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Screening and Detection SARS-CoV-2 RNA from Buffy Coats



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Protocol status: In development

We are still developing and optimizing this protocol



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Abstract

This SOP has been developed to minimize or eliminate employee occupational exposure to SARS-CoV-2 virus from blood samples of healthy volunteers obtained from the New York Center (NYBC) or from outpatients at HSS. Both are screened for symptoms and potential exposure at the point of care but are not tested for COVID-19. The CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel is intended for the qualitative detection of nucleic acid from the 2019-nCoV in upper and lower respiratory specimens (such as nasopharyngeal or oropharyngeal swabs, sputum, lower respiratory tract aspirates, bronchoalveolar lavage, and nasopharyngeal wash/aspirate or nasal aspirate). This protocol is a modified version of the CDC protocol meant to screen for Novel Coronavirus (2019-nCoV) in RNA isolated from whole blood. The Real-Time RT-PCR is for the identification of 2019-nCoV RNA from buffy coats obtained from supposedly healthy donors from New York Blood Center (NYBC). The 2019-nCoV RNA is generally detectable in upper and lower respiratory specimens during infection while rarely detected in circulating blood. Positive results are indicative of active infection with 2019-nCoV and blood should be discarded as per the . Negative results do not preclude 2019-nCoV infection and should not be used as the sole basis of management decisions. The product contains oligonucleotide primers and dual-labeled hydrolysis probes (TaqMan®) and control material used in rRT-PCR for *in vitro* qualitative detection of 2019-nCoV RNA. The panel is designed for specific detection of the 2019-nCoV (two primer/probe sets). An additional primer/probe set to detect the human RNase P gene (RP) in control samples. RNA is purified from whole blood in one-step Real-Time RT-PCR on an Applied Biosystems 7500 Real-Time PCR Instrument. Detection of viral RNA in whole blood aids in the safe operation of research labs working with human blood samples.

Attachments



[EUA-CDC-nCoV-IFU.pdf](#)

1.1MB



[purelink_blood_total...](#)

346KB

Guidelines

This standard operating procedure provides instructions for:

- Safely handling of human blood
- Appropriate waste disposal
- Precautions for setting up the CDC-SARS-CoV-2 PCR

Hazards, Safety Controls and Risk

A. Hazards

Biological hazards

- o Exposure to infectious agents
- o Biological spill

B. Controls

- o Conduct work in BSL2
- o Dispose of waste correctly

C. Risk Assessments

- Risk assessments have been undertaken and the risks after the controls are in place are LOW.
- We will obtain blood from healthy volunteer from the NYBC and outpatients from HSS who follows the FDA guidelines for collection consisting in checking the body temperature, travel and symptoms history of the donor.
- Presence of SARS-CoV-2 in the healthy donor blood will be checked by PCR and the sample used only if tested negative
- Many routine laboratory procedures can potentially generate aerosols and droplets that are often undetectable. Laboratory workers may not be aware that such particles can be generated during many laboratory procedures and that these particles could be inhaled or could cross-contaminate work surfaces, materials, and equipment.
- The following laboratory procedures have been associated with the generation of infectious aerosols and droplets: centrifugation, pipetting, vortexing, mixing, shaking, removing caps, decanting liquids, loading syringes, manipulating needles, syringes or sharps, aspirating and transferring blood and body fluids, sub-culturing blood culture bottles, spilling specimens, and cleaning up spills. Rotor-sealing lids will be used to reduce the risk of exposure to laboratory personnel. Centrifuging of blood specimens is performed using click seal biocontainment lids that will be loaded and unloaded in a BSL2. Sample vortexing will be performed inside a BSL2.
- All technical procedures should be performed in a way that minimizes the generation of aerosols and droplets. For procedures with a high likelihood to generate aerosols or droplets we will use a certified Class II Type A1 Biological Safety Cabinet (BSC) and additional precautions to provide a barrier between the specimen and researchers.
- Glass shields (inside and outside of hoods), work surfaces and equipment will be decontaminated with appropriate disinfectants, including sodium hypochlorite (bleach) (e.g. 1,000 ppm (0.1%) for general surface

disinfection and 10,000 ppm (1%) for disinfection of blood spills, 62-71% ethanol or 0.5% hydrogen peroxide. This is to be done before and after use by every user.

- All generated waste and contaminated materials will be considered biohazardous and will be placed in BSL2 in leak-proof, properly designated and sealed containers. Sealed containers will be placed in biohazard bags for subsequent collection, decontamination and disposal. All dry waste is contained in red hard sided biohazard containers. All waste is then autoclaved before disposal.
- In case of a spill, Environmental Health and Safety (EHS) will be contacted and spills will be cleaned up according to the guidelines detailed in section E

D. PPE

- o Long-sleeved laboratory gown and closed shoes
- o Safety glasses
- o Gloves
- o Masks (N95 respirator) only for inactivating of blood samples for presence of SARS-CoV2 virus testing

E. Emergencies (if applicable)

- Accidents and spills: For reference, see the WCMC EHS Bloodborne Pathogen Exposure Control Plan (BBP ECP): http://weill.cornell.edu/ehs/static_local/pdfs/3.1BBP.pdf and Biological Spill Planning and Response Manual.
- In case of a spill or exposure refer to the EHS EXPOSURE AND SPILL RESPONSE GUIDE, notify your supervisor and call EHS (646-962-7233). In case of emergency always call NYP Security first (212-746-0911)
- For biological spills which do not involve injury, are contained, pose little hazard to personnel, and for which you have the proper training and proper protective equipment to do the cleanup, you can clean the spill. For all other biological spill situations, including those for which you have any questions or doubts about your ability to clean up the spill, contact Environmental Health and Safety (EHS) (646-962-7233).

F. Waste management

All waste generated from confirmed contaminated donor inside of the BSL2 labs must be decontaminated or packaged for autoclaving within these rooms. Please refer the EHS Waste Disposal Procedures Manual for reference on the proper disposal of biological waste:

http://weill.cornell.edu/ehs/static_local/pdfs/5.2WasteDisposal.pdf

- Before aspirating any liquids, be sure that the vacuum tubing is connected to a large flask that contains 1/5 volume of concentrated bleach. Also check that the vacuum flask is connected to a secondary flask, and that both flasks are inside a secondary container large enough to hold the maximum volume of the flasks in case of a spill. The tubing between the secondary flask and the house vacuum system must contain a Vacu-guard filter (0.22um). Make sure the flasks are labeled with a Tissue Culture Waste Label.
- If the primary vacuum flask is more than 1/2 full, add 1/3 volume of concentrated bleach and allow it to sit in BSC or fume hood for 1 hour before pouring it down the sink. Obtain a clean vacuum flask for your waste and connect it as is described above in 3.C.i.

- Aspirate all liquids from flasks/tubes/plates that will be discarded (no liquids in red bags). Blood must be diluted prior to aspirating so it doesn't clog the tubing.
- After aspirating all liquid waste, aspirate an additional 10ml of bleach solution into the vacuum flask to disinfect vacuum tubing.

G. Solid (non-sharps) Waste

- All used flasks, tubes and plates must be rinsed with bleach (at least 10% concentration) and discarded in a red biohazard bag. Disposable funnels should sit in bleach filter side down, for one minute before discarding.
- All biohazard waste shall be double bagged and decontaminated by autoclaving
- New York State requires that all red bags be marked with the name and address of the facility where the regulated medical waste is generated. Only use approved pre-printed red bags. Pre-printed red bags are available from the Housekeeping department servicing your area.
- Standard red bags are not suitable for autoclaving. Generators must purchase autoclave-safe red bags. Once autoclaving is complete, allow autoclave bags to cool and then place the autoclave bag into the approved red bag to satisfy the facility name and address requirements.

H. Training and Competency:

Any person handling human blood must undergo training.

Training consists of:

- Read and discuss documentation (appropriate SOPs)
- Observation of a competent trainer demonstrating the correct procedure
- Trainer to observe trainee performing procedure
- Refresher training when required ie. Change to SOP

Competency must be demonstrated

Spills within the Biosafety Cabinet

A. Clean up of a small spill (<25 ml):

- Make sure the cabinet continues to operate. Wait 5 min. to allow aerosols to be pulled through the HEPA filter.
- Decontaminate the surfaces within the cabinet, wearing protective clothing, gently cover the spill with absorbent paper towel soaked with 15% bleach, starting at the perimeter and working towards the centre; allow sufficient contact time (20 min) before clean up.
- Discard soaked paper towels in a biohazard bag. Wipe up residual mess.

B. Clean up of a large spill (>25 ml):

- Make sure the cabinet continues to operate. Wait 30 min. to allow aerosols to be pulled through the HEPA filter.
- Wait 30 min. for aerosols to settle. Meanwhile, notify the University Biosafety Officer, Lab Head and Departmental Safety Officer. If the spill has escaped the BSC, proceed as for a spill outside the BSC II.
- Cover split liquid with Virkon-S powder, leave for 20 min. and use paper towels to clean up, starting at the perimeter and working towards the centre; decontaminate all equipment supplies, or surfaces that were potentially contaminated.

- If a large quantity is spilled, the entire cabinet, including fans, filters, airflow plenums, will need to be formaldehyde decontaminated.
- An incident report must be submitted to PI.

Spills outside of containment

- Avoid inhalation of any airborne material by holding your breath, immediately remove and discard contaminated disposable protective equipment and dispose in the biohazard waste, and leave the laboratory. Warn others in the area. Ask a coworker for help if needed, so as to prevent spreading the hazardous material even further.
- Seek medical attention if needed.
- Re-entry into the laboratory should be delayed for a period of at least 30 minutes to allow aerosol generated by the spill to settle.
- Applicable personal protective clothing should be worn when entering the laboratory to clean the spill area.
- Utilize a biological spill kit to clean up the spill; this should be prepared prior in anticipation of an incident.
- Place paper towels or other absorbent material on the spill to absorb liquids.
- Without splashing, carefully pour disinfectant (10% bleach or 70% Isopropanol) over the wet towels in the spill area and cover with a second layer of paper towels to absorb all disinfectant.
- Disinfect all other materials near the spill area that may have been affected.
- Let sit 30 minutes before discarding wet paper towels and affected materials in biohazard bag (careful not to drip onto other surfaces).
- Work area must be fully disinfected before resuming work.
- Report all incidents to lab manager.

Variables that effect RNA yields during Whole Blood RNA purification

Whole blood, plasma, and sera represent, by far, the most commonly used sample types in the diagnostic field. Because PCR inhibitors in blood samples ~ have been described, generally, it is accepted that a careful purification of nucleic acids is required from such samples before PCR analysis can be performed. Cellular or viral RNA, present in vivo in a protected form in cells or virus, is not readily accessible to all PCR reagents. This barrier is overcome by lysing the cells or virus.

Erythrocytes lysis: Since whole blood contains a high number of non-nucleated erythrocytes, purifying total RNA from whole blood without the removal of erythrocytes results in low RNA yields and clogging of purification columns. The depletion of abundant erythrocytes is therefore a key step in the purification of whole blood total RNA and is performed by selective lysis of erythrocytes using hypotonic shock. The erythrocytes are resuspended in a hypotonic buffer that causes an influx of water into erythrocytes and ruptures the erythrocyte cell membranes. Leukocytes are not affected by the hypotonic shock and are easily separated from lysed erythrocytes by centrifugation.

Warnings and Precautions related PCR assays

- Amplification technologies such as PCR are sensitive to accidental introduction of PCR product from previous amplifications reactions. Incorrect results could occur if either the clinical specimen or the real-time reagents used in the amplification step become contaminated by accidental introduction of amplification product (amplicon). Workflow in the laboratory should proceed in a unidirectional manner.
- Maintain separate areas for assay setup and handling of nucleic acids.
- Always check the expiration date prior to use. Do not use expired reagent. Do not substitute or mix reagent from different kit lots or from other manufacturers.
- Change aerosol barrier pipette tips between all manual liquid transfers.
- During preparation of samples, compliance with good laboratory techniques is essential to minimize the risk of cross-contamination between samples, and the inadvertent introduction of nucleases into samples during and after the extraction procedure. Proper aseptic technique should always be used when working with nucleic acids.
- Maintain separate, dedicated equipment (e.g., pipettes, microcentrifuges) and supplies (e.g., microcentrifuge tubes, pipette tips) for assay setup and handling of extracted nucleic acids.
- Wear a clean lab coat and powder-free disposable gloves (not previously worn) when setting up assays.
- Change gloves between samples and whenever contamination is suspected.
- Keep reagent and reaction tubes capped or covered as much as possible.
- Primers, probes (including aliquots), and enzyme master mix must be thawed and maintained on cold block at all times during preparation and use.
- Work surfaces, pipettes, and centrifuges should be cleaned and decontaminated with cleaning products such as 10% bleach, "DNAZap™" or "RNase AWAY®" to minimize risk of nucleic acid contamination. Residual bleach should be removed using 70% ethanol.
- RNA should be maintained on cold block or on ice during preparation and use to ensure stability.
- Dispose of unused kit reagents and human specimens according to local, state, and federal regulations.

Reagent Storage, Handling, and Stability

- Store all dried primers and probes and the positive control, nCoVPC, at 2-8°C until re-hydrated for use.
- Store liquid HSC control materials at $\leq -20^{\circ}\text{C}$.
- Always check the expiration date prior to use. Do not use expired reagents.
- Protect fluorogenic probes from light.
- Primers, probes (including aliquots), and enzyme master mix must be thawed and kept on a cold block at all times during preparation and use.
- Do not refreeze probes.
- Controls and aliquots of controls must be thawed and kept on ice at all times during preparation and use.

Primer and Probe Preparation:

1. Upon receipt, store dried primers and probes at 2-8°C.
2. Precautions: These reagents should only be handled in a clean area and stored at appropriate temperatures (see below) in the dark. Freeze-thaw cycles should be avoided. Maintain cold when thawed.
3. Using aseptic technique, suspend dried reagents in 1.5 mL of nuclease-free water (50X working concentration) and allow to rehydrate for 15 min at room temperature in the dark.



4. Mix gently and aliquot primers/probe in 300 μ L volumes into 5 pre-labeled tubes. Store a single aliquot of primers/probe at 2–8°C in the dark. Do not refreeze (stable for up to 4 months). Store remaining aliquots at $\leq$ –20°C in a non-frost-free freezer.

2019-nCoV Positive Control (nCoVPC) Preparation:

1. Precautions: This reagent should be handled with caution in a dedicated nucleic acid handling area to prevent possible contamination. Freeze-thaw cycles should be avoided. Maintain on ice when thawed.
2. Resuspend dried reagent in each tube in 1 mL of nuclease-free water to achieve the proper concentration. Make single use aliquots (approximately 30 μ L) and store at $\leq$ –70°C.
3. Thaw a single aliquot of diluted positive control for each experiment and hold on ice until adding to plate. Discard any unused portion of the aliquot.

No Template Control (NTC) (not provided)

1. Sterile, nuclease-free water
2. Aliquot in small volumes
3. Used to check for contamination during specimen extraction and/or plate set-up

Equipment Preparation

Clean and decontaminate all work surfaces, pipettes, centrifuges, and other equipment prior to use.

Decontamination agents should be used including 10% bleach, 70% ethanol, and *DNAzap*[™] or *RNase AWAY*[®] to minimize the risk of nucleic acid contamination.



Materials

MATERIALS

- ✕ Vortex Mixer
- ✕ PureLink[®]; Total RNA Blood Kit **Thermo Fisher Catalog #K156001**
- ✕ Buffycoats / LeukoPaks – Buffy coats made from whole blood
- ✕ 2019-nCoV CDC RUO Primers and Probes **IDT Catalog #10006713**
- ✕ 2019-nCoV CDC RUO Plasmid Controls **IDT Catalog #10006625**
- ✕ 70% ethanol
- ✕ Promega GoTaq[®] Probe 1- Step RT-qPCR System **Promega Catalog #A6121**
- ✕ Micropipettes (2 or 10 μ L 200 μ L and 1000 μ L)
- ✕ Multichannel micropipettes (5-50 μ L)
- ✕ Racks for 1.5 mL microcentrifuge tubes
- ✕ 2 $\times$ 96-well -20°C cold blocks
- ✕ 7500 Fast Dx Real-Time PCR Systems with SDS 1.4 software (Applied Biosystems; catalog #4406985 or #4
- ✕ Molecular grade water nuclease-free
- ✕ 10% bleach (1:10 dilution of commercial 5.25-6.0% hypochlorite bleach)
- ✕ Aerosol barrier pipette tips
- ✕ 0.2 mL PCR reaction plates (Applied Biosystems; catalog #4346906 or #4366932)
- ✕ 1.5 mL microcentrifuge tubes (DNase/RNase free)
- ✕ 50-500 μ L whole blood (up to 3.5×10^6 leukocytes)
- ✕ 96-100% ethanol
- ✕ Microcentrifuge capable of centrifuging $>8000 \times g$
- ✕ Ice bucket
- ✕ Sterile RNase-free tube (~15 ml) for performing erythrocyte lysis (next page)
- ✕ Lysis Buffer (L5) for lysis of erythrocytes (supplied with the kit)
- ✕ Lysis Buffer (L3) for lysis of leukocytes (supplied with the kit)
- ✕ Wash Buffer (W4) (supplied with the kit)
- ✕ Wash Buffer (W5) with ethanol (see below)
- ✕ Spin Cartridges with collection tubes Wash Tubes and Elution Tubes (supplied with the kit)

- 1 Transfer the buffy bag from a sealed NYBC container to a BSL2 hood. Decontaminate the surface of the bag by spraying generous amount of 70% Ethanol. Using a 21 or 22 gauge needle fitted on a 5-10 ml syringe carefully collect ~100 - 200 µl of blood by carefully puncturing the top of the tubing of buffy bag. 
- 2 Erythrocyte lysis is a critical step in the recovery of RNA from whole blood and has to be performed carefully without generating aerosols. Using the same syringe aspirate 5 volumes of Lysis Buffer (L5) to every 1 volume of fresh whole blood . For example, add 500 µl of Lysis Buffer (L5) to 100 µl fresh whole blood sample. Using one-handed scoop method cap the syringe followed by incubation for 10 minutes in hood to allow hypotonic lysis of RBC.   
- 2.1 **NEEDLES SHOULD NOT BE RECAPPED, BENT, REMOVED OR OTHERWISE MANIPULATED BY HAND.** 
 - *However, if it is essential that a needle be recapped due to the nature of the work, the use of a mechanical device or the one-handed scoop method must be used.*

One-Handed Scoop Method

 1. *Place the cap on the desk or other flat surface with something firm to “push” the needle cap against.*
 2. *Holding the syringe with needle attached in one hand, slip the needle into the cap without using the other hand.*
 3. *Push the capped needle against a firm object to “seat” the cap onto the needle firmly using only one hand.*
- 3 Transfer the contents from the syringe into a 1.5 ml sterile RNase-free microfuge tube. Incubate the contents on ice for 10 minutes. Within the BSL2 hood, vortex the tube briefly 2-3 times during the incubation step to allow complete lysis of erythrocytes. The solution turns translucent. 

Note: Guanidine isothiocyanate present in lysis buffer is a chaotropic agent that helps in lysis of the cells and virus particles and is capable of protecting the RNA from endogenous RNases.
- 4 Centrifuge the tube at 4°C at 400 x g for 10 minutes. Remove the supernatant completely and discard the supernatant. Do not discard the pellet as the pellet contains leukocytes. 
- 5 Resuspend the leukocyte pellet in volumes of Lysis Buffer (L5). Mix well by vortexing briefly. For example, use 200 µl Lysis Buffer (L5)/100 µl of whole blood from Step 1.
- 6 Centrifuge the tube at 4°C at 400 x g for 5 minutes. Remove the supernatant completely and discard the supernatant. Do not discard the pellet as the pellet contains leukocytes. 



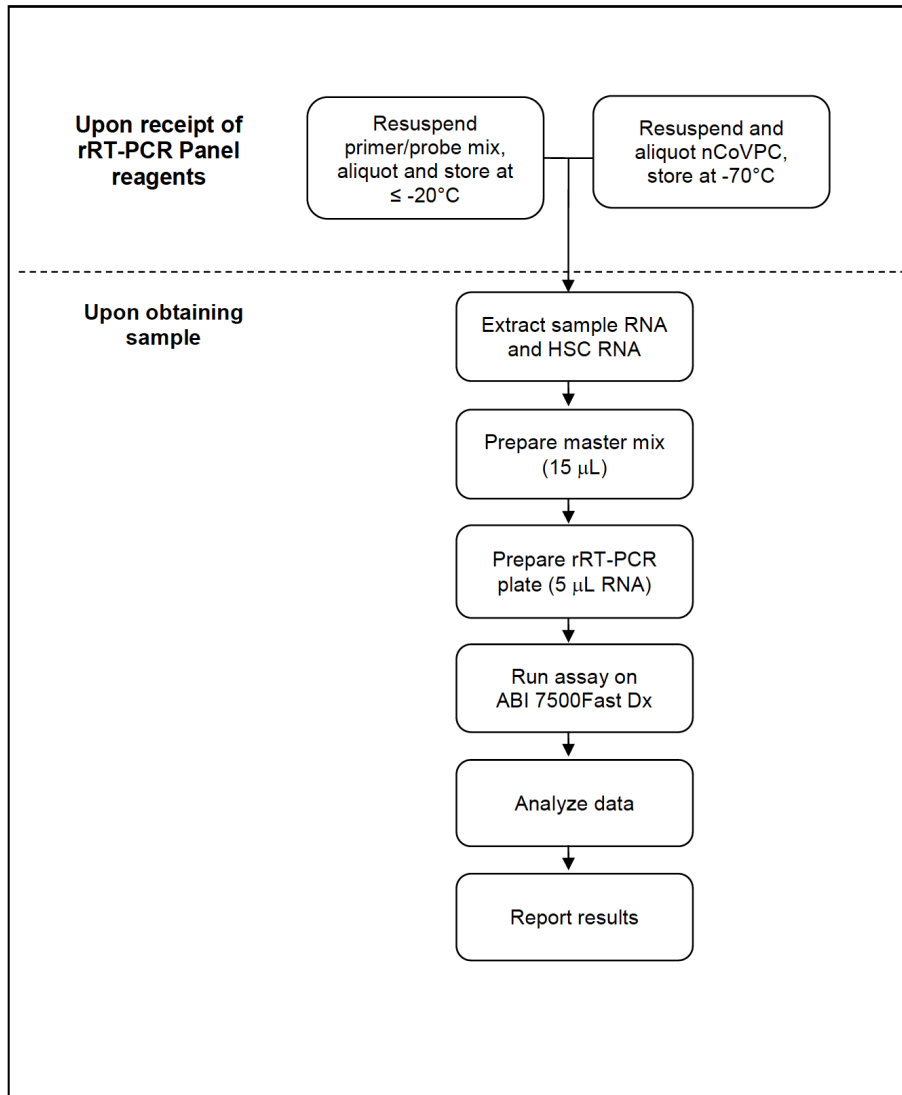
- The leukocyte pellet should be white with no traces of red. If the pellet is significantly red, wash the pellet with Lysis Buffer (L5).
- 7 Resuspend the leukocyte pellet in 350 μ l Lysis Buffer (L3). Mix well by vortexing briefly to completely resuspend the pellet ensuring the absence of any cell clumps. 
 - 8 Add 350 μ l 70% ethanol to the tube and mix well by vortexing briefly. 
 - 9 Remove a Spin Cartridge in a Collection Tube from the package. Transfer the leukocyte lysate from Step 7, above, to the Spin Cartridge. 
 - 10 Centrifuge the Spin Cartridge at 8,000 $\times$ g for 1 minute at room temperature. 
 - 11 Discard the flow through and place the Spin Cartridge into the collection tube.
 - 12 Add 700 μ l of Wash Buffer (W4) supplied in the kit to the Spin Cartridge. 
 - 13 Centrifuge Spin Cartridge at 8,000 $\times$ g for 30 seconds at room temperature. 
 - 14 Proceed to DNase I digestion if you need to remove genomic DNA or proceed directly to Step 16.
 - 15 Optional (DNase Digestion): To remove genomic DNA from the samples, add 80 μ l of DNase I solution (page 12 for a recipe) to the Spin Cartridge. Incubate at room temperature for 15 minutes.
 - 16 Add 500 μ l of Wash Buffer (W4) to the Spin Cartridge. If DNase I digestion is performed, incubate for 5 minutes at room temperature. 
 - 17 Centrifuge the Spin Cartridge at 8,000 $\times$ g for 30 seconds at room temperature. Discard the flow through. 
 - 18 Add 500 μ l of Wash Buffer (W5) with ethanol. 
 - 19 Centrifuge the Spin Cartridge at 8,000 $\times$ g for 30 seconds at room temperature. Repeat Step 18 once. 



- 20 Discard the flow through and place the Spin Cartridge into the Wash Tube supplied with the kit and centrifuge the Spin Cartridge at 8,000 x g for 1 minute at room temperature to remove any residual Wash Buffer (W5). 
- 21 Place the Spin Cartridge in a clean 1.7-ml Elution Tube supplied with the kit.
- 22 Add 20-50 µl of sterile, RNase-free water (supplied with the kit) to the center of the cartridge. 
- 23 Incubate at room temperature for 1 minute. Centrifuge the Spin Cartridge at 8,000 × g for 1 minute at room temperature. 
- 24 The elution tube contains your purified total RNA. Remove and discard the cartridge. Store the total RNA at -80°C or use total RNA for the desired downstream application.
- 25 The following is the schematic of CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR: 



Summary of Preparation and Testing Process



CDC Real-Time RT-PCR



Reaction Master Mix and Plate Set Up

Note: Plate set-up configuration can vary with the number of specimens and workday organization.

NTCs and nCoVPCs must be included in each run.

- In the reagent set-up room clean hood, place rRT-PCR buffer, enzyme, and primer/probes on ice or cold-block. Keep cold during preparation and use.
- Mix buffer, enzyme, and primer/probes by inversion 5 times.

- Centrifuge reagents and primers/probes for 5 seconds to collect contents at the bottom of the tube, and then place the tube in a cold rack.
- Label one 1.5 mL microcentrifuge tube for each primer/probe set.
- Determine the number of reactions (N) to set up per assay. It is necessary to make excess reaction mix for the NTC, nCoVPC, HSC (if included in the RT-PCR run), and RP reactions and for pipetting error. Use the following guide to determine N: • If number of samples (n) including controls equals 1 through 14, then $N = n + 1$.
- If number of samples (n) including controls is 15 or greater, then $N = n + 2$
- For each primer/probe set, calculate the amount of each reagent to be added for each reaction mixture ($N = \# \text{ of reactions}$).

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Promega GoTaq® Probe 1- Step RT-qPCR System

Step #	Reagent	Vol. of Reagent Added per Reaction
1	Nuclease-free Water	$N \times 3.1 \mu\text{L}$
2	Combined Primer/Probe Mix	$N \times 1.5 \mu\text{L}$
3	GoTaq Probe qPCR Master Mix with dUTP	$N \times 10.0 \mu\text{L}$
4	Go Script RT Mix for 1-Step RT-qPCR	$N \times 0.4 \mu\text{L}$
	Total Volume	$N \times 15.0 \mu\text{L}$

29

Set up reaction strip tubes or plates in a 96-well cooler rack.

30

Dispense 15 μL of each master mix into the appropriate wells going across the row as shown below:

31

Figure 1: Example of Reaction Master Mix Plate Set-Up

	1	2	3	4	5	6	7	8	9	10	11	12
A	N1	N1	N1	N1	N1	N1	N1	N1	N1	N1	N1	N1
B	N2	N2	N2	N2	N2	N2	N2	N2	N2	N2	N2	N2
C	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP
D												
E												
F												
G												
H												

32

Prior to moving to the nucleic acid handling area, prepare the No Template Control (NTC) reactions for column #1 in the assay preparation area.

33

Pipette 5 μ L of nuclease-free water into the NTC sample wells (**Figure 2**, column 1). Securely cap NTC wells before proceeding.

34

Gently vortex nucleic acid sample tubes for approximately 5 seconds. Centrifuge for 5 seconds to collect contents at the bottom of the tube. After centrifugation, place extracted nucleic acid sample tubes in the cold rack.

35

Samples should be added to columns 2-11 (column 1 and 12 are for controls) to the specific assay that is being tested as illustrated in **Figure 2**. Carefully pipette 5.0 μ L of the first sample into all the wells labeled for that sample (i.e. Sample "S1" down column #2). *Keep other sample wells covered during addition. Change tips after each addition.* Securely cap the column to which the sample has been added to prevent cross contamination and to ensure sample tracking. Repeat the same steps for the remaining samples.

36

Figure 2. 2019-nCoV rRT-PCR Diagnostic Panel: Example of Sample and Control Set-up

	1	2	3	4	5	6	7	8	9	10	11 ^a	12
A	NTC	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	nCoV PC
B	NTC	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	nCoV PC
C	NTC	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	nCoV PC
D												
E												
F												
G												
H												

^aReplace the sample in this column with extracted HSC if necessary

- 37 If necessary, add 5 µL of Human Specimen Control (HSC) extracted sample to the HSC wells (**Figure 2**, column 11). Securely cap wells after addition. NOTE: Per CLIA regulations, HSC must be tested at least once per day. Cover the entire reaction plate and move the reaction plate to the positive template control handling area.
- 38
- Pipette 5 µL of nCoVPC RNA to the sample wells of column 12 (**Figure 2**). Securely cap wells after addition of the control RNA. **NOTE:** *If using 8-tube strips, label the TAB of each strip to indicate sample position. **DO NOT LABEL THE TOPS OF THE REACTION TUBES!***
 - Briefly centrifuge reaction tube strips for 10-15 seconds. After centrifugation return to cold rack. **NOTE:** *If using 96-well plates, centrifuge plates for 30 seconds at 500 x g, 4°C.*
- 39 Create a Run Template depending on the available real-time PCR instrument available in the lab and run the machine. Refer to the CDC- document for the detailed set up of the real-time PCR machine.

40

Promega GoTaq® Probe 1-Step RT-qPCR System

- In Stage 1, Set to 15 min at 45°C; 1 Rep.
- In Stage 2, Set to 2 min at 95°C, 1 Rep.
- In Stage 3, Step 1 set to 3 sec at 95°C.
- In Stage 3, Step 2 set to 30 sec at 55.0°C.
- In Stage 3, Reps should be set to 45.
- Under **Settings** (**Figure 12**), bottom left-hand box, change volume to 20 µL.
- Under **Settings**, **Run Mode** selection should be **Standard 7500**.
- Step 2 of Stage 4 should be highlighted in yellow to indicate data collection (see **Figure 12**).

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Primers and Probes:

Catalog #2019-nCoV-EUA-01 Diagnostic Panel Box #1:

<i>Reagent Label</i>	<i>Part #</i>	<i>Description</i>	<i>Quantity / Tube</i>	<i>Reactions / Tube</i>
2019-nCoV_N1	RV202001 RV202015	2019-nCoV_N1 Combined Primer/Probe Mix	22.5 nmol	1000
2019-nCoV_N2	RV202002 RV202016	2019-nCoV_N2 Combined Primer/Probe Mix	22.5 nmol	1000
RP	RV202004 RV202018	Human RNase P Forward Primer/Probe Mix	22.5 nmol	1000

Positive Control (either of the following products are acceptable)

Catalog #2019-nCoV-EUA-01 Diagnostic Panel Box #2:

<i>Reagent Label</i>	<i>Part #</i>	<i>Description</i>	<i>Quantity</i>	<i>Notes</i>
nCoVPC	RV202005	2019-nCoV Positive Control (nCoVPC) For use as a positive control with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel procedure. The nCoVPC contains noninfectious positive control material supplied in a dried state and must be resuspended before use. nCoVPC consists of <i>in vitro</i> transcribed RNA. nCoVPC will yield a positive result with each assay in the 2019-nCoV Real-Time RT-PCR Diagnostic Panel including RP.	4 tubes	Provides (800) 5 µL test reactions

CDC-Primer and probe information

Interpretation of Results and Reporting

Extraction and Positive Control Results and Interpretation

No Template Control (NTC)

The NTC consists of using nuclease-free water in the rRT-PCR reactions instead of RNA. The NTC reactions for all primer and probe sets should not exhibit fluorescence growth curves that cross the threshold line. If any of the NTC reactions exhibit a growth curve that crosses the cycle threshold, sample contamination may have occurred. Invalidate the run and repeat the assay with strict adherence to the guidelines.

2019-nCoV Positive Control (nCoVPC)

The nCoVPC consists of in vitro transcribed RNA. The nCoVPC will yield a positive result with the following primer and probe sets: N1, N2 and RP.

Human Specimen Control (HSC) (Extraction Control)

When HSC is run with the CDC 2019-nCoV rRT-PCR Diagnostic Panel (see previous section on Assay Set Up), the HSC is used as an RNA extraction procedural control to demonstrate successful recovery of RNA as well as extraction reagent integrity. The HSC control consists of noninfectious cultured human cell (A549) material. Purified nucleic acid from the HSC should yield a positive result with the RP primer and probe set and negative results with all 2019-nCoV markers.

Expected Performance of Controls Included in the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel

Control Type	External Control Name	Used to Monitor	2019 nCoV_N1	2019 nCoV_N2	RP	Expected Ct Values
Positive	nCoVPC	Substantial reagent failure including primer and probe integrity	+	+	+	< 40.00 Ct
Negative	NTC	Reagent and/or environmental contamination	-	-	-	None detected
Extraction	HSC	Failure in lysis and extraction procedure, potential contamination during extraction	-	-	+	< 40.00 Ct

If any of the above controls do not exhibit the expected performance as described, the assay may have been set up and/or executed improperly, or reagent or equipment malfunction could have occurred. Invalidate the run and re-test.

2019-nCoV rRT-PCR Diagnostic Panel Results Interpretation Guide

The table below lists the expected results for the 2019-nCoV rRT-PCR Diagnostic Panel. If a laboratory obtains unexpected results for assay controls or if inconclusive or invalid results are obtained and cannot be resolved through the recommended re-testing, please contact CDC for consultation and possible specimen referral. See pages 10 and 40 for referral and contact information.

2019 nCoV_N1	2019 nCoV_N2	RP	Result Interpretation ^a	Report	Actions
+	+	±	2019-nCoV detected	Positive 2019-nCoV	Report results to CDC and sender.
If only one of the two targets is positive		±	Inconclusive Result	Inconclusive	Repeat testing of nucleic acid and/or re-extract and repeat rRT-PCR. If the repeated result remains inconclusive, contact your State Public Health Laboratory or CDC for instructions for transfer of the specimen or further guidance.
-	-	+	2019-nCoV not detected	Not Detected	Report results to sender. Consider testing for other respiratory viruses. ^b
-	-	-	Invalid Result	Invalid	Repeat extraction and rRT-PCR. If the repeated result remains invalid, consider collecting a new specimen from the patient.

^aLaboratories should report their diagnostic result as appropriate and in compliance with their specific reporting system.

^bOptimum specimen types and timing for peak viral levels during infections caused by 2019-nCoV have not been determined. Collection of multiple specimens from the same patient may be necessary to detect the virus. The possibility of a false negative result should especially be considered if the patient's recent exposures or clinical presentation suggest that 2019-nCoV infection is possible, and diagnostic tests for other causes of illness (e.g., other respiratory illness) are negative. If 2019-nCoV infection is still suspected, re-testing should be considered in consultation with public health authorities.