A contribution to the feasibility of sample pooling for molecular SARS-CoV-2 diagnostics

ADI STEINRIGL*, SANDRA REVILLA-FERNANDEZ AND FRIEDRICH SCHMOLL

Austrian Agency for Health and Food Safety, Robert Koch-Gasse 17, 2340 Moedling, Austria

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Summary

Pooling of patient samples for molecular SARS-CoV-2 testing by RT-PCR has been suggested as a strategy to economically increase sample throughput. We provide experimental data suggesting that positive samples up to Cq-values of 32 can be pooled up to 64-fold without loss of diagnostic sensitivity. Furthermore, we show that the pooling process is technically feasible even without major laboratory automation.

Zusammenfassung

Das Poolen von Patient:innenproben im Zuge der molekularen SARS-CoV-2 Diagnostik mittels RT-PCR wurde als wirtschaftlich effektive Strategie zur Erhöhung des Probendurchsatzes vorgeschlagen. Wir präsentieren hier experimentelle Daten, die zeigen, dass positive Proben bis zu Cq-Werten von 32 in Poolgrößen bis 1:64 ohne Verlust der diagnostischen Sensitivität gepoolt werden können. Zudem zeigen wir, dass der Prozess des Poolens auch ohne fortgeschrittene Laborautomatisierung technisch machbar ist.

Introduction

Due to the ongoing coronavirus disease 2019 (COVID-19) pandemic, there has been a massive surge in demand for molecular SARS-CoV-2 testing. Due to widespread shortcomings in the availability of SARS-COV-2 RT-PCR testing in the early phase of the pandemic, many veterinary laboratories, such as the AGES Institute for Veterinary Disease Control, Mödling, have been involved in RT-PCR testing of human samples. Nevertheless, further possibilities to increase sample throughput while saving on valuable consumables have been pursued. In 2020, a theoretical study has been published, highlighting the economical benefits of a sample pooling strategy [1]. A potential drawback of pooling is that infected individuals with very low viral loads might be missed, due to diluting out the analyte. Furthermore, the pooling process itself poses the risk of sample confusion. Nevertheless, pooling has been used early on for retrospective screening of a large number of individuals in the US [2]. A number of publications has since dealt with the pooling issue, some of which have tested selected pool sizes only (such as pools of five, or ten samples), whereas others have tested a wider range of possible pool sizes [3-10)]. The latter is especially important, as the optimal pool size depends on the sensitivity of the test and the assumed virus prevalence in the sampled population [11]. Here, we present experimental data demonstrating the influence of pool size on detection of samples with widely varying viral loads and show that the pooling approach can easily be realised in the laboratory, as proven by a partially blindfolded pooling experiment.

Material and methods

In experiment one, sixteen SARS-CoV-2 RNA positive clinical upper respiratory swab samples with widely varying quantification cycle (Cq)-values were selected from a cohort of samples taken in March 2020 within the frame of COVID-19 diagnostic testing in Austria. Based on the initially documented Cq-values, samples were arbitrarily classified into strong positive (Cq < 20; n=2), positive (Cq 20 - 29; n = 5), weak positive (Cq 30 - 35; n = 4) and borderline positive (> Cq 35; n= 5). Extracted nucleic acid fom theses samples was serially diluted in pooled SARS-CoV-2 RNA negative samples obtained during a screening programme. RNA was prepared using a commercial kit (BioExtract® SuperBall®, BioSellal, France). Two-fold serial dilutions of each positive sample in pooled negative SARS-CoV-2 RNA were performed from 1/2 up to 1/64, in order to reconstruct the respective pool sizes. Detection of SARS-CoV-2 RNA was then performed by RT-qPCR with 42 cycles of amplification, using the LightMix® Modular SARS and Wuhan CoV E-gene mix (TIBMOLBIOL, Germany) and SuperScript™ III Platinum® One-Step qRT-PCR System with ROX (ThermoFisher, Austria) on an Applied Biosystems 7500 Fast Real-time PCR system (ThermoFisher). Undiluted primary RNA samples and derived pools were always tested in the same run. To demonstrate the absence of SARS-CoV-2 RNA in the negative pool, the latter was tested in multiple (seven to nine) replicates per RT-qPCR run (two runs in total).

In experiment two, Eighty samples (phosphate buffered saline) were prepared, of which 9 were spiked with SARS-CoV-2 positive clinical samples with different viral loads (three replicates for each clinical sample). The identity of these samples (spiked/unspiked) was unknown to the laboratory personnel, who performed the further testing. Laboratory staff were then instructed to test the 80 samples in pools of 5 samples (by manual pooling, using a multichannel pipet) for the presence of SARS-CoV-2 RNA, to subsequently test samples contributing to positive pools individually and finally to identify the initially positive samples. SARS-CoV-2 RNA was extracted and detected as described for the first experiment.

Results

Experiment one was designed to systematically investigate the impact of increasing pool sizes on SARS-CoV-2 RNA detection by RT-qPCR. All samples classified as strong positive and positive (see Material and methods) were detected up to a pool size of 64 (Table 1). Two out of four weak positive samples (Cq 30 - 35) were still detectable up to the same pool size; however, all weak positive samples were detected up to a pool size of four. Among the borderline positive samples (>Cq 35), only one sample tested positive in a pool size of 16, whereas the sample with the highest Cq-value (Cq 38) was not detectable even in the lowest pool size. In general, we observed the expected inverse correlation between the largest detectable pool size and the Cq-values of the undiluted samples, a result that was also reported by others [3, 6, 10]. Borderline positive results were observed in some pools, despite a negative result in the preceding smaller pool size. These results are likely due to a stochastic association between analyte concentration and RT-qPCR result, as regularly observed in samples with extremely low RNA loads. Alternatively, such results could be explained by cross-contamination or unspecific amplification; however this is unlikely, since all amplification curves had a similar shape (Figure) and all no-template controls and replicates of the negative RNA pool tested negative. In summary, the first experiment demonstrated that pooling of samples up to a Cq of 32 was possible up to a pool size of 64, without impacting the diagnostic sensitivity. Furthermore, 6/8 samples with Cq-values > 32 were still detectable up to a pool size of four.

Table 1: RT-qPCR results (Cq-values) obtained by testing undiluted and pooled RNA samples.

	Pool size							
Sample ID	**not diluted**	**1/2**	**1/4**	**1/8**	**1/16**	**1/32**	**1/64**	**negative pool**
Co52-163	17	18	19	20	21	22	22	No Cq
Co52-076	19	20	21	22	23	24	25	No Cq
Co52-127	21	22	23	24	25	26	27	No Cq
Co52-183	23	23	25	26	27	28	29	No Cq
Co52-004	28	29	30	31	32	33	34	No Cq
Co52-017	28	29	31	32	33	34	36	No Cq
Co52-058	29	30	31	33	33	35	36	No Cq
Co52-177*	32	34	35	37	37	38	39	No Cq
Co52-038	34	35	35	37	38	39	39	No Cq
Co52-080	35	37	36	37	No Cq	No Cq	No Cq	nd
Co52-062	34	36	37	No Cq	No Cq	39	No Cq	No Cq
Co52-090	36	38	38	40	39	No Cq	No Cq	No Cq
Co52-173	36	38	37	38	No Cq	41	No Cq	No Cq
Co52-018	37	38	39	No Cq	No Cq	No Cq	No Cq	No Cq
Co52-023	37	37	No Cq	38	No Cq	39	No Cq	No Cq
Co52-160	38	No Cq	No Cq	No Cq	No Cq	No Cq	No Cq	No Cq

*Sample Co52-177 was tested in both runs to monitor reproducibility of results; nd: not determined

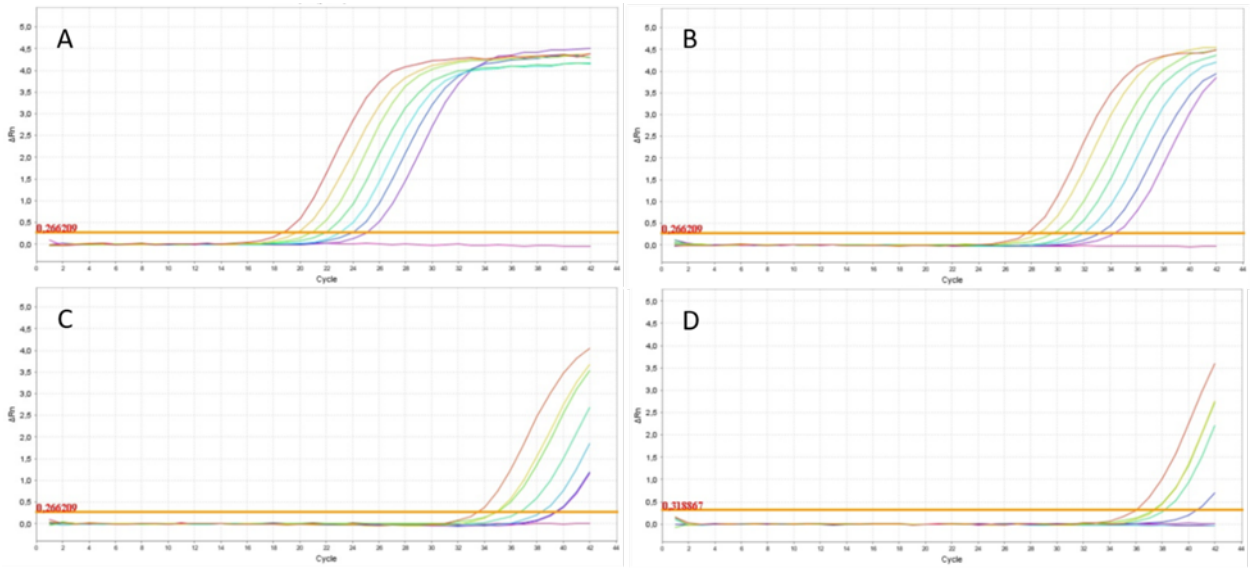

Figure. RT-qPCR amplification plots of undiluted and pooled samples (pool size ranging from 2 to 64) from: (A) strong positive, (B) positive, (C) weak positive and (D) borderline positive specimens.

In experiment two, the whole SARS-CoV-2 detection process including pooling, RNA extraction and RT-qPCR as well as the capability of laboratory staff to correctly identify individual spiked positive samples was investigated for a pool size of five. Mean SARS-CoV-2 Cq-values in the spiked samples were 27, 33 and 39, respectively (Table 2). All pools containing spiked samples in the positive and weak positive Cq-value range (n = 6) as well as the individual samples contributing to these pools were correctly identified. None of the three pools containing individual samples with borderline positive SARS-CoV-2 RNA loads (Cq 37 - 40) was detected as positive. Results from this second experiment confirmed that even weak positive samples can be pooled in a moderate pool size of five, whereas borderline positive samples are unlikely to be detected after pooling. Furthermore, correct identification of individual positive and negative samples by laboratory staff was demonstrated.

Table 2: Results of pooling experiment (experiment two; pool size of 5). SARS-CoV-2 RNA positive samples are in bold font

Spiked sample (Cq-value)	**Pooled samples (Cq-value)**	**Identified positive sample (Cq-value)**
1 (27)	**1-5 (30)**	**1 (28)**
7 (34)	**6-10 (35)**	**7 (35)**
No spike	11-15 (No Cq)	
No spike	16-20 (No Cq)	
23 (26)	**21-25 (29)**	**23 (28)**
30 (37)	26-30 (No Cq)	
32 (40)	31-35 (No Cq)	
38 (34)	**36-40 (37)**	**38 (35)**
No spike	41-45 (No Cq)	
No spike	46-50 (No Cq)	
55 (27)	**51-55 (29)**	**55 (27)**
No spike	56-60 (No Cq)	
63 (39)	61-65 (No Cq)	
No spike	66-70 (No Cq)	
74 (33)	**71-75 (36)**	**74 (34)**
No spike	76-80 (No Cq)	

Discussion

In this study, we confirmed that pooling of upper respiratory swab samples does not impact diagnostic sensitivity in samples covering a wide range of viral loads. However, pooling is likely to result in negative test outcomes in the case of borderline positive samples. Furthermore, we demonstrate that pooling can also be successfully performed without costly automated liquid handling procedures. A potential limitation of this study is the use of a single SARS-CoV-2 detection method. In general, any method for nucleic acid extraction and SARS-CoV-2 RNA detection will have an individual test sensitivity and thus influence the possible pool size [11]. Therefore, any such method must be evaluated carefully.

The observed loss of analytical sensitivity by pooling must be carefully weighed up against the benefit of higher sample throughput, which might eventually lead to earlier discovery of new infection clusters. Literature suggests that low viral loads (high Cq-values) in upper respiratory tract samples usually appear during later stages of infection [12]. Moreover, infectious virus is unlikely to be recovered from samples with viral loads below 5.4 log10 copies/ml, corresponding to a Cq-value of about 29.5 [13-14]. This was supported by a recent study investigating Austrian health care workers with prolonged SARS-CoV-2 RNA shedding [15]. Thus, a false negative test resulting from pooling of a sample with a very low SARS-CoV-2 RNA load is unlikely to result in infectious clusters being missed.

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