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CLIAHUB Automated RNA Extraction & RT-PCR Protocol V2

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1 Works for me dx.doi.org/10.17504/protocols.io.bfi2jkge

Chan Zuckerberg Biohub

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ABSTRACT

CLIAHUB How-To.pdf

Github: (to be added)

Laboratory testing for COVID-19 infection is an important part of both the individual patient care and public health responses to this emerging outbreak. Results are used to guide containment efforts, including isolation and contact tracing, and make clinical diagnosis for supportive management and experimental therapies (there is no known efficacious treatment available for COVID-19 infection as of March 2020). Real-time reverse transcriptase PCR (rRT-PCR) testing is a well-established method to detect viral RNA in clinical samples, and several labs around the world have designed and validated primers and probes for this purpose that are also used in this assay (4 and 5).

This assay is intended to qualitatively detect COVID-19 viral RNA in patient samples to enable the diagnosis of COVID-19 disease. Testing should be performed for patients with signs and symptoms of potential COVID-19 infection. Positive results would generally indicate active infection but do not rule out co-infection with other viruses, bacteria or other pathogens. Negative test results do not absolutely rule out infection, and results should be interpreted in the clinical context.

The oligonucleotide primers and probes for detection of SARS-CoV-2 were selected from regions of the Nucleoprotein (N gene)and the Envelope protein (E gene). An additional primer/probe set to detect the human RNase P gene (RP) in control samples and clinical specimens is also included in the panel.

RNA isolated and purified from upper respiratory specimens is reverse transcribed to cDNA and subsequently amplified on the Bio-Rad CFX Real Time PCR Machine. In the process, the probes anneal to a specific target sequence located between the forward and reverse primers. During the extension phase of the PCR cycle, the 5' nuclease activity of Taq polymerase degrades the probe, causing the reporter dye to separate from the quencher dye, generating a fluorescent signal. With each cycle, additional reporter dye molecules are cleaved from their respective probes, increasing the fluorescence intensity.

Detection of viral RNA not only aids in the diagnosis of illness but also provides epidemiological and surveillance information.

DOI

dx.doi.org/10.17504/protocols.io.bfi2jkge

PROTOCOL CITATION

Amy Lyden, Emily Crawford, Vida Ahyong, Manu Vanaerschot, Paula Hayakawa Serpa, Preethi Raghavan, Joana Cabrera, Michael Borja, Spyros Darmanis, Eric Chow, Joseph Derisi 2020. CLIAHUB Automated RNA

Citation: Amy Lyden, Emily Crawford, Vida Ahyong, Manu Vanaerschot, Paula Hayakawa Serpa, Preethi Raghavan, Joana Cabrera, Michael Borja, Spyros Darmanis, Eric Chow, Joseph Derisi (07/06/2020). CLIAHUB Automated RNA Extraction & RT-PCR Protocol V2. https://dx.doi.org/10.17504/protocols.io.bfi2jkge

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MATERIALS

NAME	CATALOG #	VENDOR
Quick-DNA/RNA Viral MagBead	R2140 / R2141	Zymo Research
Biorad "F" Foil PCR Plate Microseal	MSF1001	
N-gene E-gene and RNAse P primers and probes (see steps qPCR section for sequences)		
1mL Fisherbrand 96 deep well plates	12-566-611	
2mL Fisherbrand 96 deep well plates	12-566-612	
Sigma 100% Ethanol	E7203	
Bio-rad Hard-shell thin-wall 384 well PCR plates	HSP3905	
Zymo 2x DNA/RNA Shield	R1200-125	
Zymo RNA Prep Buffer	R1060-2-100	
Bio-rad Hard-shell low-profile 96 well skirted PCR plates	HSP9601	
Zymo DNAse I	E1011	
Sigma Isopropanol	190764-4L	
Beta-mercaptoethanol	97622-10X1ML	
TTP Labtech Dragonfly		
TTP Labtech Syringes	4150-07200	
TTP Labtech Reservoirs	4150-07202	
Thermo Multidrop	5840300	
Thermo Multidrop Cassettes (standard)	24072670	
Integra Viaflow 96/384 Base Unit	6031	
Integra 96 Channel Pippetting Head	6103	
Integra 300uL Sterile, Filter, Low Retention Tips	6535	
NEB Luna Universal Probe One-Step RT-qPCR kit	E3006E	
Thermo-Fisher Nuclease-Free Water	AM9930	
Agilent Bravo Liquid Handling Platform		
Agilent Bravo 250uL Pipette Tips, Sterile, Filtered, for 96LT head	19477-022	
Agilent Bravo 96LT Head	G5498B#042	
Alpaqua 96S Super Magnet Plate	A001322	
Eppendorf Centrifuges		
Nunc™ 96-Well Cap Mats	276005	
Biorad "B" Optical Adhesive Seal	MSB1001	

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NAME	CATALOG #	VENDOR
Thermo Fisher Heat Seals for ALPS	AB-0757	
Thermo Fisher ALPS 50 V-Manual Heat Sealer	AB-1443A	
Orbital shaking station for Agilent Bravo	G5498B#033	
Hamilton STARlet		
Hamilton Set of 6X Carriers for 24 tubes	173499	
Hamilton Tip Carrier	182085	
Hamilton Precision Tab Carrier, DWP, L5.	93522-01	
Hamilton CO-RE tips 480 High Volume Tips 1000uL Conductive Filter Tips	235905	
Handheld Barcode Scanner		
Thermo Fisher RNAse Zap	AMM9782	
Clinical speciments, barcoded and containing swab in 1X DNA/RNA shield or swab in UTM, VTM, Amies medium, PBS, or saline inactivated with 1X DNA/RNA Shield		

SAFETY WARNINGS

While inactivated samples in DNA/RNA Shield can be handled in BSL-1, confirm that samples have been collected in 1X DNA/RNA shield and not another common form of transport media like UTM, VTM, etc.

Reagent Plate Preparation

1 All reagent plates should be barcoded. Record barcode of the plate and lot number of the reagents used in each plate (ie record the lot numbers of DNAse, DNAse Digestion Buffer and water used to make DNAse master mix). It is also recommended that you label the reagents with a human readable label and number the reagents to faciliate matching with the automation pipeline on the Bravo.

2 **Zymo DNA/RNA Shield** - Add 500uL of 2x Zymo DNA/RNA Shield into 2mL deep well plates (for addition of samples not collected in shield) in a PCR Workstation. Aliquot using the Thermo Multidrop liquid dispenser. Seal with ThermoFisher blue rubber seals and store at room temperature.

3 **Proteinase K** - Add 1.2 ml Proteinase K Storage Buffer to reconstitute per 20 mg lyophilized Proteinase K to make a 20mg/mL stock. Aliquot 3uL Proteinase K at 20mg/mL, using the Integra Viaflo 96 into a 1mL 96 deep well plate. Seal and store at -20C, up to a week prior to nucleic acid extraction.

4 **Viral DNA/RNA Buffer** - Add 1 ml beta-mercaptoethanol (BME) per 200 ml Viral DNA/RNA Buffer, final concentration 0.5% (v/v). Aliquot 450uL of Viral DNA/RNA Buffer with BME using the Thermo Multidrop in 1mL 96 deep well plate Seal and store at RT protected from light, up to a week prior to nucleic acid extraction.

5 **Magbeads** - Vortex MagBeads and aliquot into a 1mL deep well source plate. Mix with Integra Viaflo 96. Aliquot 30uL using the Integra Viaflo 96 into Bio-Rad 96 PCR plate. You must mix the MagBeads between each aliquot to avoid settling. Seal and store upside down to avoid settling, at room temperature up to a week prior to nucleic acid extraction.

6 **MagBead DNA/RNA Wash 1 & 2** - Add 80mL of isopropanol to the MagBead DNA/RNA Wash 1 concentrate. Add 120mL of isopropanol to the MagBead DNA/RNA Wash 2 concentrate. Aliquot 550uL of DNA/RNA Magbead Wash Buffers 1 and 2 using the Thermo Multidrop into 1mL 96 deep well plate. Heat seal and store at room temperature in a flammables cabinet, up to a week prior to nucleic acid extraction.

7 **Ethanol** - Aliquot 1.1mL of 100% ethanol, using the Thermo Multidrop, into a 1mL 96 deep well plate plate. Heat seal and store at room temperature in a flammables cabinet, up to a week prior to nucleic acid extraction.

DNAse I - Add 275 µl DNase/RNase-Free Water to reconstitute the lyophilized DNase I at 1 U/µl. Mix by gentle

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8 inversion. Aliquot 55uL of DNAse I in DNAse Digestion buffer, using the Thermo Multidrop in Bio-Rad 96 PCR plate. Seal and store at 4C for for up to a week.

9 **RNA Prep Buffer** - Aliquot 550uL of RNA Prep Buffer using the Thermo Multidrop into 1mL 96 deep well plate. Heat seal and store at room temperature, up to a week prior to nucleic acid extraction.

10 **Water** - Aliquot 100uL of DNAse/RNAse free water using the Thermo Multidrop into Bio-Rad 96 PCR plate, sealed and stored at room temperature, up to a week prior to nucleic acid extraction

11 **Human RNA extraction control (HRC)** - Prepare a 1x PBS solution from the 10x stock solution using nuclease-free water. Prepare 1:1 PBS:Shield solution by diluting 1x PBS in 2x Shield. Dilute extracted HeLa RNA 1 in 1000 in PBS:Shield to a final concentration of 120 ng/ml. Mix by inverting tube and spin down to remove bubbles. Prepare aliquots (2x 500 µl per plate needed), store at 4C for up to 7 days.

12 **Positive control (PC)**: Dilute high titer patient sample (10^7 cp/ml, already in Shield) to 10^5 cp/ml in HRC (see above). Mix by inverting tube and spin down to remove bubbles. Prepare aliquots (2x 500 µl per plate needed), store at 4C for up to 7 days.

13 **PBS**: Prepare 1x PBS solution from the 10x stock solution using nuclease-free water

14 **NTC**: Use nuclease-free water as NTC.

qPCR MM Preparation

15 **N-gene primers and probe** (SARS-CoV-2) - manufactured by IDT in template-free facility

Primer/probe name	Primer/probe sequence	Fluorophore
NIID_2019-nCOV_N_F2	AAATTTTGGGGACCAGGAAC	-
NIID_2019-nCOV_N_R2	TGGCACCTGTGTAGGTCAAC	-
NIID_2019-nCOV_N_P2-FAM	ATGTCGCGCATTGGCATGGA	FAM

N-gene primers and probe

16 **E-gene primers and probe** (SARS-CoV-2) - manufactured by IDT in template-free facility

Primer/probe name	Primer/probe sequence	Fluorophore
SARS-CoV-2_E_Fwd	ACAGGTACGTTAATAGTTAATAGCGT	-
SARS-CoV-2_E_Rev	ATATTGCAGCAGTACGCACACA	-
SARS-CoV-2_E_Prb-FAM	ACACTAGCCATCCTTACTGCGCTTCG	FAM

E-gene primers and probe

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17 **RNAseP primers and probe** (human control) - manufactured by IDT in template-free facility

Primer/probe name	Primer/probe sequence	Fluorophore
RNAse P Forward	AGATTTGGACCTGCGAGCG	-
RNAse P Reverse	GAGCGGCTGTCTCCACAAGT	-
RNAse P P-HEX	TTCTGACCTGAAGGCTCTGCGCG	HEX

RNAse P primers and probe

18 **Preparation of primer/probe mixes** - the primer/probe mix will be diluted 1/20 in the final reaction (0.5 µl of primer/probe mix per 10 µl reaction). Primer/probe mixes are prepared as follows:

E-gene primer/probe mix	*Stock concentration (µM)*	*Desired final concentration in reaction (nM)*	*Required concentration in primer/probe mix (nM)*	*Dilution factor from stock*	*Volume for 1 reaction (µl)*
SARS-CoV-2_E_Fwd	200	400	8000	25	0.02
SARS-CoV-2_E_Rev	200	400	8000	25	0.02
SARS-CoV-2_E_Prb-FAM	100	200	4000	25	0.02
Nuclease-free water		-	-	-	0.44
Total (µl)					0.5

E-gene primer/probe mix

N-gene primer/probe mix	*Stock concentration (µM)*	*Desired final concentration in reaction (nM)*	*Required concentration in primer/probe mix (nM)*	*Dilution factor from stock*	*Volume for 1 reaction (µl)*
NIID_2019-nCOV_N_F2	200	500	10000	20	0.025
NIID_2019-nCOV_N_R2	200	500	10000	20	0.025
NIID_2019-nCOV_N_P2	100	200	4000	25	0.02
Nuclease-free water		-	-	-	0.43
Total (µl)					0.5

N-gene primer/probe mix

RNaseP primer/probe mix	*Stock concentration (µM)*	*Desired final concentration in reaction (nM)*	*Required concentration in primer/probe mix (nM)*	*Dilution factor from stock*	*Volume for 1 reaction (µl)*
Rnase P Fwd	200	200	4000	50	0.01
Rnase P Rev	200	200	4000	50	0.01
RNAseP Probe HEX	100	50	1000	100	0.005
Nuclease-free water		-	-	-	0.475
Total (µl)					0.5

RNaseP primer/probe mix

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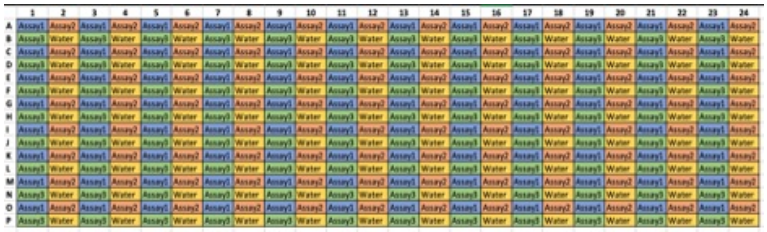
19 **Preparation of mastermix** - one plate will contain 96 individual reactions of 10 µl for one primer/probe set (960 µl), but an additional 20 µl are prepared to ensure enough material is available for plating (total of 980µl). The full composition of the qPCR reaction is as follows (note that sample is not added at this point). INSERT TABLE

Per reaction			**Per plate**		
Number of reactions	*1*		*Number of plates*	*1*	
Luna Universal Probe 1step qPCR mix (2x)	5		Luna Universal Probe 1step qPCR mix (2x)	490	
Luna WarmStart RT Enzyme	0.5		Luna WarmStart RT Enzyme	49	
Primer/probe mix (µl)	0.5		Primer/probe mix (µl)	49	
Sample (µl)	4		Sample (µl)	392	
Total (µl)	10		Tota l(µl)	980	
			Volume without sample	588	

Master Mix preparation

20 Filling qPCR plates with mastermix (6 µl/well) - The mastermix is plated in Bio-Rad 384 well PCR plates using the Dragonfly (6 µl per well) as follows:



Assay 1 = N-gene assay
Assay 2 = E-gene assay
Assay 3 = RNAseP assay

21 Freezing qPCR plates at -20C - spin down plate(s) at 1000 rpm for 1 min, cover plate(s) using F foil and store plate(s) at -20C until use.

Transfer Sample Tubes to Plate on the Hamilton or via pipette

22 **Pipette transfer for shield samples only - perform in a PCR or BSC hood**

22.1 Transfer 600uL of appropriate controls in the layout outlined in the image below into a 96-well 2mL deep well plate.

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	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC 1							PC 1	HRC 1	PBS 1	NTC 3	NTC 4
B												
C												
D												
E												
F												
G												
H	NTC 2							PC	HRC 2	PBS 2	NTC 5	NTC 6

Control layout

22.2 Carefully remove swab from samples and dispose in biohazard. Tweezers, upside-down pipette tips and protective gauze may all be useful in this process. Change gloves if sample gets on it.

22.3 Transfer 600uL of up to 84 samples into the remaining empty wells, recording barcodes and well location during sample addition. Avoid cross-contamination at this step and inspect samples for high viscosity, snot or other irregularities which may lead to difficulties liquid handling.

23 **Pipette transfer for non-shield samples (PERFORM IN APPROPRIATE BSL SPACE IN BSC HOOD)**

23.1 Grab pre-prepared plate with 500uL of 2X DNA/RNA shield. Remove 500uL of shield from wells which should have controls (see step 22.1). Add 600uL of indicated control.

23.2 Carefully remove swab from samples and dispose in biohazard. Tweezers, upside-down pipette tips and protective gauze may all be useful in this process. Change gloves if sample gets on it.

23.3 Transfer 500uL of up to 84 samples into the remaining empty wells, recording barcodes and well location during sample addition. Pipette well to mix with 2X DNA/RNA shield. Avoid cross-contamination at this step and inspect samples for high viscosity, snot or other irregularities which may lead to difficulties liquid handling.

24 **Sample transfer via Hamilton** - the following steps will refer to operation of a Hamilton protocol to transfer samples in tubes with at least 2mL of volume to a 96 well 2mL deep well plate. Custom pathing was implemented to ensure that pipette tips never passed over original tubes. In addition, samples are transferred in groups of 8, aligned in a single column. Pipette tips approach from the top of the plate, to try to contain any potential contamination to a column-wise fashion.

25 Decontaminate the workstation - Spray down the area and all surfaces with RNAseZap and wipe down. Spray down the area and all surfaces with 70% ethanol and wipe down.

26 In a hood, carefully remove swab from samples and dispose in biohazard. Tweezers, upside-down pipette tips and protective gauze may all be useful in this process. Change gloves if sample gets on it.

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27 Turn on computer and turn on Hamilton robot. Open up ‘Sample Reformatting’ shortcut on the desktop

28 Press the play button on top to open up Hamilton GUI.

29 Select site and enter name under “User name” and press “continue”

30 **Ensure the following on the GUI:**

- Correct number of patient samples is entered (this does not include control tubes)
- If samples will be recapped choose “Recapping - With Custom Pathing”.
- If samples are not going to be recapped choose “No Recapping - No Custom Pathing”
- Check “Load carrier and scan barcodes on labware” to ensure that the labware is barcoded and a csv file is generated.
- If you are transferring to only one deep well plate, turn on checkbox 3 and input 600 uL.
- If you are transferring to 2 deep well plates, turn on checkbox 3 and 4.
- If there is at least 2 mL of sample in each tube, enter 600 uL next to boxes 3 and 4.
- If there is less than 2 mL of sample in each tube, consult your supervisor to determine what volumes to transfer.
- Select tube type based on inner diameter of sample tube. For tubes with an inner diameter of ~12 mm, choose “small tube”. For tubes with an inner diameter of ~13.5, choose “large tube”.
- Check “Process transfer from control tubes” to ensure that controls are plated into the deep well.

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An example of Hamilton GUI - note that user should select recapping with custom pathing and transfer 600uL instead of 1000uL

31 Load tip rack carrier in lanes 1-6. Load all labware on the autoload tray until you hit the stopper.

32 Load the tip rack carrier with Hamilton 1000 µL conductive filter tips. Calculate the number of tips to load by using this formula: (number of tubes * number of plates) + 16

33 Use the GUI to select tip locations based upon the tip boxes you loaded onto the carrier

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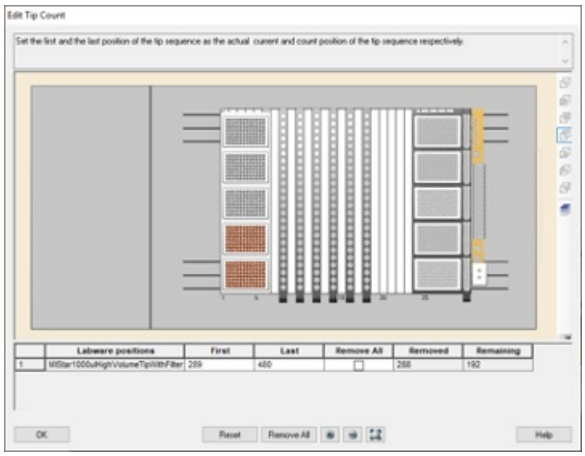

Example of Hamilton GUI showing two full racks of tips available for use

34 Load tubes into tube rack carriers - Use tube rack carriers labeled 8, 10, 12, 14, 16, and 18. These numbers correspond to the lanes they should be loaded onto on the Hamilton. Load tubes such that the barcode is visible between the windows of the tube carrier.

35 Load control tubes

NTC:
a. Tube rack carrier 8, pos 1
b. Tube rack carrier 8, pos 8
c. Tube rack carrier 18, pos 1
d. Tube rack carrier 18, pos 8
e. Tube rack carrier 18, pos 9
f. Tube rack carrier 18, pos 16

PC:
a. Tube rack carrier 14, pos 9
b. Tube rack carrier 14, pos 16

HRC:
a. Tube rack carrier 16, pos 1
b. Tube rack carrier 16, pos 8

PBS:
a. Tube rack carrier 16, pos 9
b. Tube rack carrier 16, pos 16

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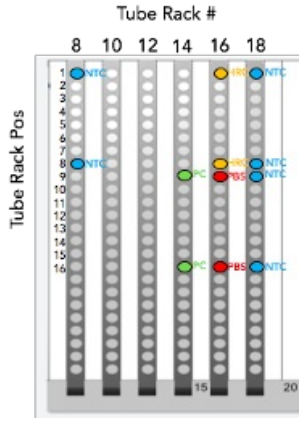
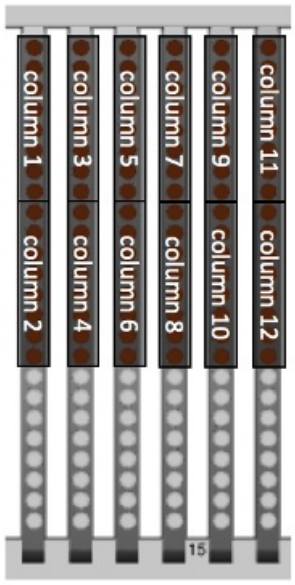


Racking of control tubes

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC1							PC1	HRC1	PBS1	NTC3	NTC4
B												
C												
D												
E												
F												
G												
H	NTC2							PC2	HRC1	PBS2	NTC5	NTC6

Resulting control layout on 96 well plate

36 Load the sample tubes into empty slots of the tube rack carrier. Begin with loading the rack on lane 8. Load the tubes in each rack starting at position 1 and ending at position 16. If a position is occupied by a control tube, move onto the next position. Load the remaining racks in the same manner. The racking will result in the columns on a deep well plate as shown in the image below.



Rack order determinates columns on 96 well plate

37 Uncap the racked tubes.

38 Load the racks onto the corresponding lanes on the robot.

39 **Labware loading and barcoding on robot deck**

- Follow the GUI to confirm that the controls are loaded into the correct positions of the tube rack carrier.
- Tube racks will be automatically loaded and barcoded. Confirm that barcodes are read. If any barcodes raise an

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error, unload the carrier and use the handheld barcode scanner to input the correct barcode.
- Follow the GUI to confirm that the deep well plates are on the correct positions of the carrier.
- The robot will then automatically load the deep well carrier and read the plate barcode.

40 Once the deep well carrier is loaded, the protocol will begin. Watch the protocol to ensure there is no dripping of sample onto the deck or the plate. Take note of any samples which do drip or do not have a pipette tip lower into the tube - the latter means no sample was transferred.

41 After protocol is complete, dispose of waste appropriately and wipe down the deck with 70% ethanol. Seal your sample plate with a blue rubber seal. Recap all tubes.

42 A CSV with barcodes for each 2mL deep well plate processed will be exported.

Preparing for RNA Extraction on the Agilent Bravo

43 Decontaminate the workstation - spray down the area and all surfaces with RNaseZAP and wipe down. Spray down the area and all surfaces with 70% ethanol and wipe down.

44 Take out Proteinase K from -20C. Allow it to thaw prior to starting extraction.

45 Gather all other reagents EXCEPT the DNase I plate and qPCR MM plate. Inspect the reagent plates to make sure they appear to have equal volumes in each well.

46 Pick up an empty sealed barcoded Full skirt Bio-Rad PCR plate for RNA

47 Spin down (1 minute at 215 rcf) all plates EXCEPT the RNA and Magbead plates. Note: when prompted, spin down DNase I and qPCR plate with the same centrifugation settings.

48 Organize plates in the order they'll be used throughout the extraction.

49 Spin down the sample plate (stored at 4C). Leave sealed until everything else is ready.

50 Turn on the Agilent Bravo instrument and open the VWorks software.

51 Open up the RNA extraction form which links to the protocol.

Example VWorks RNA Extraction Form

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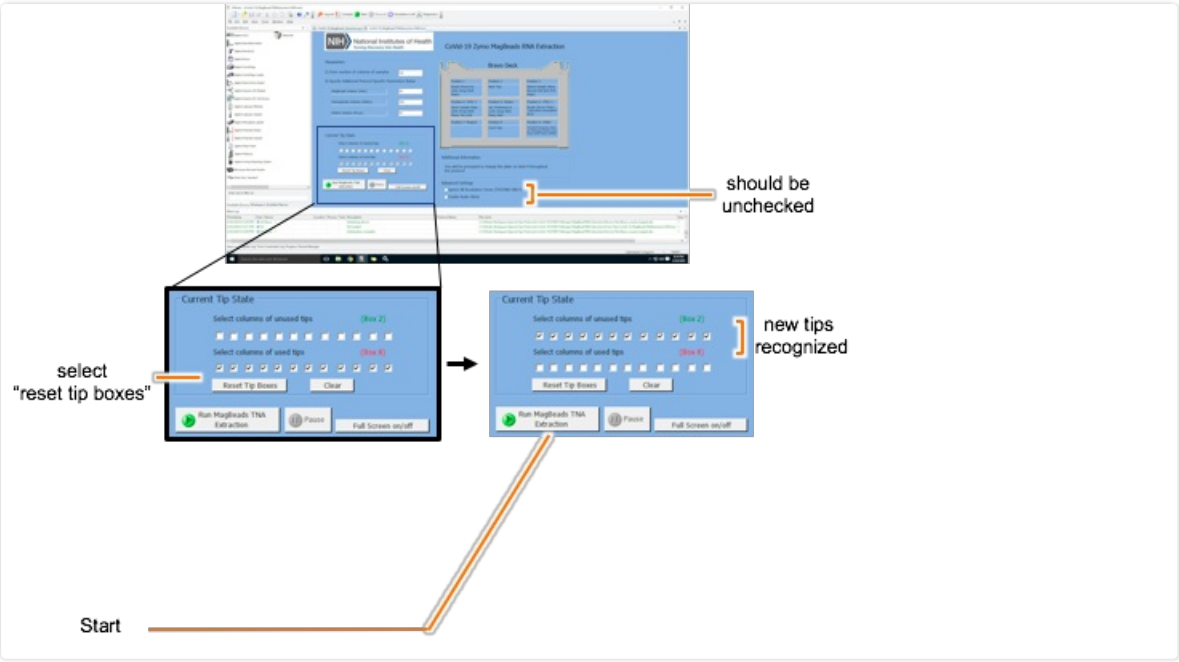
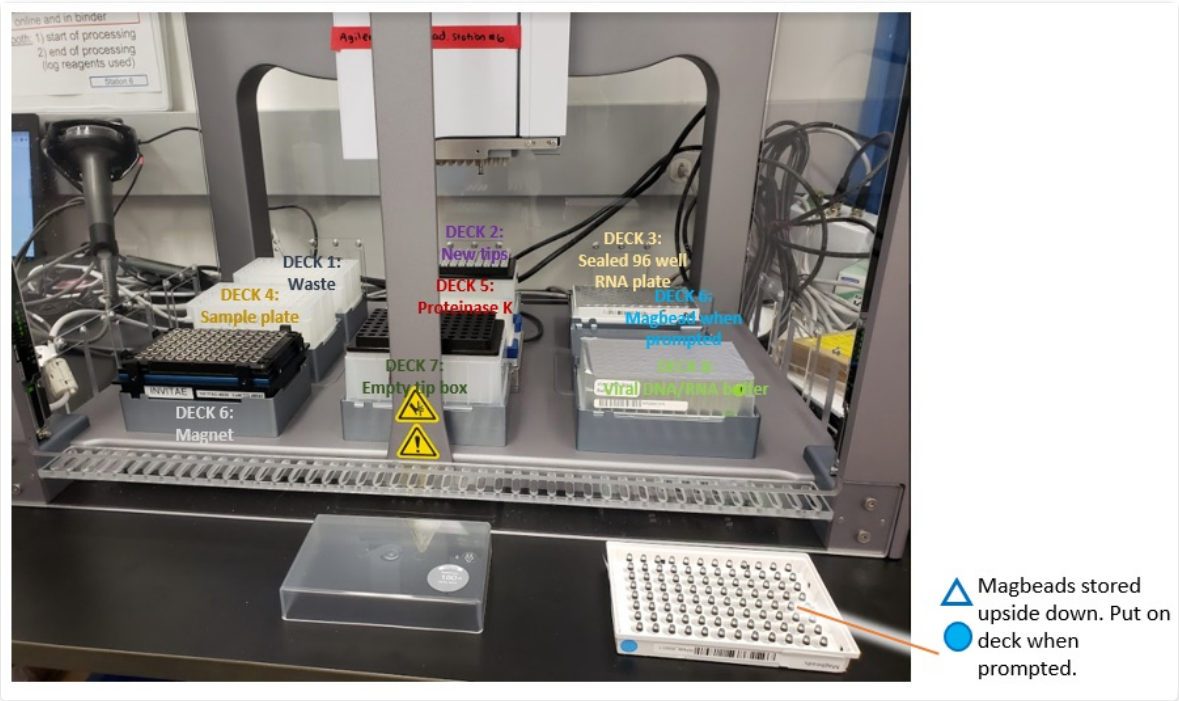


52 Set up the deck according to the VWorks form file deck layout. Make sure you have a 2mL deep well plate for waste, foil seals for re-sealing reagent plates when finished, optical clear plate seals for the qPCR plate at the end, blue rubber seals for the sample plate, waste bags, and Agilent tip boxes on hand.

Example Deck Set-Up



53 Press "Run Protocol" on the form file.

Citation: Amy Lyden, Emily Crawford, Vida Ahyong, Manu Vanaerschot, Paula Hayakawa Serpa, Preethi Raghavan, Joana Cabrera, Michael Borja, Spyros Darmanis, Eric Chow, Joseph Derisi (07/06/2020). CLIAHUB Automated RNA Extraction & RT-PCR Protocol V2. https://dx.doi.org/10.17504/protocols.io.bfi2jkge

54 During the protocol, follow the prompts to unseal reagent plates and take out reagent plates from the freezer at the appropriate time. Be sure to dispose of reagent plates, liquid waste, and tips in the appropriate chemical waste bins.

RNA Extraction on the Agilent Bravo

55 The Bravo transfers 180uL of stock sample into a 1ml deep well plate with pre-aliquoted Proteinase K on Deck 5.

56 The Bravo picks up 360uL (from deck 9) of the mix per well from a prepared stock plate and mixes it with the samples (deck 5).

57 The Bravo mixes the Magbeads (deck 6).

58 It aspirates 20uL of beads (deck 6) to be mixed with the sample in Proteinase K + Viral Buffer Mix (deck 5).

59 The beads and sample are mixed (deck 5) for 10mins.

60 The plate is moved to a magnet (deck 7) to incubate for 5mins.

61 The supernatant (deck 7) is removed to waste (deck 1).

62 The reagent plate (deck 9) is swapped with Magbead DNA/RNA Wash Buffer 1.

63 The Bravo picks up 500uL (from deck 9) of the Wash 1 Buffer and mixes it with the samples (deck 5).

64 The plate is moved to a magnet (deck 7) to incubate for 5mins.

65 The supernatant (deck 7) is removed to waste (deck 1).

66 The reagent plate (deck 9) is swapped with Magbead DNA/RNA Wash Buffer 2.

Citation: Amy Lyden, Emily Crawford, Vida Ahyong, Manu Vanaerschot, Paula Hayakawa Serpa, Preethi Raghavan, Joana Cabrera, Michael Borja, Spyros Darmanis, Eric Chow, Joseph Derisi (07/06/2020). CLIAHUB Automated RNA Extraction & RT-PCR Protocol V2. https://dx.doi.org/10.17504/protocols.io.bfi2jkge

67 The Bravo picks up 500uL (from deck 9) of the Wash 2 Buffer and mixes it with the samples (deck 5).

68 The plate is moved to a magnet (deck 7) to incubate for 5mins.

69 The supernatant (deck 7) is removed to waste (deck 1).

70 The reagent plate (deck 9) is swapped with 100% ethanol - enough for two washes.

71 The Bravo picks up 500uL (from deck 9) of the ethanol and mixes it with the samples (deck 5).

72 The plate is moved to a magnet (deck 7) to incubate for 2mins.

73 The supernatant (deck 7) is removed to waste (deck 1).

74 The reagent plate (deck 9) is currently 100% ethanol.

75 The Bravo picks up 500uL (from deck 9) of the ethanol and mixes it with the samples (deck 5).

76 The plate is moved to a magnet (deck 7) to incubate for 2mins.

77 The supernatant (deck 7) is removed to waste (deck 1).

78 The reagent plate (deck 9) is swapped with DNase Mix (DNase + Digestion buffer + water)

79 The Bravo picks up 50uL (from deck 9) of the DNase mix and mixes it with the samples (deck 5).

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80 The plate incubates (deck 5) for 15mins at room temperature.

81 The reagent plate (deck 9) is swapped with RNA Prep Buffer.

82 The Bravo picks up 500uL (from deck 9) of the RNA Prep Buffer and mixes it with the samples (deck 5).

83 The plate is mixed (deck 5) for 10mins.

84 The plate is moved to a magnet (deck 7) to incubate for 5mins.

85 The supernatant (deck 7) is removed to waste (deck 1).

86 The reagent plate (deck 9) is swapped with 100% ethanol - enough for two washes.

87 The Bravo picks up 500uL (from deck 9) of the ethanol and mixes it with the samples (deck 5).

88 The plate is moved to a magnet (deck 7) to incubate for 2mins.

89 The supernatant (deck 7) is removed to waste (deck 1).

90 The reagent plate (deck 9) is currently 100% ethanol.

91 The Bravo picks up 500uL (from deck 9) of the ethanol and mixes it with the samples (deck 5).

92 The plate is moved to a magnet (deck 7) to incubate for 2mins.

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93 The supernatant (deck 7) is removed to waste (deck 1).

94 The reagent plate (deck 9) is swapped with 100% nuclease-free water.

95 The Bravo picks up 45uL (from deck 9) of water and mixes it with the samples (deck 5).

96 The plate is moved to a magnet (deck 7) to incubate for 5mins.

97 The RNA eluted in water (deck 7) is collected and dispensed into a final plate (deck 3).

98 Spin down the plate (1 minute at 215 rcf) and immediately proceed to transferring your extracted RNA into a pre-prepared 384-well qPCR plate.

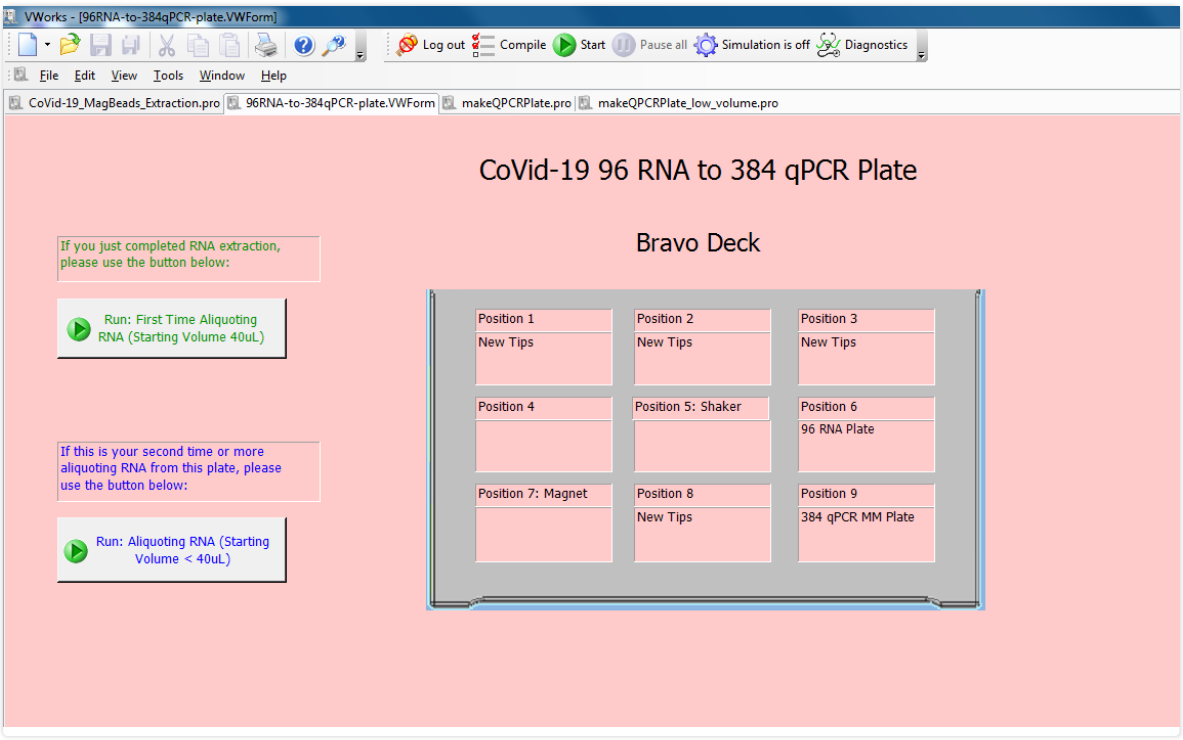
Setting up qPCR

99 Seal qPCR plate with Biorad B optical seal. Do not touch the top of the seal to avoid leaving marks. Remove the tabs.

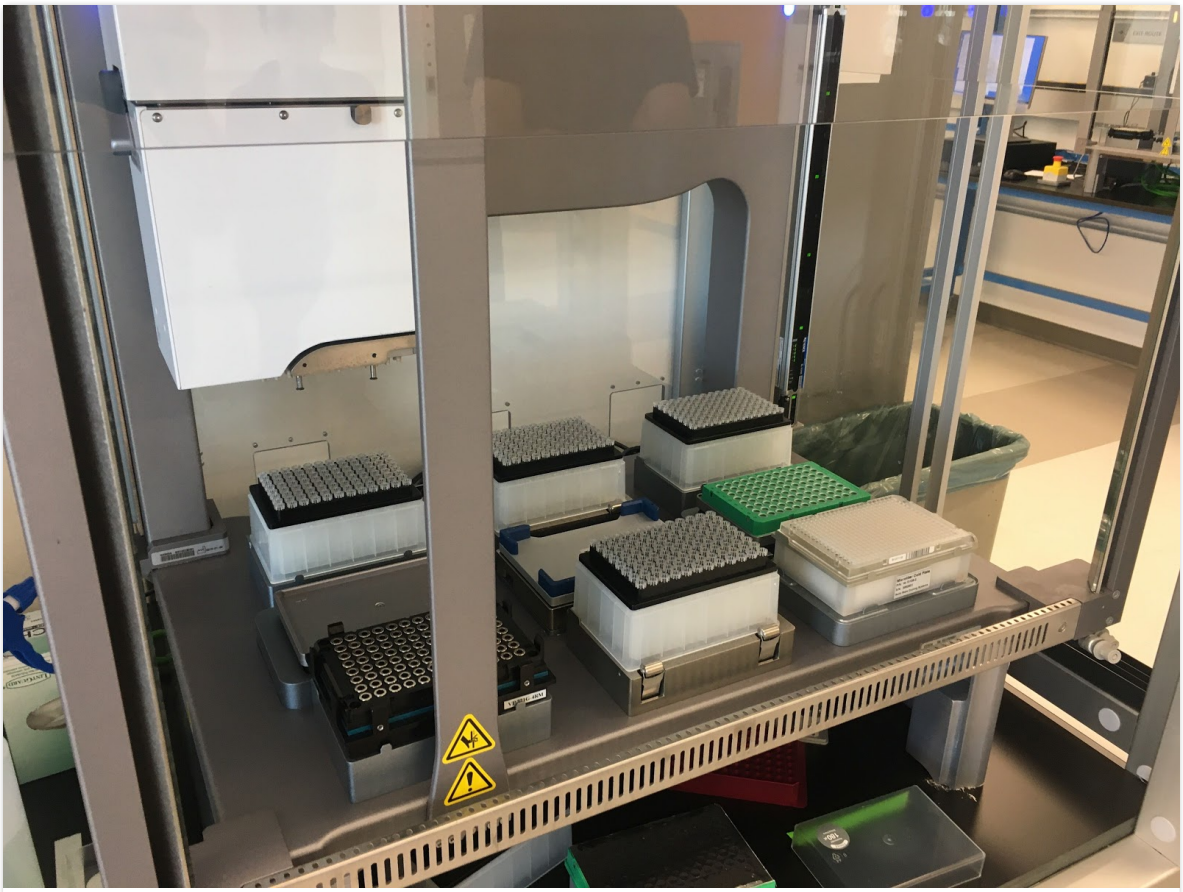
100 Clean off Bravo deck.

101 Open qPCR plating VWorks form, and set up deck according to the form - including adding your spun down RNA plate from the extraction protocol onto Deck 6 and the thawed and spun down qPCR MM plate on Deck 9.

Citation: Amy Lyden, Emily Crawford, Vida Ahyong, Manu Vanaerschot, Paula Hayakawa Serpa, Preethi Raghavan, Joana Cabrera, Michael Borja, Spyros Darmanis, Eric Chow, Joseph Derisi (07/06/2020). CLIAHUB Automated RNA Extraction & RT-PCR Protocol V2. https://dx.doi.org/10.17504/protocols.io.bfi2jkge



Example qPCR plating form in VWorks Software



Example Bravo Deck Set-Up for qPCR plating

Citation: Amy Lyden, Emily Crawford, Vida Ahyong, Manu Vanaerschot, Paula Hayakawa Serpa, Preethi Raghavan, Joana Cabrera, Michael Borja, Spyros Darmanis, Eric Chow, Joseph Derisi (07/06/2020). CLIAHUB Automated RNA Extraction & RT-PCR Protocol V2. https://dx.doi.org/10.17504/protocols.io.bfi2jkge

102 Press "Run protocol".

103 The Bravo will aliquot 4uL of RNA each into A1 quadrant, B1 quadrant and A2 quadrant.

104 Spin down qPCR plate for 1 minute at 215 rcf.

105 Run qPCR protocol on BioRad CFX thermocycler. Ensure detection for both FAM and HEX channels.

Step	Stage	# Cycles	Temperature	Time	
RT	1	1	55 oC	10 min	
Initial denaturation	2	1	95 oC	1 min	
Amplification	3	45	95 oC	10 seconds	
			58 oC	30 seconds	

Thermocycling conditions for NEB Luna RT-qPCR

106 Freeze RNA at -80C.

Interpreting Results

107 **Positive control** - PC reaction should produce a positive result with a Ct value within the following expected ranges: N gene < 38, E gene < 38, RNAse P gene < 38. If the PC does not exhibit positivity within the expected range, the run is invalid.

108 **Negative controls** - NTC should be negative and exhibit no fluorescence growth curves that cross the threshold line. If a false positive occurs with one or more of the primer and probe NTC reactions, sample contamination may have occurred. PBS should be negative and exhibit no fluorescence growth curves that cross the threshold line. If a false positive occurs with one or more of the primer and probe PBS or NTC reactions, sample contamination may have occurred.

109 **RNAse P** should be positive at Ct ≤38 for all negative clinical samples and HRC. Acceptable results for the RP in patient samples indicates the presence of sufficient nucleic acid from the human RNase P gene, and thus, acceptable specimen quality. Failure of RP detection in HRC may indicate improper assay set up or instrument malfunction. Failure of RP in patient samples but detection in HRC may indicate extraction failure, PCR inhibition, or absence of sufficient human cellular material. Samples that are positive for viral detection do not require amplification of RP target to be valid

110 **Human RNA Control** - HRC should be negative for SARS-CoV specific primer/probe sets. A fluorescence growth curve for N gene or E gene that crosses the threshold line may indicate contamination of reagents or cross contamination of samples. If the HRC exhibits a positive signal for Coronavirus specific primer/probe sets, the run is invalid.

111 **Patient samples**

N	E	RP	Interpretation	Action

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Pos	Pos	Pos or Neg	Detected	Report as Detected
1 of 2 pos, or 1 pos with Ct value > 38.0		Pos or Neg	Indeterminate	Repeat with new extraction; if same result Report as Indetermin ate
Neg	Neg	Pos	Not Detected	Report as Not Detected
Neg	Neg	Neg	Invalid	Repeat with new extraction; if same result Report as Invalid

Results interpretation summary

111.1 **Negative**: a specimen is negative if none of the SARS-CoV-2 markers (N gene, E gene) exhibit growth curves that cross the threshold AND RNase P (RP) is positive (DOES cross the threshold line).

111.2 **Positive**: a specimen is positive for SARS-CoV-2 if both markers (N gene, E gene) exhibit growth curves that cross the threshold line with Cp value < 40.0. RNase P (RP) may or may not be positive - either is valid

111.3 **Indeterminate**: If only one of the SARS-CoV-2 markers is positive or if both are positive but one or both have Ct > 40.0, the result is inconclusive for SARS-CoV-2 and should be repeated. If repeat is the same, report as Indeterminate.

111.4 **Invalid**: If all targets (N gene, E gene, RNAse P) do not cross the threshold line, the result is invalid and should be repeated. If repeat is the same, report as Invalid.

112 Bring any unexpected results to the supervisor or section director before sending the result file for reporting.

a. Any unexpected control result.
b. Cluster of positive results on a plate map with 2 or more adjacent positive wells.
c. Plates with more than 4 Indeterminate or Invalid results.
d. Plates with more than 10 positive results.

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