positive samples with an average Ct value of 24.5 ± 3.1 were tested [22], the virus could be detected in a pool with sizes up to 30. When a pool contained two or more positive samples, larger than 15 samples in a pool could be used [16]. There was a lack of validation concerning the fact that an increase in the number of samples in a pool could lead to false negatives. We found that positive samples with Ct values around 35 can result in false-negative results if the pool size exceeded 11. Therefore, possible false-negative results should be taken into account when a pooling assay is used to screen a population with low viral load such as asymptomatic people. The maximum number of samples in a pool would vary depending on the target gene, the commercial kit used, and the characteristics of the population tested. Thus, the present experimental results should be validated and modified before implementing the pooling test in individual institutes.

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As the efficiency of the pooling test is dependent on the prevalence (P), the design of the pool needs to be tailored to different scenarios. The prevalence of the disease in the tested population is not always known because testing has been primary restricted to individuals with moderate to severe symptoms [23] due to the limited availability of tests [24]. However, it could be estimated using the previous surveys of individual samples [25], the rate of symptomatic patients, or alternative methods such as serological screening [26, 27] or wastewater titer monitoring [28]. The CDC recommends the use of prevalence based on a rolling average of the positivity rate of their SARS-CoV-2 testing over the previous 7 - 10 days [10]. In Daejeon, Korea from April 1, 2020 to November 10, 2020, the mean daily positivity rate was 0.66 with a range of 0 to 7.4. The test strategy may require modification according to the prevalence. For example, the theoretical optimal pool size calculated with the Dorfman equation according to the rolling average of the positivity rate varied from 8 to 50. As the prevalence rises, the use of pooling might need to be limited and the pool size should be reduced as shown in **Table 2**. Since the prevalence changes as the pandemic progress, healthcare personnel may need to monitor the prevalence and change the pool size accordingly, and each institute should use a standardized methodology for estimating the prevalence.

In addition to a simple two-step pooling test, multi-dimensional methods can reduce the total number of tests needed by adding extra rounds of pooling tests [29]. In this method, the time would be increased by adding a third step to the test, so overall efficiency needs to be evaluated. A one-step method was introduced to reduce the time waiting for the result from the first round. The one-step method uses only one round of testing by distributing samples into a matrix of overlapping groups.

The pooling assay has some limitations. The diagnostic integrity of an individual specimen can not be ensured because it is combined with other specimens before testing [8], the quality of the swab collection can affect the integrity of the specimens, and inadequate individual specimens might not be eliminated from the pooled specimen. This means that the healthcare personnel should monitor and control the quality of the samples collected in their institute. Another limitation is the risk of inevitable false-negative results. This could be a critical problem in some clinical situations such as regular screening processes for healthcare workers, hospitalized patients, factory workers, and military units. To reduce the risk of false-negative results, we propose a gray zone in interpreting the test result. For example, if the Ct value of the pooled sample is between 38 and 40, it is judged as an indeterminate result, not a positive nor negative result. The pooled samples in the gray zone are recommended to be re-tested or split into smaller samples to test individually. As shown in the linear regression of the experimental results (**Fig. 2**), the increase in Ct value by two is equivalent to a dilution effect by about eight times; therefore, introducing a gray zone would sufficiently reduce the risk of false-negative results. Additional amplification can also reduce the risk of false-negative results. If we increase the threshold of positive FAM and HEX signal values from 38 to 40, then all experimental pools in the experiments can be judged as positive until the pool size increase up to 15. In general, RT-PCR kits and protocols vary internationally; thus, the use of the pooling method described above requires additional validation for each specific setting.

In conclusion, we found that the number of samples in a pool should not be exceed 11 when the pooling test is used to screen the COVID-19 in a population with low viral loads. Also, the number of samples in a pool should be optimized with mathematical simulations and modified as the prevalence changes. We hope our proof-of-concept for a population with low

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viral loads will help the healthcare workers to properly validate the pooling test properly and develop effective strategies.

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