University of Tübingen

Institute of Tropical Medicine

Clinical Trial Platform

Process Description

SOP011b-ITM CTP

Qualitative detection of SARS Coronavirus-2 (SARS-CoV-2) by reverse transcription quantitative PCR assay

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| Author: | \_\_\_\_\_\_\_\_ | \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ |
| **Albert Lalremruata** | Date | Signature |

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| --- | --- | --- |
| Review: | \_\_\_\_\_\_\_\_ | \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ |
| **T.P. Velavan** | Date | Signature |

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| Approval: | \_\_\_\_\_\_\_\_ | \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ |
| **Meral Esen** | Date | Signature |

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| Applicability: | This Standard Operating Procedure (SOP) is applicable to all personnel involved in detection of Coronavirus (COVID-19) by RT-qPCR assay. |

**Document History:**

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| --- | --- |
| **Version-No.** | **Description of changes (eventually reasons of changes)** |
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# Purpose

The purpose of this Standard Operating Procedure (SOP) is to describe the methodology to detect SARS Coronavirus -2 (SARS-CoV-2) by real-time PCR using nucleic acid extracted from clinical study participants (see SOP010b-ITM CTP for RNA extraction).

# Definitions

|  |  |
| --- | --- |
| UKT | Universitätsklinikum Tübingen |
| CTP | Clinical Trial Platform; the unit of ITM that performs and manages ITM’s clinical trials activity |
| SOP | Standard Operating Procedure |
| RNA | Ribonucleic Acid |
| ITM | Institute of Tropical Medicine |
| LC 480 | LightCycler® 480 Instrument II |
| RT-qPCR | Reverse Transcription Quantitative PCR |
| Cp | Crossing point (also known as cycle threshold) |
|  |  |

# Responsibilities

The QIAgility is designed for automated liquid handling actions that enables high-precision PCR assay set up. The instrument is intended for use by users trained in molecular biological techniques and the operation of the instrument only. All personnel involved in real-time PCR assay for detection and quantification of coronavirus are responsible to follow all aspects detailed in this SOP. Laboratory head, group leader molecular biology experts, statistician, and lab managers are responsible for providing training on real-time PCR assay and analysis of results.

# Procedure

Ensure that there is no risk of contamination. To remove DNA/RNA from the worktable and to eliminate cross-contamination, the QIAgility must be routinely decontaminated by wiping the worktable with a soft cloth soaked in a cleaning agent [Gigasept instru AF (Schülke & Mayr GmBH)] and by UV irradiation. Clean the working surface, pipette sets, tube racks etc. with RNase decontamination solutions (RNase-Exitus Plus™ or equivalent). Always wear clean protective clothing and change gloves frequently whenever contamination with RNases is possible (e.g. after touching non-cleaned surfaces, door handles, keyboards, etc.). Use RNase & DNase-free certified disposable tubes and filter tips. This SOP describes how to set up and perform the real-time PCR assay in a 384-well plate format using automated liquid handling system (QIAgility, Qiagen) and the real-time PCR instrument (LightCycler® 480 Instrument II, Roche).

**Note: For manual preparation of master mix and pipetting for real-time PCR, please follow procedures from section 4.5.7**

## Preliminaries

* Ensure LightCycler® 480 Instrument II has been turned on before preparing the PCR master mix.
* Remove the purified RNA tubes from -80°C, allow the sample to thaw at room temperature and spin briefly using a centrifuge.
* Ensure that all PCR kit components have thawed completely before use. Briefly vortex and centrifuge to ensure uniform mixing and collection of the tube contents.
* Set the appropriate plate adapters (384 well/96 well) in the correct positions and put the appropriate plastic wares in the defined positions.
* Calibrate both plate/tube position and height. Use 200 µL tips for positions and 50 µL tips for heights.
* Prepare a pipetting scheme/sample sheet using Microsoft Excel spreadsheet.
* Copy and paste the Loading Scheme into LC480 software version 1.5.1 sample editor.
* After the run, first check the results of the controls. Amplification signal in a negative control indicates contamination in the reagents or cross-contamination. Determine source of contamination and repeat experiment.
* The RNA Extraction Control is required to validate the extraction and PCR assays.
* Monitor and document instrument performance using The LightCyler® 480 control kit (cat# 04710924001) at least once in a year

## Template

RNA (extracted according to SOP010b-ITM CTP).

## Materials and reagents

* RealStar® SARS-CoV-2 RT-PCR Kit 1.0 (Altona Diagnostics, cat# 821003)
* 384-well plates for LC480 (Roche, cat# 4729749001)
* 50 µl conductive filtered tips (Qiagen, cat# 990512)
* Pipettes set (P1000, P100, P10)
* Disposable filter tips
* DNase and RNase-free tubes
* Nuclease-free water
* The LightCyler® 480 control kit (Roche, Cat No 04710924001)

## Instrument

* QIAgility (Qiagen)
* LightCycler® 480 Instrument II (Roche)
* PCR workstation
* Centrifuge

## Procedural description

### Setting up QIAgility

* Switch on the QIAgility with the power button located at the rear of the instrument and ensure the lid is closed.
* Switch on the laptop connected to the QIAgility instrument.
* Double-click on the “QIAgility” icon on the desktop.
* Click on the “Recent” tab from the welcome dialog screen. Click “Browse” and navigate to QAS Run file template “384well plate with 96-well EMTR plate” (This PC/Local Disk (C)/ Program Files/QIAgility/Data). Click “Open”.
* The main software window will appear and configure the worktable layout with the following function:
* M1: “Mix” (B to E wells for 1.5mL tube and A for 5 mL tube)
* A1 & A2: “Tip” (Fill-in 50 µl conductive filtered tips here)
* B1 & B2: “Tip” (Fill-in 50 µl conductive filtered tips here)
* C1: “Sample” (place sample tubes here)
* C2: “Reaction” (place a 384-well plate with adapter here)

### Preparing a master mix for reaction setup

* Prepare a master mix as mentioned in step 4.5.7 in a PCR workstation and configure the QIAgility as follows
* Click on the well in the mastermix block (workspace position M1) that contains the mastermix tube. The “Mix Selection” right-hand pane will appear.
* Select “Use Master Mix”. Enter a name for the master mix in the “Master Mix name” field.
* Click on the “Configure Mix” button. The “Master Mix Component Selection” window will appear.
* Enter the master mix volume per reaction (enter **10** µL).
* Enter the default sample volume (enter **5** µL). The total reaction volume will then be displayed (15 µL) and click “OK”.
* Then, select “Use pre-mixed Master Mix?” from the “Mix” pane. The corresponding well in the Master Mix Block (M1) will now be colored yellow.

### Creating a sample bank

* Click on the sample rack (workspace position C1) and the “Sample Banks” dialog box will appear.
* Select “New Bank” and enter a name for the new sample bank.
* Select all samples for amplification
* Click on the “Add Selection” button and then “Done”. The selected samples will now appear in color in the workspace position C1.

### Setting up a reaction list

* Click on a reaction plate (workspace position C2) and the “Reaction List” right-hand pane will appear.
* Click on the “Add” button.
* Select a sample bank from the drop-down menu in the “Samples” panel.
* Select the components of the reaction. Available master mixes are listed in the “Mixes” panel.
* Define the number of replicate from the “Target wells Replicates”.
* Uncheck “Mix on ejection”.
* Click “Ok”.

### Set tip usage options

* Double-click on the bar “Tip re-use” to open the “Tip Usage” dialog box.
* Select “Re-use tips where possible” from the drop-down menu.
* Select “Allow multiple ejections from a single liquid pick-up”.
* Click “Ok”.

### Starting a run

* Click on the run icon (green button) on the toolbar.
* The “Save as” dialog box will appear. For saving the run file, enter a name for the run file and click on the “Save” button.
* The pre-run “Checklist” dialog box will appear.
* If the run has been set up correctly, the checklist will display blue message warnings and must be checked to continue. Red messages indicate errors and the user intervention is required before the run can be started.
* Click on the “Ok” button to start the run.

### Master mix setup

* All reagents should be thawed completely, mixed and kept on ice at all time.
* Prepare a master mix on ice based on the number of samples intended for the assay. Include extra volume while preparing the master mix to account for pipetting losses and evaporation. Minimum 2 replicates are recommended for each sample.
* Every real-time PCR assay must include positive and negative PCR control reactions in at least 2 replicates.
* Use no-template extraction control (NTC) from a non-infected healthy donor.
* Use internal control (IC) included in the kit. This can be used either as an extraction control or PCR inhibition control (see SOP10b)
* Prepare one step RT-qPCR master mix as shown in the table 1.

Table 1. Master mix

|  |  |
| --- | --- |
| Components | Per reaction (µl) |
| Master A | 2.5 |
| Master B | 7.5 |
| Volume master mix | 10 |

* Automate the pipetting of master mix and sample into each reaction well (384-well plate) using QIAgility as describe from step 4.5.1. The final reaction of the assay is shown in the table 2

Table 2. Reaction mix

|  |  |
| --- | --- |
| Components | Per reaction (µl) |
| Master mix | 10 |
| Sample or Positive control | 5 |
| Total reaction volume | 15 |

* Seal and centrifuge the plate at 1600xg for 1 minute.

### Amplification conditions

* Load reaction plate onto the LightCycler® 480 Instrument II.
* Create the thermal cycling conditions specified in the table 3

Table 3. Thermal Profile

|  |  |  |
| --- | --- | --- |
| **Steps** | **Temperature (°C)** | **Time (min:sec)** |
| Reverse transcription | 55 | 20:00 |
| Denaturation | 95 | 02:00 |
| Amplification and Cycling (45X) | 95 | 00:15 |
| 55 | 00:45 |
| 72 | 00:15 |
| Cooling | 48 | 00:10 |

* Before clicking “Start Run” make sure to select the right filter settings for acquiring signals. Define the three fluorescence detectors as shown in table 4

Table 4. Dectection channels

|  |  |  |
| --- | --- | --- |
| Target | Target genes | Reporter dye/channel |
| B-βCOV specific RNA | E gene | FAM |
| SARS-COV-2 specific RNA | S gene | Cy5/618-660 |
| Internal control | IC | HEX/465-533 |

### Data analysis and interpretation

Perform data analysis using the second derivative maximum method. The negative control (NTC) must show no signal.

Table 5. Interpretation

|  |  |  |  |
| --- | --- | --- | --- |
| Result Interpretation | B-βCoV (Target E)  FAM | SARS-CoV-2 (Target S) Cy5 | Internal control  JOE |
| COVID-19 Positive | **+** | **+** | **+** |
| COVID-19 Negative | **+** | **-** | **+** |
| COVID-19 Negative | **-** | **-** | **+** |
| Reaction failure (presence of inhibitors). Repeat test with a new sample | **-** | **-** | **-** |

# Safety

Specimens and reagents containing materials from humans are potentially contagious and need to be handled accordingly. Always wear protective clothing and gloves. When needle stick or cut injuries occur safety regulations of the UKT apply. Instructions and emergency numbers and contact details of the respective safety officer are displayed near the entry door of all laboratories at ITM where infectious material is handled.

# References

* RealStar® SARS-CoV-2 RT-PCR Kit 1.0 (Altona Diagnostics GmbH)
* QIAgility® User Manual (Version June 2013)
* SOP010b-ITM CTP: Sampling, storage and RNA extraction of swab specimens