SARS-CoV-2 detection for diagnosis purposes in the setting of a research molecular biology lab

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Abstract

The emergence of SARS-CoV-2 virus and the exponential growth of COVID-19 cases confront the public health system to a major crisis. The diagnostic capacity is essential to monitor the epidemic. To date, antibody-based detection methods are not available. Thus, the critical identification of the contagious asymptomatic carriers requires the isolation of viral nucleic acids and amplification by PCR. However, the shortage of specific proprietary reagents or the lack of automated platforms has seriously hampered diagnostic throughput in many countries.

Here, we provide a procedure for SARS-CoV-2 detection for diagnosis purposes from clinical samples in the setting of a basic research molecular biology lab. The procedure details the

necessary steps for the daily analysis of up to 500 clinical samples with a team composed of 12 experienced researchers.

1. Introduction

The protocol described here aims at providing a fast and easy to duplicate procedure for SARS-CoV-2 detection for diagnosis purposes from clinical specimens.

It is based on methodology available in each lab equipped with routine devices and reagents used in molecular biology. By organizing the work-flow of experienced technicians, PhD students or post-doc researchers, the strategy aims at increasing the diagnostic capacity. Indeed, one team is able to carry out up to 500 SARS-CoV-2 tests a day. To achieve this, the team of scientists must be composed of 12 researchers. They have to work in appropriate biosafety conditions with basic equipment. One team requires access to 2 laminar flow cabinets, 4 laboratory chemical hoods, 8 high speed refrigerated centrifuges (14,000 g), and two real time PCR instruments.

Probe and primers for SARS-CoV-2 detection were validated and published in (Corman et al., 2020).

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2. Human resources and materials

2.1. Sample collection, tracking and labelling

1) One logistician.

2.2. Inactivation of viral infectivity

1) Two researchers (or any person qualified to handle a molecular biology experiment).
2) Biosafety level 2 hood.
3) Guanidinium thiocyanate solution (TRI Reagent from Sigma-Aldrich or TRIzol from Invitrogen or QIAzol from QIAGEN).
4) Internal Control (IC):
   RNA extract from cell culture supernatant of Schmallenberg virus-infected cells. The Internal Control can be provided by the UNamur URVI lab upon request (damien.coupeau@unamur.be). Briefly, BHK-21 cells were infected with SBV (Schmallenberg virus) strain BH80/11-4. Forty-eight hours post infection, the cell culture supernatant was collected and RNA extracted with QiAmp Viral Mini Kit (Qiagen). A 10^4 fold dilution is used as spike-in RNA (i.e. Internal Control).
5) Umonium Medical Spray (Huckert's International).

2.3. RNA extraction

1) Eight researchers (or any person qualified to handle a molecular biology experiment).
2) Vortex apparatus.
3) Four to eight high speed 1,5ml tube refrigerated centrifuges.
4) Chloroform (Sigma-Aldrich).
5) GlycoBlue (Thermofisher Scientifc).
6) Isopropanol (Sigma-Aldrich).
7) Ethanol 75% (Sigma-Aldrich). In a falcon 50 ml tube, add 12,5 ml of RNAse free water to 37,5ml of ethanol 96-100% (Sigma-Aldrich).
8) RNAse free water.

2.4. Taqman RT-qPCR for SARS-CoV-2

1) Two researchers (or any qualified person to handle a molecular biology experiment).
2) 5X Master Mix containing DNA polymerase, $MgCl_2$ (5.5 mM final concentration) and dNTPs (Eurogentec Takyon One-Step No Rox Probe 5X MasterMix dTTP).
   Important note: use a low ROX or high ROX PCR Master Mix if your PCR machine requires ROX normalization.
3) Euroscript II RT (50 u/µl) and RNAse inhibitor (20 u/µl) (Euroscript II Reverse Transcriptase/RNAse inhibitor provided in the Eurogentec Takyon One-Step No Rox Probe 5X MasterMix dTTP).
4) Additive (provided in the Eurogentec Takyon One-Step No Rox Probe 5X MasterMix dTTP).
5) RNase free water.
6) Primers and Probe Mixes:

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SARS-CoV-2 primers and probe (published in (Corman et al., 2020)):
E_Sarbeco_Fw: 5'- ACAGGTACGTTAATAGTTAATAGCGT-3'
E_Sarbeco_Rev: 5'- ATATTGCAGCAGTACGCACACA-3'
E_Sarbeco_Probe: 5'-(FAM)ACACTAGCCATCCTTACTGCGCTTCG(BHQ1)-3'

Internal Control primers and probe (published in (Fischer et al., 2013)):
IC_Fw: 5'-TTGCCGTTTGATTTTGAAGTTGTG-3'
IC_Rev: 5'-TCAGGGATCGCAAATTAAAGAACC-3'
IC_Probe: 5'-(FAM)TCATCCGTGCTGACCCTCTGCGAG(BHQ1)-3'

Prepare Primers and Probe Mixes by mixing in a 1.5 ml tube:

Primers and Probe Mix for SARS-CoV-2
10 µl of E_Sarbeco_Probe (100 µM)
+ 20 µl of E_Sarbeco_Fw (100 µM)
+ 20 µl of E_Sarbeco_Rev (100 µM)
+ 950 µl of RNAse free water

Primers and Probe Mix for IC (SBV)
+ 10 µl of IC_Probe (100 µM)
+ 20 µl of IC_Fw (100 µM)
+ 20 µl of IC_Rev (100 µM)
+ 950 µl of RNAse free water

Primers and Probe Mix (5X mix) contains primers at 2 µM and probe at 1 µM.

7) Positive Controls (PC):
   Heat inactivated virus stock (diluted a 10.000 times) of SARS-CoV-2-infected Vero cells was provided by the UZ/KU Leuven national reference center. RNA was extracted using classic guanidinium thiocyanate-phenol-chloroform extraction (as for the clinical specimens). Briefly, RNA from 100 µl of the diluted cell culture supernatant was extracted and resuspended in 100 µl RNAse free water. This RNA extract is thereafter called positive control 1 (PC1). Positive control 2 (PC2) is a 10-fold dilution of PC1.
8) Internal Control (IC), as described in section "2.2. Inactivation of viral infectivity".
9) Negative Control (NC):
   RNAse free water.
10) Two Quantitative PCR machines (Roche Light Cycler 480 or equivalent, make sure the PCR master mix is compatible with your machine notably regarding ROX normalization).

2.5. Communication of the data

1) One logistician

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3. Methods

3.1. Sample collection, tracking and labelling

Tagged clinical specimens will be shipped from the reference lab to the labs of molecular biology. The clinical specimens are If a delay in extraction is expected, store specimens at 4°C overnight and notify the responsible at the reference center.

There are different types of clinical specimen tubes (Figure 1). In some case, the clinical specimen has been mixed with a transport solution (Figure 1, panel A). These specimens are adequate for direct processing. In other case, the clinical specimen has been collected on a dry swab or on a swab on gel (Figure 1, panel B). These specimens are less appropriate. To analyze those specimens, the swab must be dipped in 500 µl of PBS and 100 µl will be used for RNA extraction. Keep track of specimens collected with those tube when analyzing the data.

The Logistician is responsible of the reception and encoding of the clinical specimens. He connects the patient sample and its external identification tag issued by the reference lab with the tracking number. The tracking number will follow the sample all over the procedure and will be unique to that sample. In other words, if a hundred samples were analyzed at the first day of operation, the first sample of the second day will receive the tracking number 101.

The Logistician prepares ahead a collection of labels with the tracking number written on it.
Four labels per treated sample will be necessary.

- the 1st label is sticked to the original sample
- the 2nd label is sticked to the sample's sheet (pink sheet)
- the 3rd label is sticked to the first 1.5 ml tube for inactivation of viral infectivity
- the 4th label is sticked to the second 1.5 ml tube for RNA precipitation and resuspension.

The label must be sticked on the tube cap to allow visualization of the sample.

3.2. Inactivation of viral infectivity

Clinical specimen processing will be performed in a class II biological safety cabinet by experienced researcher wearing appropriate protective equipment (FFP2 or FFP3 mask, safety goggles, gown and gloves).

The whole process is carried out using filtered tips. Researcher must change of filtered tips between each pipetting. A tracking form follows the samples at each steps of the protocol (Figure 2).

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1) Vortex nasopharyngeal swabs, aspirates or BAL fluids briefly.
2) Spin the clinical specimen collection tube at 200 g, 1 min. To make sure the sample (transport media inside the clinical sample collection tube) is at the bottom of the tube. Use a centrifuge with sealed buckets. Clean the centrifuge with Umonium Medical Spray or equivalent antiviral disinfectant.
3) Under a BSL2 hood, transfer 100 µl of the sample in the corresponding labelled 1,5 ml tube containing 1ml of guanidinium thiocyanate solution (Trizol or equivalent) supplemented with 5 µl of Internal Control. Mix by inverting the tube.
   This procedure instantly inactivates viral infectivity.
   The 1,5 ml tubes containing 1 ml of guanidinium thiocyanate solution supplemented with 5 µl of Internal Control must be prepared ahead under a chemical hood (vapors of guanidinium thiocyanate solution are toxic). These aliquots can be stored at -80°C if necessary.
4) Before the samples can get out of the hood, spray the tubes with quaternary ammonium solution (Umonium Medical Spray or equivalent). Wipe the tubes.
   Samples are now handled without any biosafety issue. Organize the inactivated samples by series of 21 samples + 1 tube called Extraction Control or EC. The EC tube contains 1 ml of guanidinium thiocyanate solution supplemented with 5 µl of Internal Control ONLY; i.e. without clinical sample. RNA extraction will be performed by batch of 22 tubes (21 samples + 1 EC).
   If necessary, samples can be stored at -20 °C (or -80 °C) for further processing.

3.3. RNA extraction

Inactivated samples (identified by their unique tracking numbers) are treated in parallel by a team of experienced researchers or lab technicians. One researcher is typically able to handle 2 to 3 batches of 21 samples in one day. To reach 504 extractions a day, a team should be composed of 8 researchers working with 4 chemical hoods (hood large enough to host two researchers).

The whole process is carried out using filtered tips. This step is prone to cross-contamination between samples: researcher must change of filtered tips between each pipetting. Clean chemical hood and pipettes between each batch of extraction. For each extraction batch, keep track of the operator name and hood used.

Prepare ahead 21 Eppendorf tubes with the corresponding sample labels + 1 Eppendorf tube for the IC only (spiked-in RNA without clinical sample).

In a 5 ml Eppendorf tube, prepare 4,8 ml of chloroform (use chloroform resistant plastic).

In a Falcon 15 ml tube, prepare 12 ml of isopropanol

In a Falcon 50ml tube, prepare 24ml of 75 % ethanol.

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In a 1,5 ml tube, prepare 720 ul of RNAse free water.

It is critical to prepare these aliquots in a dedicated room with dedicated pipettes. Clinical specimen, RNA extract and, of course PCR, product must never be introduced in that room.

Just before starting RNA extraction, supplemented with 72 µl of GlycoBlue.

To process a batch of 22 tubes (21 samples + 1 EC) (Work-flow in Figure 3):

1) Vortex the samples 10 sec and incubate 5 min at room temperature.
2) Add 200 µl of chloroform and vortex for 10 sec.
3) Vortex the samples 10 sec and incubate 5 min at room temperature.
4) Centrifuge at 12,000 g for 10 min at 4 °C.
5) During the centrifugation:
6) Transfer 500 µl of the colorless upper aqueous phase (containing the RNA) in the second 1.5 ml tube, avoid contact with the ring or the lower organic phase (pink). Important note: the organic and aqueous phase can be inverted, i.e. the organic pink phase can be above the clear aqueous phase. Add 100ul of RNAse free water to the sample, vortex, centrifuge at 12,000 g for 10 min at 4 °C and resume from step 7).
7) Add 500 µl of the mix isopropanol-GlycoBlue.
8) Vortex for 10 sec and incubate 5 min at room temperature.
9) Centrifuge at 12,000 to 14,000 g for 10 min at 4 °C.
   The RNA pellet forms a blue pellet on the bottom of the tube (Figure 3). If no pellet is visible, restart extraction from the inactivation step.
10) Discard the supernatant.
11) Add 900 µl of 75 % ethanol.
12) Gently mix by inverting the tubes.
13) Centrifuge at 12,000 to 14,000 g for 10 min at 4 °C.
14) Aspirate slowly the supernatant with a 1 ml pipette by avoiding contact with the blue pellet (slow pipetting allows the ethanol to drain along the tube wall).
15) Use a narrow tip (e.g. gel loading tip) to remove residual ethanol.
16) To dry the pellet, leave the tube open under the chemical hood until complete ethanol evaporation. This step lasts about 5 min. Do not over dry the RNA by letting the sample dries more than 10 min.
17) Resuspend the pellet in 30 µl of RNAse free water.
18) Incubate at room temperature until complete resuspension of the blue pellet.
19) If necessary, RNA can be stored at -80C for further processing.

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3.4. Taqman RT-qPCR for SARS-CoV-2

RNA samples (identified by their unique tracking numbers) are transferred to the molecular biology platform. To analyze by PCR 504 samples (12 plates, 42 samples per plate), the team should count 2 researchers each running six 96 well plates.

The whole process is carried out using filtered tips. Researcher must change of filtered tips between each pipetting.

Composition of the SARS-CoV-2 PCR Mix:

Volume in µl	per reaction	1 plate	2 plates	3 plates	4 plates	5 plates
5X Master Mix	4	200	400	600	800	1000
Euroscript II (RT) and RNAse inhibitor	0.2	10	20	30	40	50
Primers and Probes Mix SARS-CoV-2	4	200	400	600	800	1000
RT Additive	0.2	10	20	30	40	50
RNAse free water	7.6	380	760	1140	1520	1900
Sample or PC or NC or EC	4					

Composition of the Internal Control (SBV) PCR Mix:

Volume in µl	per reaction	1 plate	2 plates	3 plates	4 plates	5 plates
5X Master Mix	4	200	400	600	800	1000
Euroscript II (RT) and RNAse inhibitor	0.2	10	20	30	40	50
Primers and Probes Mix IC (SBV)	4	200	400	600	800	1000
RT Additive	0.2	10	20	30	40	50
RNAse free water	7.6	380	760	1140	1520	1900
Sample or PC or NC or EC	4					

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Disposition of Samples on the PCR plate:

- S stands for Sample, 42 Samples per plate (2 batches of 21 samples)
- NC for Negative Control (water)
- PC1 for Positive Control 1, PC2 for Positive Control 2 (RNA from SARS-CoV-2-infected cells)
- EC1 for Extraction Control of the first extraction batch, EC2 for Extraction Control of the second extraction batch.

A hard copy of the PCR plate is kept for the validation process (Figure 4).

		1	2	3	4	5	6	7	8	9	10	11	12
SARS	**A**	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
	B	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24
	C	S25	S26	S27	S28	S29	S30	S31	S32	S33	S34	S35	S36
	D	S37	S38	S39	S40	S41	S42	EC1	EC2		PC1	PC2	NC water
IC	**E**	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
	F	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24
	G	S25	S26	S27	S28	S29	S30	S31	S32	S33	S34	S35	S36
	H	S37	S38	S39	S40	S41	S42	EC1	EC2				NC water

It is critical to prepare the PCR mixes in a dedicated room with dedicated pipettes. Clinical specimen, RNA extract and, of course PCR, product must never be introduced in that room.

1) First deposit 16 µl of the PCR Mix per well.
2) Next, add 4 µl of sample per well (or 4 µl of water for NC, or 4 µl of PC, or 4 µl of EC).
3) Stick the adhesive fil.
4) Spin the PCR plate to collect the PCR mix at the bottom of the well, 200g, 1min.

Thermal protocol

- 48 °C 10 min
- 95 °C 3 min
- 45 cycles: 95 °C 15 sec, 58°C 30 sec

We consider the plate as VALID only if Ct values for

- NC (SARS) > 45 and NC (IC) > 45
- and PC1 (SARS) < 30 and PC2 (SARS) < 35
  Otherwise the plate must be re-run

We consider an extraction batch as VALID only if Ct value for

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- EC (SARS) > 45
  Otherwise the extraction batch must be redo (EC (SARS) < 45 indicates cross-contamination and would lead to false positive results)

We consider the sample as VALID only if

- ΔCt Sample (IC) – EC (IC) < 3.3
  this allows us to exclude samples whom the RT-qPCR is inhibited.
  Otherwise the original clinical specimen must be diluted 5 times in PBS and RNA must be re-extracted. Diluted samples should be marked in the tracking sheet, in order that the reduced sensitivity of the assay can be reported. In case of persistent inhibition, the sample is classified as "undetermined due to inhibition of the diagnostic procedure"

For a valid sample (from a valid extraction batch and a valid plate):
The result is "positive" if Ct value for Sample (SARS) < 40
The result is "negative" if Ct value for Sample (SARS) > 45
The result is "undetermined" if Ct value for Sample (SARS) is between 40 and 45.

The validation process is summarized on Figure 5.

Excel files with tracking numbers are used to communicate the results.

If no longer needed, clinical specimens must be autoclaved and discard appropriately.

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Competing Interests
The authors have declared that no competing interests exist.

Author Contributions
NB, NL, SL, AP, SP, FP, L-AR, LW, KW, EA, LL, LC, MVR, PB: Conceptualization, Investigation.
DC, BM, NAG: Conceptualization, Investigation, Supervision, Original Draft Preparation, Writing – review & editing.

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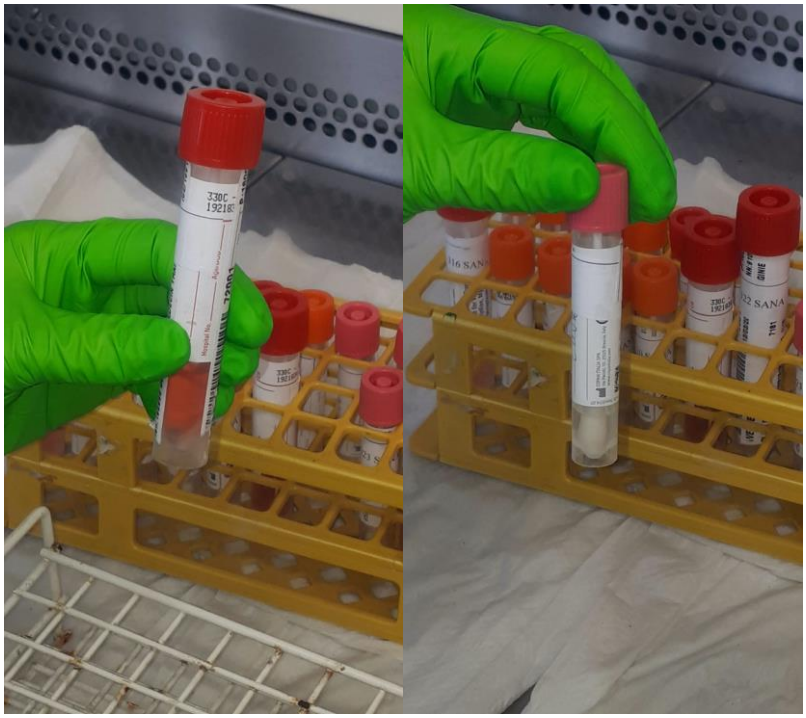
Figure 1: Type of clinical specimen tubes

A. Clinical specimen in transport media



B. Clinical specimen on dry swab or gelose

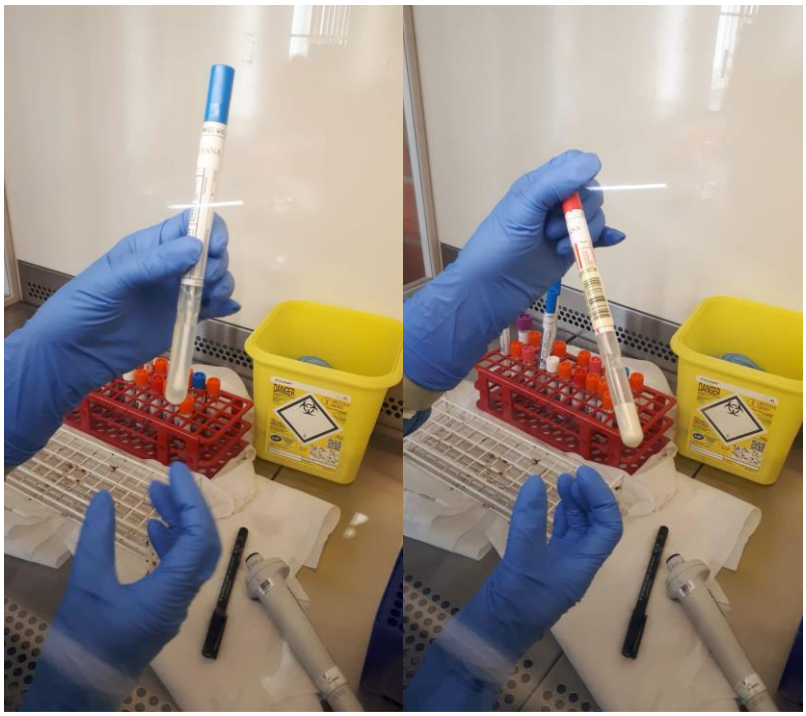


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Figure 2: Tracking form

Tracking form

Samples processed :	Date of clinical specimens' reception:

UNPACKING and LABELING		**Logistician:**
INACTIVATION	**Date:**	**Researcher:**

Comments:

EXTRACTION	**Date:**	**Researcher:**
chemical hood used n°		hood cleaning:

Comments:

RT-qPCR	**Date :**	**Researcher:**
PCR bench n° PCR machine n°		bench cleaning:

Comments

DATA VALIDATION	**Date:**	**Researchers:**

Comments

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Figure 3: RNA extraction work flow

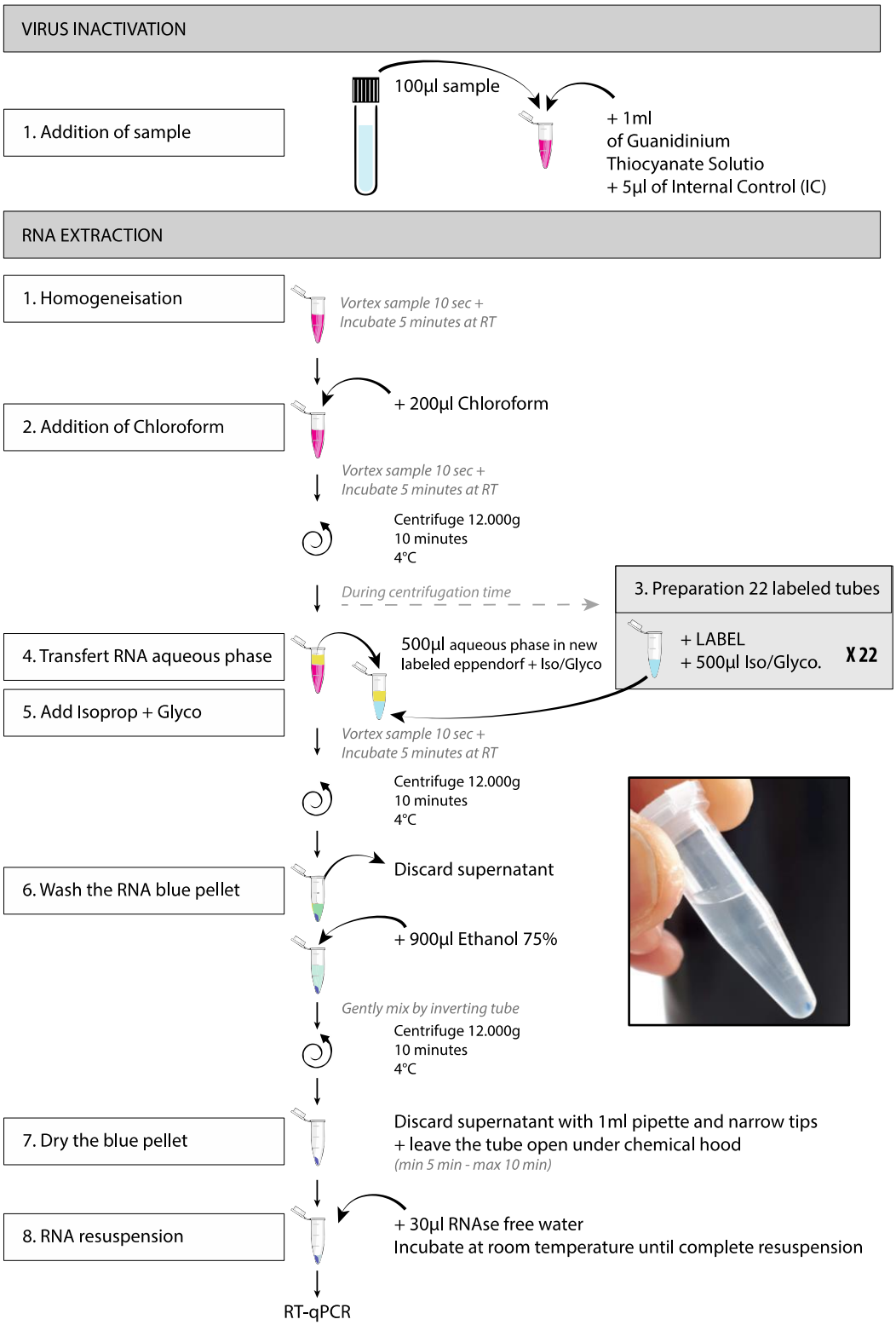

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Figure 4: PCR loading form

UNamur research labs								Date of processing …					
Samples n°... to n°…								Name of operator……					
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
SARS PCR Mix	**A**												
	B												
	C												
	D							EC1	EC2		PC1	PC2	water
IC PCR Mix	**E**												
	F												
	G												
	H							EC1	EC2				water

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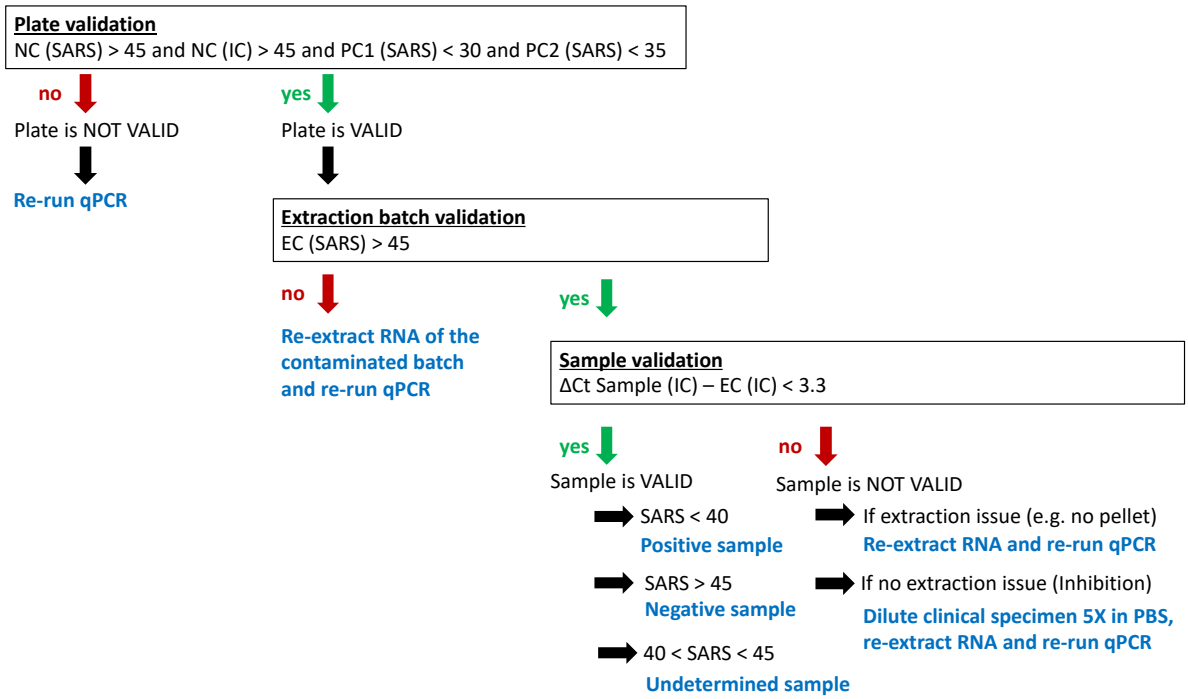
Figure 5: Validation process of the RT-qPCR data



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