# REAL-TIME RT-PCR COVID-19 TESTING

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## 1. Scope

This document describes the requirements to be complied by laboratories offering real-time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) test for SARS-CoV-2 detection.

The guideline, requirement and performance criteria outlined in this document are intended for comparable, accurate and reproducible results.

## 2. Background

Detection of viral RNA through Nucleic Acid Amplification Test (NAAT) is considered as the referral standard for the diagnosis of Covid-19. NAAT includes Real-Time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) which is considered as "gold" standard, Loop-mediated isothermal amplification (LAMP), Transcription mediated amplification (TMA) and Strand displacement amplification (SDA).

Malaysia has previously set for the testing assay to have at least two (2) different targets on the COVID-19 genomes, of which at least one target is confirmatory for the COVID-19 virus. With the recent updates and widespread SARS-CoV-2 circulation, currently only one (1) confirmatory target is required to diagnose COVID-19.

There are numerous SARS-CoV-2 PCR kits available in the Malaysian market that have met the Medical Device Authority (MDA) requirements. An approval from the MDA is based on the evaluation performance of each kit with a cut off 100% sensitivity and 100% specificity.

## 3. General Requirements

a) The laboratories performing RT-PCR must be under supervision by a clinical microbiologist or pathologist with molecular training/experience officially appointed by the laboratory (refer to Appendix 1 - **TOR for the clinical microbiologist/pathologist)**

b) New laboratories intending to offer RT-PCR need to obtain approval from Jawatankuasa Pasukan Khas Makmal COVID-19. Refer to Guidelines for Application Procedure for RT-PCR COVID-19 Testing

## 4. Personnel

The personnel conducting the test procedure shall have a minimum Diploma in Medical Laboratory Technology. Adequate training and competency in nucleic acid testing methods shall also be documented (etc logbook and competency assessment).

The laboratory shall have qualified, skilled and experienced signatory (ies) to validate data and troubleshoot problems. Approved signatory (ies) shall have a degree or higher in medicine or basic science), trained and competent in the nucleic acid method with at least one year or more laboratory working experience and 3 months experience in molecular testing.

Personnel that can validate results are as following:

1) Pathologist
2) Medical Officer
3) Science Officer
4) Research Officer

The lab should have at least one personnel that have qualifications in microbiology.

## 5. Accommodation and environmental conditions

The laboratory shall have dedicated areas for specimen reception, pre and post analysis to minimize cross-contamination. There shall be separate room for pre-PCR, reagent preparation and PCR amplification. The laboratory that has fully automated system, such as from extraction to master-mix preparation, need not have separate room.

*Specimen storage*

There shall be a designated space for storage of specimens.

Specimens shall not be placed in the same storage as the reagents. Pre-testing samples shall not be kept together with post-testing samples.

*Reagents storage*

Reagents shall be stored at the appropriate temperature as recommended by the manufacturer. There shall be an inventory on the date of receive, lot number, expiry date and date of the kit in use.

## 6. Equipment

All freezers and chillers shall have daily temperature chart monitoring.

All equipment including biosafety cabinet class II shall be operated and maintained according to the manufacturer's instructions.

Autoclaves shall be operated by trained personnel.

## 7. Test method and method validation

7.1 The laboratory shall use kits that have been approved by Medical Device Authority(MDA).

7.2 The laboratory shall perform verification of the test method before offering the test. Records of all verifications shall be safely stored for future reference.

7.3 Each new lot of the kit or any changes in reagents or procedure shall be verified for performance before use in testing. It should be documented and records kept by the laboratories.

## 8. Specimen collection, packaging and transport

8.1 Staff shall be trained for appropriate personal protective equipment (PPE) usage, specimen collection, storage, packaging and transport before collecting the samples.

8.2 Request forms and specimen shall be labeled with at least 2 unique identifiers to ensure traceability of specimens.

8.3 The traceability of all sampling activities from receipt through preparation, proper analysis, reporting of results , storage to disposal of the samples shall be documented.

8.4 All swab samples shall be placed in viral transport media (VTM) or universal transport media and kept at 2-8°C before processing.

8.5 Samples shall be packed in triple packaging and transported at 2-8 $^{0}$C. The request forms shall be placed separately on the outside package.

8.6 All positive samples shall be kept at -80°C for at least one week.

8.7 Clinical samples can be pooled prior to performing RT-PCR for SARS-CoV-2 detection. Details on the clinical sampling pooling for the SARS-CoV-2 RT-PCR are as the following. Refer to **Appendix 3**

## 9. Examination procedure

9.1 The laboratory shall have a standard operating procedure for conducting the real-time RT- PCR.

9.2 Specimens shall be processed in biosafety cabinet class II and handled by staff wearing proper PPE.

9.3 All worksheets related to the testing shall be kept and maintained for retention of record.

## 10. Assuring the quality of test results

10.1 Each test run shall include positive and negative control.

10.2 The Laboratory shall participate in the External Quality Assurance Programme upon application. The laboratory shall achieve at least 80% overall correct results. If the result is unsatisfactory , the laboratory shall give evidence of corrective actions taken.

10.3 For non accredited laboratories, the laboratories shall register for MSISO15189 within 1 year from approval date. Existing accredited laboratories shall include molecular testing SARS-CoV-2 into their scope of accreditation during their next assessment.

10.4 All COVID-19 RT-PCR testing laboratories shall be audited by the committee when necessary.

10.5 Random audit can be done on all laboratories that have been approved and laboratories that does not comply to the SOP will be taken off the Annex 4a and reapplication is needed.

## 11. Reporting the results

11.1 All results shall be reviewed and validated by authorized personnel (approved signatories) prior to release.

11.2 All interpretation of results shall follow kit insert by manufacturer

11.3 All results shall be keyed into the *Sistem Informasi Makmal Kesihatan Awam* Outbreak (SIMKA Outbreak) or E-Covid according to the current directive by Ministry of Health.

11.4 It is mandatory to report all results into SIMKA Outbreak or E-Covid within 24 hours.

## 12. Waste management

The laboratory shall have waste management procedure in place.

All clinical samples and consumables used shall be autoclaved or incinerated.

There must be dedicated and suitable waste area prior to collection by third party company. Frequency of collection shall be determined by the laboratories' workload in compliance with laboratory safety.

## 13. Risk Management

The laboratory shall have risk management for performing SARS CoV2 RT PCR testing.

## 14. Post Approval

Any changes in workflow, person in charge (Clinical Microbiologist/Pathologist or laboratory manager) or structural changes or change of location must be informed via email to the JPKMCOVID-19 at covid19makmal@moh.gov.my.

**Appendix 1**

**TOR for the clinical microbiologist/pathologist**

i. Qualifications and experience: Gazetted specialist with at least 3 months experience in molecular testing
ii. Frequency of **onsite** visit: **Minimum twice a month** with attendance documentation
iii. Clinical microbiologist/pathologist appointed are to ensure all requirements are complied by the laboratory
iv. All MOH personnel who are working as clinical microbiologist/pathologist in the private facilities need to obtain permission from their hospital or institution prior to appointment.
v. Scope of supervision to include work processes, storage of samples and reagent, validation of results

**STANDARD REQUIREMENT CHECKLIST FOR CLINICAL MICROBIOLOGIST/PATHOLOGIST**

**LABORATORY NAME : .....**

**MINIMUM AREA TO BE COVERED/SUPERVISED BY CLINICAL MICROBIOLOGIST/PATHOLOGIST:**

| Related SOP /Clause | Requirement | COMMENTS |
|---|---|---|
| 1.Scope | To ensure laboratory:<br>• Have registered for MSISO15189<br>• Have participate in EQA | |
| 2.Personnel | To ensure:<br>• training and competency of all staffs. | |
| 3.Accommodation and environmental conditions | • To ensure:<br>• Dedicated areas for specimen reception, pre and post analysis to minimize cross-contamination.<br>• A separate room for pre-PCR, reagent preparation and PCR amplification.<br>• The laboratory that has fully automated system, from extraction and master-mix preparation, need not have separate room.<br>• A designated space for storage of specimens<br>• Specimens are not be placed in the same storage as the reagents.<br>• Pre-testing and post samples not kept in the same place<br>• appropriate temperature for reagents storage as recommended by the manufacturer.<br>• An inventory on the date of received, lot number, expiry date and date of the kit in use are available.<br>• Each new lot of the kit or any changes in reagents or procedure are verified for performance before use in testing and documented. | |
| 4 .Equipment | To ensure :<br>• Daily temperature chart monitoring for all freezers and chillers.<br>• All equipment including biosafety cabinet class II are | |

| | | |
|---|---|---|
| | maintained according to the planned preventive maintenance (PPM).<br>• Autoclaves are operated by trained personnel. | |
| 5 . Test method and method validation | To ensure :<br>• Kits recommended for use by MDA<br>• Method verification of the test performed and record safely stored<br>• The procedure for offering real time RT-PCR tests for COVID - 19 are followed:<br>1. Obtained an approval from Secretariat of Jawatan Kuasa Petugas Khas Makmal COVID-19 (JPKM COVID-19)<br>2. Pass blinded samples evaluation/test<br>3. All positive samples are kept at -80°C for one week. | |
| 6. Sampling | To ensure :<br>• Staff are trained for appropriate<br>• personal protective equipment (PPE) usage,<br>• specimen collection,<br>• packaging ,transport and storage before collecting the samples.<br>• Only non- expired supplies/consumables are used<br>• All swab samples placed in viral transport media (VTM) or universal transport media and kept at 2-8°C before processing.<br>• At least 2 unique identifiers on the request forms<br>• The traceability of all sampling activities from receipt through preparation, proper analysis, reporting of results, storage to disposal of the sample are documented | |
| 7. Handling of test items | To ensure :<br>• Samples are packed in triple packaging and transported at 2-8 °C.<br>• The request forms are placed separately on the outside package<br>• Processing of specimen in biosafety cabinet class II and handled by staff wearing proper PPE. | |

| | | |
|---|---|---|
| 8 Examination procedure | To ensure :<br>• Standard operating procedure for conducting the real-time RT-PCR.<br>• All worksheets related to the testing kept and maintained<br>• Results should be reviewed randomly at minimum of 2 batches/visit.<br>• Records of corrective action done, endorsed and filed<br>• Customer satisfaction surveys | |
| 9 .Assuring the quality of test results | To ensure :<br>• Each test run included positive and negative control.<br>• The interpretation of test results to follow MOH COVID-19 Guideline<br>• External Quality Assurance participation<br>• Internal audit conducted at least once a year and corrective actions taken where necessary and documented. | |
| 10. Reporting the results | To ensure :<br>• Reviewed and validated by authorized personnel<br>• Keyed all laboratory results into the Sistem Informasi Makmal Kesihatan Awam Outbreak (SIMKA Outbreak) or E-COVID according to latest directive by MOH | |
| 11. Waste Management | To ensure :<br>• Waste management procedure are in place<br>• All clinical samples and consumables are autoclaved or incinerated | |
| 12. Risk Management | To ensure :<br>• Risk management for performing SARS CoV2 RT PCR testing conducted. | |

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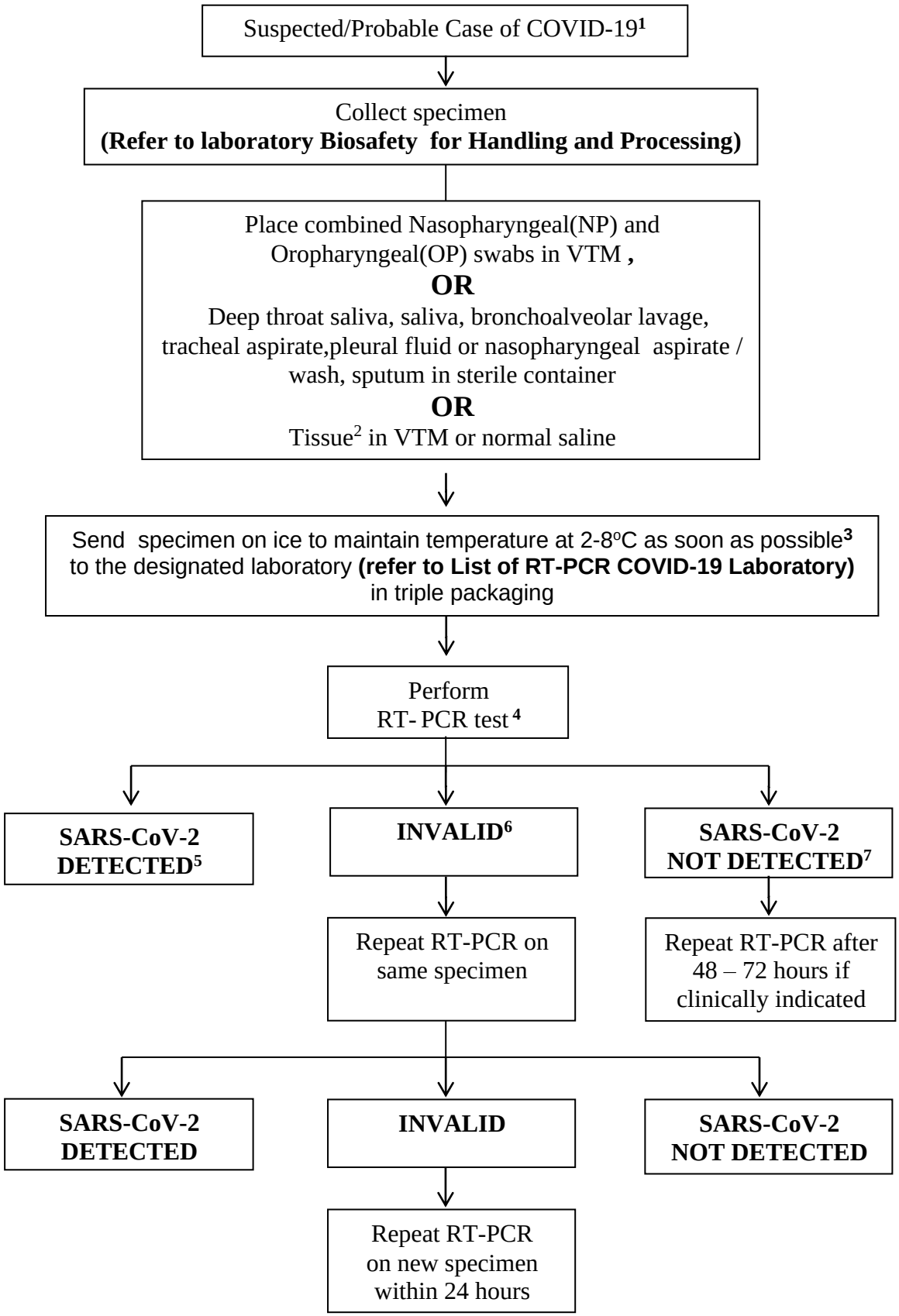
NAME OF CLINICAL MICROBIOLOGIST/PATHOLOGIST :
DATE :

**Appendix 2**

**Flow Chart for Laboratory Diagnosis of SARS-CoV-2 using Real- Time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) in Suspected/Probable Case of COVID-19**



[1] Refer to Case Definition of Covid-19 ( Refer to the latest KKM guideline)
[2] Selected tissues from post mortem cases. Send specimens to IMR, National Institute of Health (NIH).
[3] If transportation of sample is within 72 hours, store at 2-8°C. If transportation of sample is after 72 hours, store at - 80°C
[4]Use Medical Device Authority(MDA) approved RT-PCR kit . The assay shall have minimum one confirmatory target for SARS-CoV-2 (RdRp, ORF1ab or N). S gene should not be used as a single target gene for diagnosis as there is a likelihodd of missing true positive because of spike gene target failure.
[5] A positive laboratory result must be interpreted taking into consideration the clinical history, presentation and/or post mortem findings
[6] Internal control of the results are not detected.
[7] The negative result does not conclusively rule out these viruses as the causative agent of the disease for the following reason: a. Specimens were not collected at the time when the virus present, b. Specimens were not collected, stored or transported in a proper manner .

All results must be reported in SIMKA OUTBREAK or E-COVID according to the current directive by Ministry of Health.

**Appendix 3**

## Sample Pooling for RT-PCR SARS-COV-2

### 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.

### Strategies and Limitations in Using Sample Pooling

- 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.
- 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.

- 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. (between 0.2 % to 2.5%).

**Materials and Methods for Pooling of Clinical Samples**

Based on several publications and recommendations, the Virology Unit, IDRC, IMR evaluated and optimized and pooling of samples for COVID-19 RT-qPCR testing. Briefly, comparisons are between Ct values of RT-qPCR among the pooled samples versus the individual sample. There were two types of pooling investigated which are two-samples pooling and five-samples pooling. However, results indicate that currently only two-samples pooling is within acceptable limits for results. Hence only SOP for two-samples pooling is described below:

**A. 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.

## B. 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. 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 is course of concern as there will be samples with low Ct values such as Ct 36 to 38 that will be missed when pooling of 5 clinical samples carried out as any Ct value ranging from 36 to 38 will become 38 to 41 respectively and thus will be reported as negative (false negative).

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 Ct for two-pooling, 2 Ct for four-pooling and 3 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.

## C. SOP for Pooling of two clinical samples

**Only pre-defined clinical samples are used to optimize sample pooling**

1. Identify the set of samples for two-pooling. For ease of explanation, the two samples will be identify pooling as WCoV 001 and WCoV 002.
2. 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.

3. 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.
4. Proceed to do RNA extraction for two-pooling (WCoV-001 + WCoV 002) and also proceed to perform real time RT-PCR.
5. **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.
6. 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.
7. **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.

**Results and Data Analysis and reporting of results**

- **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.

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 then 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.

**Table 1: Mean delta CT value (ΔCt) for each pool, which is representing the loss of PCR sensitivity because of pooling**

| Pool Size | Mean Ct of positive sample | Mean of pooled Ct | Mean ΔCt | Acceptable/ Not Acceptable |
|---|---|---|---|---|
| Two | 30.53 | 30.93 | 0.4 (0.3-0.8) | Acceptable |
| Five | 30.90 | 33.49 | 2.6 (1.9-3.2) | Not Acceptable |

- **Reporting of results in pooled samples setting**

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

**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 between 0.2-2.5% (based on a rolling 7 days), and the laboratory to strictly follow the SOP for sample pooling. The laboratory offering pool-testing must also have a well-defined algorithm to ensure reliability of results.