Pooling protocol using AMPHABIO HT-HiThroughput PCR COVID-19 Kit

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Pooling protocol

1 Pre-amplification

1.1 Master mix preparation

- Thaw all reagents to obtain homogeneous solutions. Mix all the tubes gently with the vortex mixer and briefly spin down. Do not leave the reagents at room temperature for more than 30 minutes.
- Keep all the tubes on ice.
- Prepare the pre-amplification mix according to the formula below:

10 µl pre-amplification master mix

1.25 µl Oligo P mix

8.75 µl ultrapure, DEPC-treated water

- Mix the pre-amplification mix thoroughly by pipetting. Dispense 20 µl pre-amplification mix into each PCR well. Keep all the tubes/plates on ice.
- Add 5 µl of extracted RNA or Proteinase K-treated saliva of the corresponding samples (or negative control or positive control) to each PCR reaction, close the PCR tube, mix well, spin the tubes shortly and place the tubes back on ice.
- Transfer all the PCR tubes/plates into a conventional PCR thermocycler, e.g. vapo.protect

(Eppendorf).

1.2 Set up the thermal cycling program for the first round of amplification:

+ Reverse transcription at **50 °C** for **00:10:00**

+ Step-down from **50 °C** to **40 °C** with **1 °C** per **00:01:00**

+ Initial denaturation at **95 °C** for **00:15:00**

+ 6 cycles of **94 °C** in **00:00:15** , **60 °C** in **00:03:00** , then **72 °C** in **00:00:30**

+ 22 cycles of **94 °C** in **00:00:15** , **76 °C** in **00:03:00** , then **72 °C** in **00:00:30**

+ Final extension at **72 °C** in **00:05:00** , and then hold at **4 °C** .

2 Nested amplification (2nd round) & Melting analysis

2.1 Prepare the pooled templates

- Mix the pre-amplified products of the first round to make the pooled templates (8-channel and 12-channel pipettes, filter tips and 96-well plates are use in this step). The AMPHABIO HT-HiThroughput PCR COVID-19 Kit allows for pooling of up to 100 pre-amplified products.

2.2 Prepare the master mix

- Thaw all reagents to obtain homogeneous solutions. Mix all the tubes gently with the vortex mixer and briefly spin. Do not leave the reagents at room temperature for more than 30 minutes.
- Keep all tubes on ice.
- Prepare the reactions of the detection mix according to the formula below:

8 µl detection master mix

1 µl oligo D mix

9 µl ultrapure, DEPC-treated water

- Mix the detection mix thoroughly by pipeting. Dispense 18 µl master mix into each PCR well. Keep all the tubes/plates on ice.
- Add 2 µl of the pre-amplified product of 1st round (pooled template) to each PCR wells (filter tips are recommended to use in this step), close the PCR tubes/plates, mix well, spin the tubes/plates shortly and place the tubes/plates back on ice.
- Transfer all the PCR tubes/plates into the PCR or Realtime PCR instruments.

2.3 Set up the thermal cycling program of the 2nd round amplification

+ Initial denaturation at **95 °C** in **00:15:00**

+ 45 cycles of **94 °C** in **00:00:15** , **63 °C** in **00:00:30** , and **72 °C** in **00:00:30**

2.4 Melting analysis

- The 2nd-round amplification products are melting analyzed for acquisition of the fluorescence signals on the FAM channel to generate the melting spectrum using the realtime PCR instrument with High-resolution melting analysis or Melting curve analysis.

+ For High-resolution melting analysis, the thermal cycle is set up as below:

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Hold at **95 °C** in **00:01:00**

Then increase from **70 °C** to **95 °C** with **0.2 °C** per **00:00:02**

+ For Melting curve analysis, the thermal cycle is set up as below:

Hold at **95 °C** in **00:01:00**

Then increase from **70 °C** to **95 °C** with **0.5 °C** per **00:00:02**

*** Note**: The amplification of the 2nd round and melting spectrum analysis can be performed continuously on the same realtime PCR instrument in one run. Users also can perform the 2nd round of amplification on a conventional PCR instrument to reduce the burden of using realtime PCR instruments. Raw melting spectra of all samples in a batch is exported as a .txt file or an excel file.

2.5 *Analysis of melting spectrum using cloud-based AI model for detection of SARS-CoV-2*

- The raw melting spectra are analyzed for detection of *SARS-CoV-2* using the artificial intelligence model with the following steps:
  + Access the artificial intelligence software web application at the following link: https://htpcr.topdatascience.com/
  + Verify the registered information of the kit (once at the time of opening the kit) by scanning the QR code on a small box inside, using Google Chrome browser on the smartphone.
  + Upload melting spectra data file in .txt or excel format.
  + Click on the melting categories: “Melt” or “High-resolution melting”.
  + Declare information related to pooled samples and realtime PCR instrument
  + Click “Run prediction”.

2.6 *Result interpretation*

- The detection results of *SARS-CoV-2* are analyzed by artificial intelligence software.
- Check the results of positive control and negative control to ensure that the test results are reliable.
  + The positive control must give a positive result when analyzed by AI-powered software
  + The negative control must give a negative result when analyzed by AI -powered software
- A pooled sample will be reported as positive for Sars-CoV-2 if the test results of that pool sample and the positive control are positive, and the test result of the negative control is negative when analyzed by the AI-powered software.
- A pooled sample will be reported as negative for Sars-CoV-2 if the test result of the positive control is positive, and the test results of that pool sample and the negative control are negative when analyzed by the AI-powered software.
- If the test results of the positive control and the negative control are not correct, all pooled samples need to be re-tested.
- ***Result interpretation of pooled testing***
  + If a pooled sample (A) result is negative, then all samples in that pooled sample (A_1, A_2, A_3, … A_n) will be reported as negative for SARS-CoV-2.
  + If a pooled sample (A) result is positive, then the pre-amplified product of each sample (A_1, A_2, A_3, … A_n) in that pool will be re-tested individually by the 2nd-round amplification to determine which samples are positive.

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