AMPHABIO HT-HiThroughput PCR COVID-19 Kit Protocol V.2

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ABSTRACT

This protocol shows procedure for sample handling and detection of SARS-CoV-2 using the AMPHABIO HT-HiThroughput PCR COVID-19 Kit.

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MATERIALS TEXT

Sample processing:
- RNA extraction Kit or AMPHABIO Sample Collection Kit.
- Pipette 1000uL and filter tips
- Pipette 200uL and filter tips
- Pipette 10uL and filter tips
- Sterile tubes for storage
- Appropriate gloves and a face mask
- Biosafety cabinet
- Incubator, vortex
- Centrifuge for tubes

Amplification and pooling
- 12-channel-pipette, 8-channel pipette and filter tips
- Pipette 1000uL and filter tips
- Pipette 200uL and filter tips
- Pipette 10uL and filter tips
- Plate 96 and centrifuge for plate
- Appropriate gloves and a face mask
- Biosafety cabinet
- Conventional PCR machine and realtime PCR instrument (with a computer for data analysis).

SAFETY WARNINGS

Handling of samples which potentially contain SARS-CoV-2 should be conducted in a biosafety cabinet, under BSL-2 conditions.

ABSTRACT

This protocol shows procedure for sample handling and detection of SARS-CoV-2 using the AMPHABIO HT-HiThroughput PCR COVID-19 Kit.

Preparation

1 Input the testing batch information to cloud-based AI application and create the testing plan

- Access the web-based application of artificial intelligence at the following link using user/password provided with the test kit: https://htpcr.topdatascience.com/
- Select appropriate testing workflow:
  + "Pooled testing workflow" tab for pooled testing
  + "Individual testing workflow" tab for testing of individual samples without pooling.
- Enter "Batch size" (number of samples per batch).
- Click "Create testing plan"

- The application will generate the batch ID, the number of plates to be prepared and the plate names, the sample names, which correspond to positions of samples, the number and positions of positive controls, negative controls to be prepared for the pre-amplification step.
- The application will also create the detail plan for sample mapping during pre-amplification, as well as, sample pooling of the whole batch after pre-amplification step (more details in step 4.1).
- The information of the testing batch will be saved upon Finish.
- Upon entering the model/plate format of the realtime PCR instrument(s) needed in the step 4.2, the application will create the detail plan for adding templates in the step 4.2. below

Sample handling

2 Sample handling

The samples can be treated by either an RNA extraction Kit or the extraction-free method using the AMPHABIO Sample Collection kit.

This work should be completed under BSL-2 conditions, and handling of samples which potentially contain SARS-CoV-2 should be conducted in a biosafety cabinet.

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Step 2 includes a Step case.

Extraction-free

RNA extraction

Amplification

step case

Extraction-free

For upper respiratory samples (swabs and/or saliva) collected by the AMPHABIO Sample Collection kit (Ampharco U.S.A, Vietnam, catalogue number: 10SA122001), and stored in the ASC buffer provided with the kit:
- Samples are mixed in the ASC buffer 10X to the final concentration of 1X
- A 50-µl aliquot is then treated by adding 6.5µl of Proteinase K (20mg/ml), followed by a 10-min incubation at 95^{o}C.
- The treated samples are ready to be used as the template for Pre-amplification step of AMPHABIO HT-HiThroughput PCR COVID-19 kit, or stored at -80^{o}C until analysis.

3 Pre-amplification

3.1 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 Proteinase-K treated sample or extracted RNA template 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), MiniAmp Thermal Cyclers (ThermoFisher Scientific).

3.2 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** .

4 Nested amplification (2nd round) & Melting analysis

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4.1 Prepare the pooled templates (for pooled testing workflow only)

- Pooling the pre-amplified products in the step 3. according to the detail instruction generated by the web-based application (step 1.), which is described briefly as below:
  i) 8-sample pooling (or column pooling) to make the pooled templates for each column of a plate using the 12-channel pipette.
  ii) From the 8x12 plate, which contains 8-sample pooled templates in the step i), perform 12-sample pooling using the 8-channel pipette to make the pooled templates for each row of this 8-sample pooled plate. As a result, 96 single samples from one 96-well plate is pooled in a well after step i) and ii). (so called 96-sample pooling, or plate pooling)
- The resulting 96-sample pooled samples are used as template in the following step 4.2.

8-channel and 12-channel pipettes, filter tips and 96-well plates are recommended to use in this step

4.2 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 pipetting. Dispense 18 µl master mix into each PCR well. Keep all the tubes/plates on ice.
- Add 2 µl of either pre-amplified product from the step 3. (*for individual testing workflow*) or pooled template from the step 4.1. (*for pooled testing workflow*) after 5x dilution (in ultrapure H_2O) to each PCR wells, according the the plan and instruction created on the web-based application in the step 1. above.
- Close the PCR tubes/plates, spin the tubes/plates shortly, and place the tubes/plates back on ice.
- Transfer all the PCR tubes/plates into the conventional PCR or realtime PCR instrument.

If the realtime PCR instrument is used in this amplification step, the melting analysis mentioned in the step 4.4. below can be proceeded continuously following amplification step 4.3 within the same run of realtime PCR. Filter tips are recommended to use in this step.

4.3 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**

4.4 Melting analysis

- The 2nd-round amplification products are melting analyzed for acquisition of the fluorescence signals

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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:

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**

The amplification of the 2nd round and melting spectrum analysis can be performed continuously on the same realtime PCR instrument in one run as noted above. However, users also can perform the 2nd round of amplification on a conventional PCR instrument to reduce the burden of using realtime PCR instruments and for higher throughput of testing. Raw melting spectra of all samples in a realtime PCR run of melting analysis is exported as a .txt file or an excel file.

Data analysis

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

- The raw data of melting spectra is analyzed for detection of *SARS-CoV-2* using the web-based application of artificial intelligence available at the same link as mentioned in the step 1. (https://htpcr.topdatascience.com/).
- Melting analysis of all samples within a run could be done by following steps:
  + Navigate from the homepage to the testing batch of interest, and follow the detail instruction provided.
  + Select appropriate model/plate format of realtime PCR instrument (if required)
  + Click "Choose file" under the tab "Upload PCR melting data" (melting spectra data file in .txt or excel format)
  + Click on the appropriate melting analysis types: “Melt” or “High-resolution melting”.
  + Click “SARS-CoV-2 melting analysis”.

6 **Result interpretation**

- The AI model will *automatically* analyze the melting data and interpret results according to the types of test workflow, individual testing or pooled testing.
- The AI model will initially inform the user about the reliability of test results. The test results are valid if the results of both positive controls and negative controls are valid:
+ The positive control must give a positive result with a likelihood ≥60%
+ The negative control must give a negative result with a likelihood ≥60%
- If the test results of the positive control and the negative control are invalid, the AI model will inform to the user that the test is invalid, the nested amplifications need to be retested. If the test result still shows invalid, all samples need to be retested from the pre-amplification step.

Step 6 includes a Step case.
Individual test
Pooled testing

step case

Individual test

The web-based AI model will *automatically* analyze and report:
- A tested sample will be reported as positive for Sars-CoV-2 if the controls are valid, the Prediction of that sample is Positive, and the Likelihood is ≥60% in the Prediction result analyzed by AI-powered software.
- A tested sample will be reported as negative for Sars-CoV-2 if the controls are valid, the Prediction of that sample is Negative and the Likelihood is ≥60% in the Prediction results analyzed by AI-powered software.
- A tested sample will be reported as presumptive positive for Sars-CoV-2 if the controls are valid, the Prediction of that sample is Positive, and the Likelihood is <60% in the Prediction result analyzed by AI-powered software.

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- A tested sample will be reported as presumptive negative for Sars-CoV-2 if the controls are valid, the Prediction of that sample is Negative and the Likelihood is <60% in the Prediction result analyzed by AI-powered software.
- The presumptive positive/negative samples will be re-tested by the nested amplification. If the results still show the Likelihood <60%, the samples need to be re-tested from the pre-amplification steps or a second sampling will be required.

7 Next steps for pooled testing workflow

7.1 For negative pools

- All samples in the negative 96-sample pools will be automatically reported as negative for SARS-CoV-2.
- Finnish the testing workflow for such samples

7.2 If there are pools, which are positive, presumptive positive, or presumptive negative

- The web-based AI application *automatically* show the button to create the plan for re-test, which include detail instruction to follow, regarding:

+ Prepare the 12-sample pooling of pre-amplified products in each row using 8-channel pipette for each 96-well plate positive with SARS-CoV-2. Therefore, for each 96-well plate positive with SARS-CoV-2, the application will indicate eight 12-sample pools (row pools) and twelve 8-sample pools (column pools, created in the step 3.1) to be re-tested by the nested amplification to find which one is positive for SARS-CoV-2.

+ The application shows the number of re-tests (nested amplification - step 4.2).

+ Upon entering the model/plate format of the realtime PCR instrument(s), the application will create the detail plan for adding templates in the step 4.2.

+ The information of the testing plan will be saved upon Finish.

+ When the sub-pooled re-testing finishes, perform melting analysis as described in the step 5.

+ The web-based AI model will *automatically* identify the negative, positive pools. Based on that, the positive, negative samples will be *automatically* identified, or further individual re-test is indicated according to the following rules:

- *If a row/column pool is negative, all samples in that row/column will be reported as negative.*
- *If a row pool and a column pool are positive, the sample crossed both row and column will be reported as:*

i) Positive for SARS-CoV-2: when there is only a single row- or single column pool to be positive.

ii) Individual re-test (nested amplification - step 4.2.) of the crossed samples, when more than 1 row- and more than 1 column pool are positive.

+ Follow the detail instruction of individual re-test if required.

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