

1 Increased PCR screening capacity using a multi-replicate pooling scheme

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8 Abstract

9 Effective public health response to viral outbreaks such as SARS-CoV-2 is often informed by real-time
10 PCR screening of large populations. Pooling samples can increase screening capacity. However, when
11 a traditional pool is tested positive, all samples in the pool need individual retesting, which becomes
12 ineffective at a higher proportion of positive samples. Here, we report a new pooling protocol that
13 mitigates this problem by replicating samples across multiple pools. The resulting pool set allows the
14 sample status to be resolved more often than with traditional pooling. At 2% prevalence and 20 samples
15 per pool, our protocol increases screening capacity by factors of 5 and 2 compared to individual testing
16 and traditional pooling, respectively. The corresponding software to layout and resolve samples is freely
17 available under a BSD license (<https://github.com/phiweger/clonepool>).

19 Group testing has long been used to screen larger collections of samples, most of which are expected to
 20 test negative.¹ In pathogen outbreaks such as the current SARS-CoV-2 pandemic, this “pooling” allows
 21 screening of large populations to guide public health response and restrict spreading. However, in most
 22 laboratories, the screening capacity is limited by the number of PCR reactions that can be performed in
 23 a day. It is, therefore, desirable to maximize the number of samples that can be tested per reaction.

24 Various approaches have been proposed to do so in the context of SARS-CoV-2 RT-PCR testing.^{2,3} One
 25 problem with the traditional pooling approach, where several samples are collected and tested collectively,
 26 is that the number of positive pools that require individual retesting increases rapidly with the number
 27 of positive samples in the overall population, henceforth called “prevalence”. A high prevalence renders
 28 traditional pooling ineffective. To mitigate this, we propose to test samples in replicates and distribute
 29 them across multiple pools. The resulting “pool address” can then be used to resolve samples in one pool,
 30 given the information from other pools that contain a replicate. While some previous studies have taken a
 31 similar approach implicitly,² it has neither been investigated systematically for more replicates than two,
 32 nor is there any software that would generate and resolve the corresponding pooling layout for laboratory
 33 use.

34 We therefore introduce “clonepool”, a pooling framework to maximize the effective number of samples s_e
 35 per PCR reaction. “Effective” refers to the fact that samples in positive pools, whose status cannot be
 36 resolved in the pooled run, are assumed to be retested individually. The maximum number of samples for
 37 a given pool size p , number of pools n and number of replicates r is calculated as $s_m = \frac{pn}{r}$. The effective
 38 number of samples can then be calculated from the number of unresolved samples s as $s_e = \frac{s_m}{p+s}$.

39 The clonepool algorithm first distributes all sample replicates randomly across the available pools, with
 40 the limitation that a sample’s replicates do not co-occur in the same pool. After the pools have been
 41 tested, the algorithm attempts to resolve the samples’ status in two phases: In a first phase, all samples
 42 that have at least one replicate in a negative pool are marked negative. In the second phase, samples that
 43 (a) only occur in positive pools, and (b) have at least one replicate in a pool in which all other samples
 44 are known to be negative, are marked positive (red, orange). All other samples cannot be resolved, and
 45 need to be retested individually. The longer the set of pools a sample is distributed across, i.e., the larger
 46 its pool set, the more samples can be resolved given a particular prevalence. Of course, a higher number

of replicates comes at the price of a reduced number of samples which can be processed at a fixed number of wells. Fortunately, our results provide an efficient means to find the optimal trade-off for any given set of parameters.

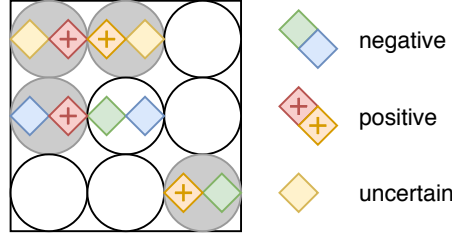


Figure 1: Illustration of the clonpool algorithm. Circles denote the wells, each containing a pool of samples (small squares). A distinct color marks all replicates of a single sample. Positive samples are flagged with “+”, negative ones remain empty. Positive pools are shaded in grey, negative ones in white. In a first phase, all samples that have at least one replicate in a negative pool are identified as negative (blue, green). In the second phase, samples that only occur in positive pools and where at least one replicate is in a pool where all other samples are negative, are recognized as positive (red, orange). All other samples cannot be resolved and have to be retested individually (yellow).

We tested the proposed clonpool algorithm using simulated data. We assumed no pipetting errors, which can be achieved, e.g., through the use of a pipetting robot. We also assume that 94 pools are available, which corresponds to a 96-well plate with two wells reserved for a positive and a negative control. Furthermore, we assume that there are no false positive or false negative PCR reactions.

Two parameters determine which pooling scheme is most effective (Fig. 2). If both the prevalence and the number of samples per pool are low, traditional pooling without replicates yields the highest number of samples per reaction. However, as the prevalence increases or more and more samples are pooled, the number of positive pools increases, causing a large number of retested samples and thus reducing the overall throughput. Using sample replicates will then allow to resolve more samples than in the traditional approach. In our testing experience, we observed a prevalence of about 5%, but this value is subject to variability, e.g., depending on a population’s pre-test probability.

The number of samples that can be pooled without affecting the PCR sensitivity is limited by the PCR cycle threshold (Ct) for the target, i.e., the cycle at which amplification becomes detectable over background noise (typically ten times the standard deviation, SD). Usually, Ct values above 35 are treated as unspecific amplification. SARS-CoV-2 amplifies at low Ct values due to high viral titers (Ct 18-25 depending on the material and number of days post-infection).^{4,5} A 20-fold dilution, i.e., pooling 20 sam-

66 ples, would cause the Ct value to increase by about 4.3 cycles ($2^x = d$, where d is the dilution and x the
 67 shift in Ct), which still lies comfortably above the detection limit.

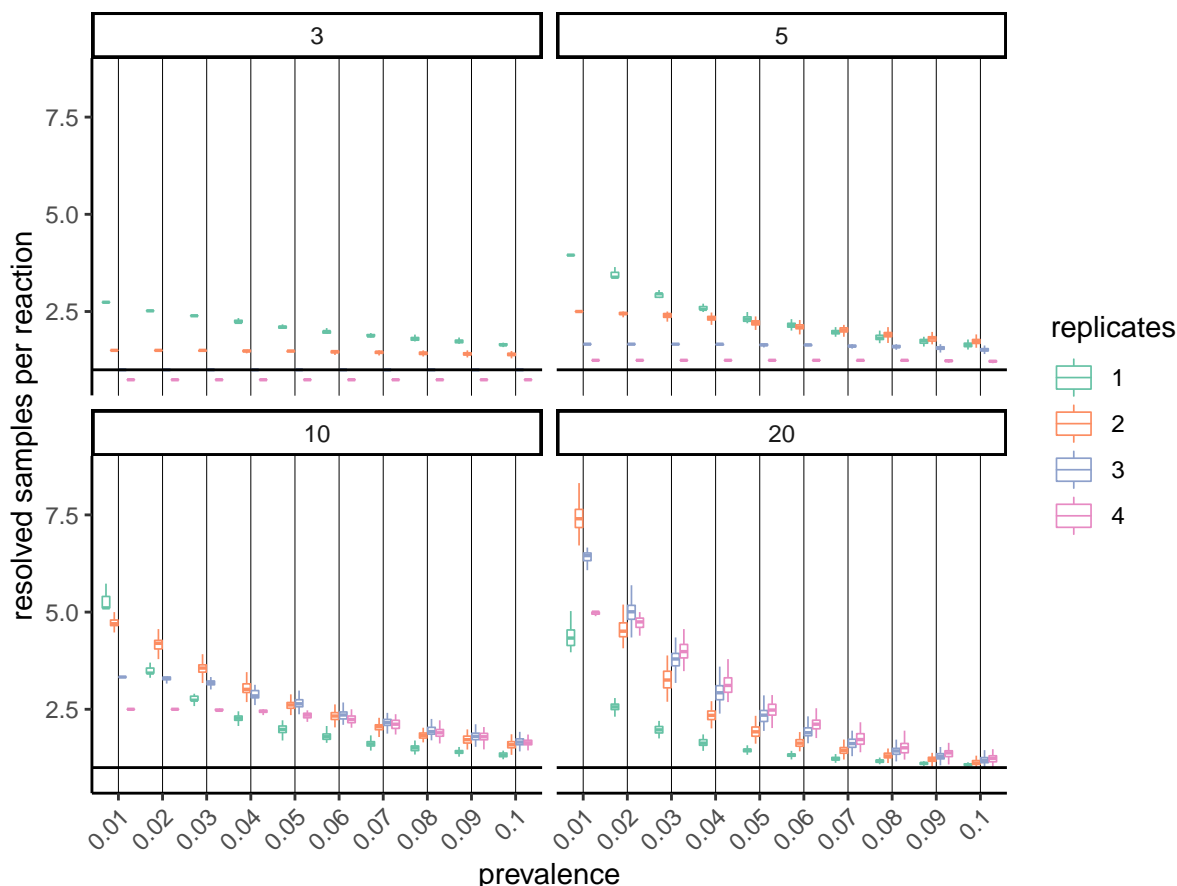


Figure 2: Simulation results for different percentages of positive samples (x-axis), replicates (colors), and pool sizes (panels). The target metric is the effective number of samples per PCR reaction, which includes the individual retesting of samples that cannot be resolved in the first pooling run.

68 At a prevalence of 5% SARS-CoV-2 positive samples, and for ten samples per pool and two replicates per
 69 sample, we simulate that 2.61 times the number of samples can be processed compared to testing samples
 70 individually (SD 0.13). This result is in line with previous estimates using a slightly different version of
 71 the 2-replicate scheme.² Using two replicates increases the effective number of samples per reaction by
 72 31% compared to pooling without replicates. At 2% prevalence and 20 samples per pool – a scenario
 73 more akin to screening large populations – 5.01 times the number of samples can be screened compared
 74 to individual testing (SD 0.28), and the increase over traditional pooling is 193%. These presented values
 75 correspond to *in silico* simulations, and require further validation in the laboratory.

76 In conclusion, our pooling protocol based on sample replicates can substantially increase the number of

77 samples per PCR reaction when screening large populations during pathogen outbreaks, such as SARS-
78 CoV-2. The protocol can be tuned to local laboratory conditions such as pool size and proportion of
79 positive samples. The accompanying software supports the protocol's implementation and routine use.

80 References

- 81 1. Dorfman, R. The detection of defective members of large populations. *Ann. Math. Stat.* **14**, 436–440
82 (1943).
- 83 2. Sinnott-Armstrong, N., Klein, D. & Hickey, B. Evaluation of group testing for SARS-CoV-2 RNA.
84 *medRxiv* (2020).
- 85 3. Eis-Huebinger, A. M. *et al.* Ad hoc laboratory-based surveillance of SARS-CoV-2 by real-time RT-PCR
86 using minipools of RNA prepared from routine respiratory samples. *medRxiv* (2020).
- 87 4. Wölfel, R. *et al.* Virological assessment of hospitalized patients with COVID-2019. *Nature* (2020).
- 88 5. Holshue, M. L. *et al.* First case of 2019 novel coronavirus in the united states. *N. Engl. J. Med.* **382**,
89 929–936 (2020).