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Ministry of Health Malaysia

2 April 2021
1. **Background**

There are three most important factors namely timeliness, accuracy and scalability when considering laboratory testing for SARS-CoV-2 as a public health response to COVID-19 pandemic. The gold standard for laboratory testing for COVID-19 is by real time RT-PCR. However, access to PCR reagents can be a major limitation for testing in many countries especially among those who are completely dependent on importation of those reagents. One of the measures in optimization of testing is by pooling clinical samples before testing.

This approach for molecular testing of Covid-19 has been evaluated by many institutes including the German Red Cross Blood Donor Service, Frankfurt and Centers for Diseases Control (CDC). Likewise, the Virology Unit, Institute for Medical Research has also optimized and validated the pooling method for molecular testing of COVID-19 in April 2020. The main aim is to accommodate high-capacity testing in a larger population. The method allows a combination of few swab samples in a single tube and subsequently tested using PCR assay. In the case of a negative result, all included samples have a reliable negative result. In the case of a positive mini-pool result, individual testing is carried out in previously pooled samples.

2. **Strategies and Limitations in Using Sample Pooling**

2.1 A pooling strategy depends on the community prevalence of virus, and pool size will need to be adjusted accordingly. CDC recommends that laboratories should determine prevalence based on a rolling average of the positivity rate of their own SARS-CoV-2 testing over the previous 7–10 days. Laboratories should use a standardized methodology or calculator that factors in the sensitivity of the assay they are using and their costs of testing to determine when the positivity rate is low enough to justify the implementation of a pooling strategy. Laboratories should also understand and, where appropriate, communicate the limitations associated with pooled testing, which are described in greater detail below.

2.2 Based on limited data, using a pooling testing procedure for SARS-CoV-2 has some limitations. In a pooling procedure, the laboratory cannot
ensure the diagnostic integrity of an individual specimen because it is combined with other specimens before testing. Specimen integrity can be affected by the quality of swab specimen collection, which could result in some swabs having limited amounts of viral genetic material for detection. Inadequate individual specimens, including those with limited amounts of viral genetic material, might not be eliminated from the pooled specimen before testing. Even if each individual specimen in a pool is adequate, the specimens in a pooled procedure are diluted, which could result in a low concentration of viral genetic material below the limit of detection. These limitations mean that monitoring the prevalence of disease and properly validating the assay and the instrumentation are important to limit the potential for false-negative results. In general, the larger the pool of specimens, the higher the likelihood of generating false-negative results.

2.3 When should pooling be used? Pooling should be used only in areas or situations where the number of positive test results is expected to be low, for example in areas with a low prevalence of SARS-CoV-2 infections (less than 5%).

3. Materials and Methods for Pooling of Clinical Samples

3.1 Target population for pool testing of clinical samples

During the optimizing of pool testing, almost 300 cases were tested using methods for pooling samples among in-patients (samples include such as NPS or OPS), for which requires that clinical samples should be from lower acuity settings including samples from out-patient. As TAT for samples that were pooled will take longer than usual (due to the need to repeat when their pooled samples are positives), pooling of samples should be avoided if involved samples from hospital including from in-patients, healthcare workers and outbreak investigation, which requires urgency of results.

3.2 Brief SOP for pooling for clinical samples

The Virology Unit, IDRC, IMR NIH have carried out evaluation of pooling
of two, and five clinical samples. The pooling evaluation was carried out based on several publications and recommendations. Results indicate that pooling of two clinical samples in single tube provided reliable PCR results with an increase of only 0.4 to 0.8 Ct value compared to the positive samples tested individually. This increase is acceptable as there is very low probability of causing false negative results.

In contrast, pooling of five clinical samples caused an increase of 2.0 to 3.2 Ct value as compared to the single samples. This increase will cause samples with high Ct values such as Ct 36 to 38 to be missed. E.g. pooling of 5 clinical samples with original Ct value ranging from 36 to 38 will become 38 to 41 respectively and thus will be reported as negative (false negative). However, based on viral culture study, the high Ct values are not infectious.

The above findings are quite similar to those found by researchers from the Victorian Infectious Diseases Reference Laboratory, Melbourne, Australia. That laboratory conducted pooling of two, four and eight samples and resulted in increase of 1.0 Ct for two-pooling, 2.0 Ct for four-pooling and 3.0 Ct for eight-pooling (Chong et al, 2020).

Due to possible human errors in interpretation of results, the laboratory that performs pool-testing must have very clear algorithm. There must be careful tracking of positive pools and accompanying worksheets to maintain traceability of results throughout the PCR workflow.

### 3.3 SOP for Pooling of two clinical samples

i. Identify the set of samples for two-pooling. For eg, for ease of understanding, provide identity of samples for two-pooling as WCoV 001 and WCoV 002.

ii. Determine the usual volume of clinical samples used for RNA extraction. The volume can range from 140 µl to 200 µl. Divide the volume into two and that volume is needed from each clinical sample for two-pooling.

iii. Pipet 70 µl (if final volume is 140 µl) each from the original sample (WCoV
001 and WCoV 002 respectively) into another sterile tube for two-pooling and label as (WCoV 001 + WCoV 002). Briefly vortex the pooled samples.

iv. Proceed to do RNA extraction for two-pooling (WCoV 001 + WCoV 002) and also proceed to perform real time RT-PCR.

v. Scenario 1: If the results of the two-pooling (WCoV 001 + WCoV 002) is positive for COVID-19 RT-qPCR, please repeat RNA extraction on individual samples (WCoV 001 and WCoV 002 each respectively) and repeat real time RT-PCR.

vi. Subsequently, please report the results of the individual results. There are possibilities of either one of the samples is positive or both the samples are positive. But there won’t be possibility of both samples becoming negative.

vii. Scenario 2: If the results of two-pooling (WCoV 001 and WCoV 002) is negative, then there is no need to repeat RNA extraction and RT-qPCR. Please proceed to report both samples as negative for COVID-19.

3.4 SOP for Pooling of five clinical samples

i. Identify the set of samples for five-pooling. For eg, for ease of understanding, provide identity of samples for five-pooling as WCoV 001 and WCoV 002, WCoV 003, WCoV 004 and WCoV 005.

ii. Determine the usual volume of clinical samples used for RNA extraction. The volume can range from 140 µl to 200 µl. Divide the volume into five and that volume is needed from each clinical sample for five-pooling.

iii. Pipet 40 µl (if final volume is 200 µl) each from the original sample (WCoV 001, WCoV 002, WCoV 003, WCoV 004 and WCoV 005. respectively) into another sterile tube for five-pooling and label clearly as (Pool of WCoV 001 till 005). Briefly vortex the pooled samples.

iv. Proceed to do RNA extraction for five-pooling (WCoV 001 till 005) and also proceed to perform real time RT-PCR.

v. Scenario 1: If the results of the five-pooling (WCoV 001 till 005) is positive for COVID-19 RT-qPCR, please repeat RNA extraction on individual
samples (WCoV 001, WCoV 002, WCoV 003, WCoV 004 and WCoV 005 each respectively) and repeat real time RT-PCR.

vi. Subsequently, please report the results of the individual results. There are possibilities of at least one sample is positive or more than one becoming positive.

vii. **Scenario 2:** If the results of five-pooling (WCoV-001 till 005) is negative, then there is no need to repeat RNA extraction and RT-qPCR. Please proceed to report all five samples as negative for COVID-19.

### 4. Results, Data Analysis and Reporting of Results

#### 4.1 Interpretations of results and data analysis

Results for each pooled sample will be tabulated using the Cycle threshold (Ct) cut-off of the real time RT-PCR kit used. The delta Ct value (ΔCt) is defined as the absolute increase in Ct value when the pooled sample was tested compared to when the positive sample was tested individually. Hence a positive ΔCt value indicated by an increase in Ct value in the pooled sample, represents the loss of PCR sensitivity due to sample pooling (Table 1).

In addition, the interaction between the prevalence of SARS-CoV-2 in the test population and the efficiency of the various pool sizes was examined for a disease’s prevalence between 0.2% to 10%. When prevalence rate increases, the probability of pooled samples requiring repeat individual testing increases which will take longer than the usual TAT. This is because the need to repeat RT-qPCR for all positive pools samples and hence takes longer TAT to release result.

The pooled testing results can be reported as suggested in Table 2.
Table 1 Mean delta Ct value (ΔCt) for each pool, which is representing the loss of PCR sensitivity because of pooling

<table>
<thead>
<tr>
<th>Pool Size</th>
<th>Mean Ct of positive sample</th>
<th>Mean of pooled Ct</th>
<th>Mean ΔCt</th>
<th>Acceptable/Not Acceptable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two</td>
<td>30.53</td>
<td>30.93</td>
<td>0.4 (0.3-0.8)</td>
<td>Acceptable (up to 5% prevalence)</td>
</tr>
<tr>
<td>Five</td>
<td>30.90</td>
<td>33.49</td>
<td>2.6 (1.9-3.2)</td>
<td>Acceptable (up to 2.5% prevalence)</td>
</tr>
</tbody>
</table>

4.2 Reporting of results in pooled samples setting

Table 2: Reporting of results in pool testing

<table>
<thead>
<tr>
<th>No</th>
<th>Results of Pooled Testing</th>
<th>Reporting</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Negative (-)</td>
<td>Report each individual in the pool as negative.</td>
</tr>
<tr>
<td>2</td>
<td>Positive (+)</td>
<td>Do not report pooled results. Proceed to test individual testing in that pooled samples and report after that.</td>
</tr>
<tr>
<td>3</td>
<td>Inconclusive (+/-)</td>
<td>Do not report pooled results. Proceed to test individual testing in that pooled samples (either + or -) and report after that.</td>
</tr>
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</table>

5. Ministry of Health Malaysia Committee on Laboratory Testing of COVID-19 Recommendations:

Based on the evaluation, MOH Committee on Laboratory Testing of COVID-19 recommends two clinical samples pooling can be done in areas with a prevalence less than 5% (based on a rolling 7 days), while five clinical samples pooling can be done in areas with a prevalence less than 2.5% (based on a rolling 7 days). In addition, the laboratory must strictly follow the SOP for sample pooling. The laboratory offering pool-testing must also have a well-defined algorithm to ensure reliability of results.
6. References


7. INQUIRIES

Any inquiries about this document can be referred to:

Unit Virologi, Pusat Penyelidikan Penyakit Berjangkit, Institut Penyelidikan Perubatan , Aras 2, Blok C7, Kompleks Institut Kesihatan Negara (NIH), No 1, Jalan Setia Murni U13/52, Seksyen U13, Bandar Setia Alam, 40170 Shah Alam Selangor, Malaysia.
Tel : 03-3362 8960
Email : virology.sa@moh.gov.my
Method for Evaluation of Sample Pooling

A) Two-pooling

100µl 100µl

200µl

RNA Extraction

Results

Negative

Report both samples as negative

Positive

REPEAT RNA Extraction using individual samples

B) Five-pooling

40µl 40µl 40µl 40µl 40µl

200µl

RNA Extraction

Results

Positive

REPEAT RNA Extraction using individual samples

Report results for each samples respectively

Negative

Report all five samples as negative

Real Time RT-PCR for COVID-19

Algorithm of testing for Sample Pooling Method