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Novel pooling strategy with sample concentration for screening of SARS-CoV-2

Reverse transcription PCR (RT-PCR) is the gold standard for rapidly confirming infection with SARS-CoV-2. However, the great demand for SARS-CoV-2 RT-PCR testing has outpaced its supply in some scenarios, such as the large-scale testing of asymptomatic subjects for public health interventions. Notably, sample pooling, that is, combining multiple samples before and after nucleic acid extraction into a single test sample, has the potential to improve the available RT-PCR testing capacity.[1 2] However, compromised sensitivity caused by the sample dilution effect can lead to higher rates of false-negative results in low-viral-load specimens, which carries a risk of missing asymptomatic carriers capable of transmitting the infection.[3–5] Thus, developing a high-throughput testing strategy with no sensitivity loss for the early detection and active monitoring of individuals potentially exposed to SARS-CoV-2 is urgently required for prevention and control of the COVID-19 pandemic. Joung *et al* has demonstrated that increasing sample input via capturing all of the viral RNA from a nasopharyngeal swab (NPS) can boost the sensitivity of SARS-CoV-2 RT-PCR.[6] In this study, we compared the effectiveness and sensitivity of the traditional sample pooling (TSP) strategy and the sample pooling concentration (SPC) strategy for COVID-19 diagnosis using the automated nucleic acid extraction system based on magnetic nanoparticles.

NPS in chemically inactivated buffer (Liferiver, Shanghai, China) obtained from patients with confirmed COVID-19 and health professionals were used for pooling. For each pool, 300 µL of NPS from three samples and 200 µL of NPS from eight samples were pooled into a single tube and mixed thoroughly. Two pooling strategies were evaluated as follows: (1) TSP strategy: part of the pooled samples, that is, 300 µL of the three-TSP pool and 200 µL of the eight-TSP pool, was subjected to RNA extraction using the Liferiver EX3600 and Autrax192 automatic nucleic acid extraction system (Liferiver); (2) SPC strategy: all pooled samples, that is, 900 µL of the three-SPC pool and 1600 µL of the eight-SPC pool, were subjected to RNA extraction using the Liferiver EX3600 and Autrax192 automatic nucleic acid extraction system (figure 1). In addition, individual analyses of the same samples were performed in parallel to the analysis of the pools. All RNA was eluted in 75 µL elution buffer. A 10 µL aliquot of RNA was used in 30 µL of reaction using a real-time fluorescent RT-PCR kit targeting the SARS-CoV-2 ORF1ab gene (BGI, Shenzhen, China) on an Applied Biosystem 7500 Real Time PCR system (Thermo Fisher Scientific).[7] A sample was defined as positive if viral RNA was detected at threshold cycle (Ct) values of ≤38, as indeterminate at Ct values of >38 but <40, or as negative at Ct values of ≥40, in accordance with the BGI kit instructions.

In total, we tested 21 three-sample and eight-sample pools for TSP and SPC (18 pools that each included at least 1 positive sample, composed of 54 and 144 individual samples; and 3 pools of all negative samples, composed of 9 and 24 individual samples) and also tested each sample individually in parallel. Of the 18 positive pools, 12 contained a single positive sample; 3 contained two positive samples; and 3 contained three positive samples (figure 2). All the pools containing only negative samples were confirmed as being negative, demonstrating 100% specificity (figure 2). For the positive TSP pools, 16/18 (88.9%) three-TSP and 17/18 (94.4%) eight-TSP were correctly detected (figure 2A). The four samples that were missed in the TSP pools each had a Ct value of ≥36. Additionally, two three-TSP and eight-TSP pools containing one or two low-positive samples (Ct >34.6) yielded indeterminate results, which indicated the need for pool retesting (figure 2). However, all 36 positive SPC pools, even those containing samples with low viral loads (Ct >36), were correctly detected.

An important consideration in sample pooling is retaining sufficient sensitivity. Theoretically, pooling three and eight samples in the TSP strategy should increase the Ct value of a single positive sample by 1.58 and 3 cycles, respectively, but this should not happen in the SPC strategy owing to sample concentration. Of the pools that contained only one positive sample, the sample dilution inherent in the TSP strategy resulted in an average loss of 1.67 (95% CI 1.39 to 1.96) Ct for the three-TSP and 2.43 (95% CI 2.15 to

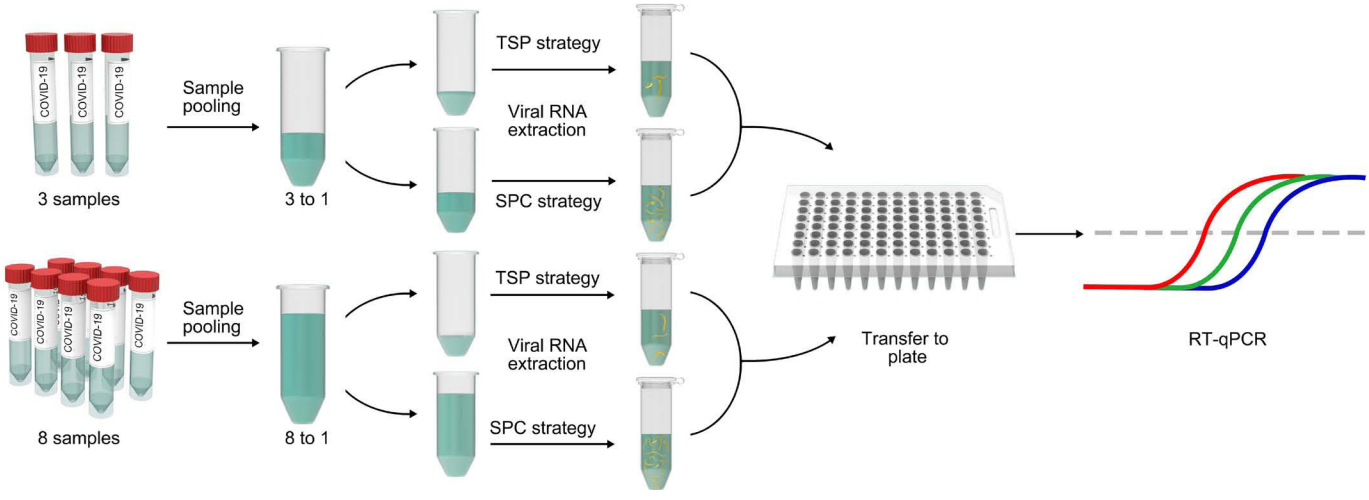

Figure 1 Schematic overview of SARS-CoV-2 RT-PCR testing applying the TSP strategy or SPC strategy. Three or eight individual nasopharyngeal samples were pooled into a single sample. RNA was extracted from the pooled sample using the TSP and the SPC strategies. The extracted RNA was transferred to a PCR plate and subjected to RT-PCR targeting the SARS-CoV-2 ORF1ab gene. RT-PCR, reverse transcription PCR; SPC, sample pooling concentration; TSP, traditional sample pooling.

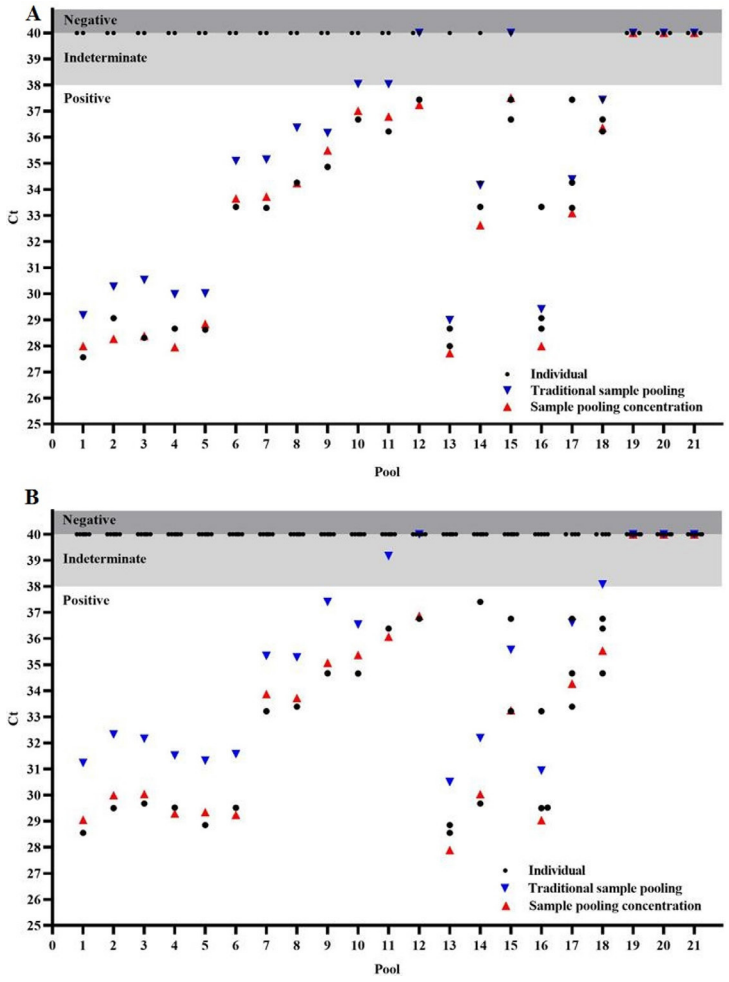

Figure 2 Changes in Ct values for individual sample testing compared with the TSP strategy and SPC strategy. A total of 21 pools (pools 1–12 each contained one positive sample; pools 13–15 each contained two positive samples; pools 16–18 each contained three positive samples; and pools 19–21 each contained all negative samples), to which the TSP strategy or the SPC strategy was applied, were tested in parallel with the corresponding individual samples. (A) Three-sample pooling. (B) Eight-sample pooling. Ct, threshold cycle; SPC, sample pooling concentration; TSP, traditional sample pooling.

2.71) Ct for the eight-TSP when compared with individual sample tests (online supplemental table S1). In contrast, only slight Ct value losses were observed when using the SPC strategy, with an average change of 0.11 (95% CI −0.19 to 0.40) Ct in the three-SPC and 0.27 (95% CI 0.04 to 0.50) Ct in the eight-SPC (online supplemental table S1). In accordance with the theoretical estimation of Ct change, the empirical average change in Ct value was significantly different between the TSP and SPC strategies ($p<0.001$, t-test). Furthermore, each of the pools that contained ≥2 positive samples showed Ct value change trends in the TSP and SPC strategies similar to those of pools containing only one positive sample when compared with individual sample tests.

Sample pooling is a major alternative strategy for large-scale SARS-CoV-2 screening in low-prevalence populations. Here, our data show that the sample dilution from traditional pooling (TSP strategy) led to a mild, but expected, loss in Ct value, in agreement with other studies.[3–5] This drop in sensitivity was responsible for all of the false-negative results in samples with low viral loads. However, use of the SPC strategy can effectively avoid sample dilution and yielded 100% accuracy with no loss of sensitivity, meaning that it may be possible to correctly identify all symptomatic and asymptomatic individuals with COVID-19. Unlike those of previous studies, in our approach, we did not need to consider the size of the pool for maximum efficiency based on COVID-19 prevalence,[3 5 8] only the capacity of the RNA extraction system in the laboratory where pooling was performed. Therefore, the SPC method as a resource-efficient strategy may facilitate early detection and elimination of COVID-19 community transmission.

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Table S1. Comparison of Ct values for individual positive samples compared to traditional sample pooling (TSP) strategy and sample pooling concentration (SPC) strategy[a]

Pool[b]	3-sample pooling					8-sample pooling				
	Individual Ct	TSP Ct	Δ Ct[c]	SPC Ct	Δ Ct[c]	Individual Ct	TSP Ct	Δ Ct[c]	SPC Ct	Δ Ct[c]
1	27.56	29.17	1.18	27.99	0.43	28.55	31.23	2.68	29.05	0.50
2	29.06	30.27	1.21	28.27	-0.79	29.50	32.32	2.82	29.99	0.49
3	28.31	30.53	2.22	28.38	0.07	29.67	32.16	2.49	30.04	0.37
4	28.66	29.98	1.32	27.95	-0.71	29.52	31.52	2.00	29.29	-0.23
5	28.62	30.01	1.39	28.84	0.22	28.85	31.32	2.47	29.34	0.49
6	33.33	35.09	1.76	33.65	0.32	29.51	31.57	2.06	29.24	-0.27
7	33.29	35.14	1.85	33.72	0.43	33.21	35.34	2.13	33.87	0.66
8	34.26	36.36	2.10	34.24	-0.02	33.39	35.28	1.89	33.72	0.33
9	34.86	36.16	1.30	35.49	0.63	34.67	37.41	2.74	35.07	0.40
10	36.68	38.04	1.36	37.01	0.33	34.66	36.54	1.88	35.37	0.71
11	36.22	38.03	1.81	36.79	0.57	36.38	39.17	2.79	36.07	-0.31
12	37.44	**40.00**	2.56	37.24	-0.20	36.76	**40.00**	3.24	36.87	0.11
13	27.99	28.99	N/A	27.72	N/A	28.55	30.50	N/A	27.89	N/A
	28.66					28.85				
14	33.33	34.16	N/A	32.63	N/A	29.67	32.19	N/A	30.03	N/A
	34.26					37.41				
15	36.68	**40.00**	N/A	37.51	N/A	33.21	35.57	N/A	33.25	N/A
	37.44					36.76				
16	29.06	29.41	N/A	27.99	N/A	29.50	30.94	N/A	29.03	N/A
	28.66					29.52				
	33.33					33.21				

17	33.29	34.38	N/A	33.09	N/A	33.39	36.61	N/A	34.27	N/A
	34.26					34.67				
	37.44					36.76				
18	36.68	37.43	N/A	36.36	N/A	36.38	38.08	N/A	35.54	N/A
	36.22					36.76				
	37.44					34.67				

[a]Ct: cycle threshold; Boldface type indicates samples that were missed when pooled; N/A: not applicable.

[b]pools 1-12 containing one positive sample, pools 13-15 containing two positive samples, pools 16-18 containing three positive samples.

[c]Values represent the difference between Ct values of the pooled group and corresponding individual positive sample.