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Two-step Reverse Transcription Droplet Digital PCR Protocols for SARS-CoV-2 Detection and Quantification

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TITLE:

Two-Step Reverse Transcription Droplet Digital PCR Protocols for SARS-CoV-2 Detection and Quantification

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SUMMARY:

This work summarizes steps on developing different assays for SARS-CoV-2 detection using a two color ddPCR system. The steps are elaborate and notes have been included on how to improve

the assays and experiment performance. These assays may be used for multiple SARS-CoV-2 RT-ddPCR applications.

ABSTRACT:
Diagnosis of the ongoing SARS-CoV-2 pandemic is a priority for all countries across the globe. Currently, reverse transcription quantitative PCR (RT-qPCR) is the gold standard for SARS-CoV-2 diagnosis as no permanent solution is available. However effective this technique may be, research has emerged showing its limitations in detection and diagnosis especially when it comes to low abundant targets. In contrast, droplet digital PCR (ddPCR), a recent emerging technology with superior advantages over qPCR, has been shown to overcome the challenges of RT-qPCR in diagnosis of SARS-CoV-2 from low abundant target samples. Prospectively, in this article, the capabilities of RT-ddPCR are further expanded by showing steps on how to develop simplex, duplex, triplex probe mix, and quadruplex assays using a two-color detection system. Using primers and probes targeting specific sites of the SARS-CoV-2 genome (N, ORF1ab, RPP30, and RBD2), the development of these assays is shown to be possible. Additionally, step by step detailed protocols, notes, and suggestions on how to improve the assays workflow and analyze data are provided. Adapting this workflow in future works will ensure that the maximum number of targets can be sensitively detected in a small sample significantly improving on cost and sample throughput.

INTRODUCTION:
Polymerase chain reaction (PCR), a well-recognized technique, has undergone several transformations since its advent to become a powerful technique capable of providing answers to nucleic acid research. These transformations have been a constant improvement of the old technique. These transformations can be summarized into three generations[1]. The first generation is conventional PCR that relies on gel electrophoresis to quantify and detect amplified targets. The second generation is quantitative real time PCR (qPCR) that can detect samples in real time and rely on a standard curve to directly quantify targets in a sample. The third generation, digital PCR (dPCR), can perform both detection, and absolute quantification of nucleic acid targets without the need of a standard curve. dPCR has also been improved further from reaction chambers being separated by the wells of a wall into emulsions of oil, water, and stabilizing chemicals within the same well as seen in droplet-based digital PCR[2]. In droplet digital PCR (ddPCR), a sample is partitioned into thousands of nanoliter-sized droplets containing individual targets that will later be quantified using Poisson statistics[2–4]. This technique gives ddPCR an edge in quantifying low abundant targets when compared to the other generations of PCR.

Recently, multiple applications have highlighted the superiority of ddPCR over the commonly used qPCR when detecting and quantifying low abundant targets[1,5,6]. SARS-CoV-2 is no exception to these applications[7–12]. Since the outbreak of SARS-CoV-2, scientists have been working on all fronts to come up with solutions on how to diagnose the virus and detect it efficiently. The current gold standard still remains to be qPCR[13]. However, RT-ddPCR has been shown to be more accurate in detecting low abundant SARS-CoV-2 targets from both environmental and clinical

samples when compared to RT-qPCR[7–12]. Most of the SARS-CoV-2 ddPCR published works depend on simplex assays with the multiplex ones depending on commercial assays. Hence, more should be done to explain how to develop multiplex RT-dPCR assays for SARS-CoV-2 detection.

In a proper assay design, multiplexing can be used to save on cost, increase sample throughput, and maximize on the number of targets that can be sensitively detected within a small sample. When multiplexing with ddPCR, one must take account of how many fluorophores can be detected in a particular system. Some ddPCR platforms can support up to three channels while others support only two channels. Hence, when multiplexing with two channels, one has to use different approaches, including higher order multiplexing to detect more than two targets[14–16]. In this work, a two color ddPCR detection system is used to show steps on how to develop different SARS-CoV-2 RT-ddPCR assays that can be adapted for different research applications.

PROTOCOL:

Ethical statement

Wuhan Institute of Virology (WHIOV) is among the labs and institutes approved by China CDC of Wuhan city to conduct research on SARS-CoV-2 and detect COVID-19 from clinical samples. Research on developing new diagnostic techniques for COVID-19 using clinical samples has also been approved by the ethical committee of Wuhan Institute of Virology (2020FCA001).

1. Sample processing workflow (Figure 1A)

NOTE: Throughout the protocol, it is important to use separate rooms with dedicated pipettes for sample handling (extraction and storage), reagent/mastermix preparation and storage, reaction mix preparation (sample plus mastermix), and detection, to avoid cross contamination. The assays to be developed can be used in the detection of clinical samples or research samples. All samples should be treated as if they can transmit infectious agents even when using safe laboratory procedures. Sample processing steps should be done in a biosafety level 2 (BSL-2) laboratory following strict BSL-2 rules, including wearing of appropriate personal protective equipment (PPE).

1.1. Sample inactivation

1.1.1. Take 1 mL of SARS-CoV-2 sample to a BSL-2 laboratory. Heat-inactivate samples at 65 ^{o}C for 30 min.

NOTE: Samples may come from various sources, hence, one should make sure the volume to be inactivated is sufficient to ensure subsequent RNA extraction. Most extraction kits require a small sample volume of up to 200 µL, hence, a sample volume of about 1 mL would be sufficient for inactivation. Surfactants, kits, and lysis buffers may also be used for direct inactivation and RNA extraction according to the labs own SOP.

1.1.2. Take samples to a biosafety cabinet (BSC) and let them stand at room temperature for 10 min to allow potential aerosols to settle. Store samples in 4 °C for up to 24 h if not processed immediately.

NOTE: No specific container is required for sample storage. Samples may be stored in the tubes or containers used during inactivation. For this work, most samples were stored in viral transport media (VTM) tubes or 1.5 mL tubes.

1.2. **RNA extraction**

NOTE: Many RNA extraction instruments and kits with specific protocols are available for ready use. Here, an automated procedure following the manufacturer's instructions is presented.

1.2.1. Take out the pre-filled 96-deep well orifice plate; invert it upside-down gently to mix the magnetic beads. After mixing, gently shake the plate on a flat surface to concentrate reagent and magnetic beads that may be on the wall to the plate's bottom.

1.2.2. Carefully tear off the aluminum foil sealing film to avoid vibration of the plate and liquid spillage. Add 20 µL of proteinase K and 200 µL of the sample per well in rows 2 and 8 of the 96-well plate. Then, place the 96-well plate on the base of the 32-channel automatic nucleic acid extraction instrument.

NOTE: Run the program on the computer within 1 h after adding the sample.

1.2.3. Insert the magnetic bar sleeve into the card slot of the 32-channel automatic nucleic acid extraction instrument. Select the program GF-FM502T5-TR"1"GF-FM502T5YH (quick version) and run it.

1.2.4. After completion of the extraction, take out the nucleic acid samples in rows 6 and 12 (about 100 µL/sample) and distribute in a clean nuclease-free 96-well. Store the samples at 4 °C for up to 24 h until use or at -20 °C for a longer time.

NOTE: It is recommended to use a 100 µL multichannel pipette to transfer samples into a new 100–200 µL plate as it can directly match the columns of the sample plate.

1.3. **cDNA generation**

NOTE: For samples stored for a long time, avoid multiple freeze/thaw cycles. Perform cDNA generation using a cDNA kit following the manufacturer's instructions.

1.3.1. In a 100 or 200 µL PCR tube placed on ice/cooling block, add 2 µL of 5x RT master mix (e.g., Perfect Real Time), 5 µL of sample RNA, and 3 µL of RNase free ddH2O per reaction (total volume 10 µL).

1.3.2. Place the PCR tube in a thermal cycler set to run at 37 °C for 15 min (reverse-transcription), 85 °C for 5 s (heat inactivation of reverse transcriptase), and 4 °C for an infinite time.

NOTE: Process the samples immediately, or store at 4 °C for up to 24 h until use or at -20 °C for up to 6 months.

2. Optimization of ddPCR assay and workflow

NOTE: Optimize the assays before/after reading the droplets. Dependent on the results, they can be optimized at any point of the work to achieve better results. Below are some common factors to be considered when optimizing ddPCR experiments.

2.1. Validate the ddPCR primers and probes (**Table 1**).

NOTE: The primers ORF1ab and N were adapted from China CDC[17], the human endogenous control gene (RPP30) from Lu et al.[18], and RBD2 from Nyaruaba et al.[13]. All primers and probes except RBD2 had already been ddPCR tested and optimized[7,8,18].

2.2. Run the ddPCR test sample controls comprising a positive control (a sample with all four targets), No Template Control (NTC; Nuclease free water or sample with no targets), and extraction/negative control (total nucleic acid extracted from a non-infectious cultured human cell or pooled samples of health volunteers).

NOTE: Standard control samples with known copies of target genes are preferred. However, in the absence of standards, use the available samples to define their controls or sample matrix. Run the controls together with test samples.

2.3. Optimize the annealing temperature (**Figure 2D**) by inserting a temperature gradient of 55 °C to 65 °C in the annealing step of PCR (**step 3.3**).

NOTE: Annealing temperature optimization helps to find the best temperature where droplet separation is maximum (easy target identification) with minimal rain. After obtaining the results, narrow down the temperature range for better optimization, e.g., 55 °C to 60 °C.

2.4. Optimize the primer and probe concentrations. If optimum separation between positive and negative droplets is not achieved in the assays, vary the primer (300–900 nM) and/or probe (100–400 nM) concentrations.

NOTE: Lowering primer/probe concentrations results in a lower target droplet amplitude and increasing it also increases the target's amplitude. This can be helpful in amplitude-based multiplexing.

2.5. Test the analytical sensitivity considering different parameters including, limit of blank (LoB), limit of detection (LoD), specificity, and sensitivity using both standard and test samples, as preferred.

3. ddPCR workflow (Figure 1B) and assay development (Table 2)

NOTE: Like other ddPCR detection systems, this workflow also consists of four steps (**Figure 1B**), including reaction mix preparation, droplet generation, PCR amplification, and droplet reading.

3.1. Reaction mix preparation

NOTE: Prepare all assays in a clean separate room and inside a BSC. Observe standard precautions, including use of clean gloves, clean pipettes, nuclease free water, and disinfectants. Aliquot reagents in separate tubes and store at -20 ^{o}C to avoid repeated freeze thawing cycles. Prepare assays as shown in **Table 2**. Primer and probe stock solutions should be stored in high concentrations of 100 µM. The working solutions can be stored in aliquots of 20–40 µM (diluted in nuclease free water).

3.1.1. Common steps in all assays

NOTE: Before preparing the assays, calculate the total volume of mastermix needed based on the number of samples. Here, each mastermix component volume was multiplied by 1.05 to account for any pipetting errors.

3.1.1.1. Thaw and equilibrate reaction materials to room temperature. Vortex the reactions components briefly (30 s) to ensure homogeneity, and briefly centrifuge to collect the contents at the bottom of the tube.

3.1.1.2. Prepare a primer-probe (PP) mix per assay by mixing specific reaction volumes as detailed in **Table 2** from the working solutions.

3.1.1.3. Prepare the mastermix by mixing 11 µL (1x) of 2x ddPCR supermix for probes (No dUTP), PP mix (dependent on assay as shown in **Table 2** from the working solution) and nuclease free water to a final volume of 19.8 µL per well. Distribute the mastermix into the wells of a nuclease-free 96-well ddPCR plate based on the number of samples.

NOTE: For the sample plate, make sure all 8 wells in one column have the solution. If only a few wells are used, e.g., 5 samples, fill the other 3 wells with 22 µL of nuclease free water or control buffer each.

3.1.1.4. To get the final volume reaction mix (22 µL), add 2.2 µL of cDNA sample per well with mastermix. Seal the plate with a disposable PCR plate sealer.

NOTE: Perform the sample additions in the designated room. Include the controls i.e., positive, NTC, and extraction. Additionally, if the RNA samples are of low concentration, increase the sample volume up to 5.5 µL.

3.1.1.5. Once the reaction mix is distributed per well, vortex briefly (15–30 s), and centrifuge (10–15 s) to collect the contents at the bottom of the plate. Proceed for droplet generation.

3.2. **Automated droplet generation (Supplementary Figure 1)**

NOTE: Different droplet generators can be used; however, this study was performed with an automated droplet generator (AutoDG). To avoid amplicon contamination, ensure the droplet generator and readers have dedicated space in separate areas. When loading consumables, it is recommended to start loading from the back of the instrument working toward the front to avoid moving hands over the consumables to avoid cross contamination.

3.2.1. Obtain all consumables needed to set up the AutoDG, including AutoDG oil for probes, DG32 Cartridges, pipette tips, cooling block, sample plate, droplet plate (new clean ddPCR plate), and a waste bin.

3.2.2. On the AutoDG touch screen, touch **Configure Sample Plate** and select columns where samples are located on sample plate and press **OK**.

NOTE: The screen will turn yellow indicating LOAD where consumables should be loaded.

3.2.3. Open the AutoDG door and load consumables in their respective places.

NOTE: The respective places where consumables should be loaded will have a yellow indicator that will later turn green if the consumables are loaded. This is similar to the touchscreen.

3.2.4. Load the sample plate and droplet generation plate.

NOTE: The droplet generation plate should be placed on the cooling block. Ensure the cooling block is purple (ready to use) and not pink (should be kept in a freezer).

3.2.5. Close the AutoDG door, ensure the screen is green (consumables loaded), and select **Oil for Probes** before pressing **Start Droplet Generation**.

3.2.6. Wait for the droplets to be generated. Open the door once the screen shows **Droplets Ready** and remove the plate containing the droplets.

3.2.7. Seal the plate with a pierceable foil seal using a plate sealer set to run at 185 °C for 5 s. Proceed to PCR amplification.

NOTE: PCR amplification should be started within 30 min after droplet generation.

3.3. **PCR amplification**

3.3.1. Insert the sealed droplet plate into a 96-deep well thermal cycler, set the sample volume to 40 µL and lid temperature to 105 °C.

3.3.2. PCR amplify the droplets using the program: 95 °C for 10 min (enzyme activation), 40 cycles of denaturation at 94 °C for 30 s and 1 min annealing/extension at 57 °C, enzyme deactivation at 98 °C for 10 min, and holding step at 4 °C indefinitely. After the PCR, read the droplets or store at 4 °C for up to 24 h.

NOTE: Use a ramp rate of 2 °C /s at all steps, as ramp rates may differ for different thermal cyclers. Hold the plate for at least 30 min at 4 °C to allow droplet stabilization before reading the droplets.

3.4. **Droplet reading**

3.4.1. Transfer the thermal cycled 96-well plate into a droplet reader, and on a computer connected to the droplet reader, open/start the accompanying software.

3.4.2. In the setup mode, select **New** and then double-click on any well to open the **Well Editor** dialog box. Select the wells to be read and choose **Experiment: ABS, Supermix: ddPCR Supermix for Probes (no dUTP), Target 1 Type: Ch1 Unknown, Target 2 Type: Ch2 Unknown**.

NOTE: Negative, blank, NTC, or positive can be selected from the target 1/2 type dropdown menu for the control samples.

3.4.3. Assign the target or sample names based on plate layout and select **Apply**. When done, select **OK**, and save the created template. Click on **Run** and when prompted choose the right color (FAM/HEX).

NOTE: Priming the system before running is recommended before the first run of the day. Also, check whether all lights in the reader are green and if not, follow the recommended application on the tool tip status.

3.4.4. After data acquisition is finished, remove the plate from the reader. Analyze data as required.

NOTE: Since preliminary results can be seen on the screen, running the positive samples in the first wells can be used for real-time quality check.

4. **Data analysis (Supplementary Figures 2 and 3)**

4.1. Check the data from all the wells for total number of droplets. If the droplet count is <10,000, then discard the results and repeat the assay. Set a cut off to accept positive and negative results. E.g., A droplet count of up to 3 or more droplets can be considered as the cut off for positive results.

NOTE: Data for all assays, including simplex, duplex, triplex probe mix, and quadruplex assays are generated using the accompanying software. However, this software is only suitable for simplex and duplex assays. For high order multiplex assays (>3 targets), an external software is needed.

4.2. **Simplex and duplex assays**

NOTE: The external software can also analyze simplex and duplex data. However, using the accompanying software is easier for these assays, hence used in this article.

4.2.1. Double click on the .qlp file to be analyzed to open it and select **Analyze**.

4.2.2. Use the threshold tools in the **1D Amplitude** and **2D Amplitude** to distinguish between positive and negative droplets for each well in the correct channel. The NTC and positive control samples can be used as a guidance for threshold setting.

NOTE: FAM results are seen in Channel 1 while HEX/VIC in channel 2.

4.2.3. After threshold setting, the results can be exported as a .csv file and further analyzed in Excel or recorded by reading directly on the results window.

4.3. **Triplex probe mix (Supplementary Figure 2) and quadruplex assays (Supplementary Figure 2)**

NOTE: Before analyzing the triplex probe mix and the quadruplex assay, download the external software. Refer to the minimal system requirements before installing.

4.3.1. Open the .qlp file by right clicking on it and choosing **Open** with the external software, or by opening the external software and clicking on the **Browse** option to locate the .qlp file in your folder, or by simply dragging the .qlp file and dropping it on the already open external software.

4.3.2. In the plate editor tab, select the wells to be analyzed on the right side of the tab.

4.3.2.1. For triplex probe mix, select **Direct Quantification (DQ)** as experiment type, select **Probe Mix Triplex** as assay information and enter target names accordingly, and then click on **Apply**.

4.3.2.2. For quadruplex, select **Direct Quantification (DQ)** as experiment type, select **Amplitude Multiplex** as assay information and enter target names accordingly, and then click on **Apply**.

4.3.3. On the left side of the 2D Amplitude tab, use the **Graph Tools** to assign specific colors to different target clusters following the **Select to Assign Cluster** window pop up suggestions.

NOTE: Preferred cluster mode can be applied based on the users' preference.

4.3.4. Once the target colors are assigned, quantification data can be read in the **Well Data Window** on the lower right. Export data to Excel/csv for further analysis using the triple-bar icon on the upper right hand of the well data table.

REPRESENTATIVE RESULTS:

In a proof-of-concept study, the multiplex assays analytical performance was tested on clinical and research samples[19]. The performance of the multiplex assays was superior to that of an RT-PCR[19]. Since low numbers of droplets may indicate a problem during droplet generation, in this article a cutoff of 10,000 droplets per well was set based on empirical data.

A good separation between positive and negative droplets with minimal rain interference can help in data analysis. Hence, in a good experiment, assay optimization is key[19]. As seen in the temperature gradient analysis results in **Figure 2D**, high annealing temperatures (e.g., 65 ^{o}C) could not clearly distinguish positive droplets from negative droplets when a duplex assay (N (FAM) and RPP30 (HEX)) was run. However, with a decrease in annealing temperature, optimal separation between positive and negative droplets was achieved. A temperature of 57 ^{o}C was found optimal. This can also be observed in other assays[19].

Using a two color (FAM/HEX) RT-ddPCR detection system, it is possible to detect one (**Figure 2A, B**), two (**Figure 2C**), three (**Figure 3A**), and four (**Figure 3B**) SARS-CoV-2 targets within a single sample. During data analysis, the accompanying software used to read droplets can only analyze simplex and duplex assays as shown in **Figure 2**. This means that for higher order multiplex assays (>3 targets), an external software should be used for data analysis as shown in **Figures 3, S2**, and **S3**. It is important to also note that the external software can also be used to analyze simplex and duplex data. For simplex and duplex data, analysis is quite simple as targets are separated as either positive or negative droplets in their respective channels as shown in **Figure 2**. A NTC sample can help in the location of negative droplets that can in turn help one set thresholds for data analysis as shown in **Figure 2A**. For the duplex assay, analysis can be done in individual channels (**Figure 2B i,ii**) or in the 2D Amplitude (**Figure 2B iii**).

For higher order multiplex assays, data analysis is not straightforward, and attention should be focused on droplet target assignment. After installing the external software, select wells to be analyzed, select the appropriate experiment type, and assign target clusters based on experiment type as shown in **Figure S2** and **S3**. The Select to Assign Cluster window pop will guide one on how to assign clusters in the higher multiplex assays. Use the graph tools to assign up to 8 droplet

clusters for the triplex probe mix assay (**Figure 3A**), and up to 16 droplet clusters for the quadruplex amplitude-based assay (**Figure 3B**).

After assigning clusters and thresholds, quantification data for each target in the form of copies/µL can be read in the well data window on the lower right of the external software. This data can be used to estimate the number of copies of targets in the starting sample. E.g., if 2.2 µL of the sample was used in a final volume of 22 µL, and the software recorded 30.5 copies/µL for ORF1ab, there were 30.5 x 22 = 671 copies of ORF1ab in the PCR mix. The mix contained 2.2 µL of original sample, hence, there were 671 copies of ORF1ab in the starting sample, and 671/2.2 = 305 copies/µL of ORF1ab in the original sample. This method can be used to estimate the concentration of each target in all assays.

TABLE LEGENDS:

Table 1: Primer and probe sequences used to develop the different SARS-CoV-2 assays.

Table 2: Final concentration of different primer and probe pairs per assay.

FIGURE LEGENDS:

Figure 1: Sample processing and droplet digital PCR workflow. (**A**) The sample processing workflow includes sample collection and transport to a BSL-2 facility, inactivation, extraction, and cDNA generation. (**B**) The droplet digital PCR workflow begins with preparation of the mastermix, loading the mastermix into a ddPCR plate and adding sample(s), generating droplets, amplifying targets inside droplets by PCR, and finally reading the amplified droplets using a droplet reader.

Figure 2: Simplex and duplex assay results, including annealing temperature optimization. (**A**) Simplex assay results when a single target (RBD2) was FAM labeled. (**B**) Simplex assay results when a single target (RPP30) was HEX labeled. (**C**) Duplex assay result of two targets in 1D and 2D (iii) Channels after N (i) was FAM labeled and RPP30 (ii) was HEX labeled. (**D**) Annealing temperature gradient (65 ^{o}C to 55 ^{o}C) results of a duplex assay labeled with ORF1ab (FAM) and RPP30 (HEX).

Figure 3: Triplex probe mix and quadruplex amplitude-based multiplex assay results. (**A**) Triplex probe mix assay results when three targets were labeled with the following ratios of FAM:HEX; RBD2 (1:0), N (0.5:0.5), and RPP30 (0:1). (**B**) Quadruplex amplitude-based assay results after four targets were labeled with the following ratios of FAM:HEX; RBD2(0.5:0), N (1:0), RPP30 (0:0.5), and ORF1ab (0:1). 1 and 0.5 are probe concentrations 250 nM and 125 nM, respectively.

SUPPLEMENTARY FILES:

Supplementary Figure 1: Droplet generation using an automated droplet generator. (**A**) Consumables needed to set up the AutoDG. (**B**) On the AutoDG touch screen, touch **Configure Sample Plate** and select columns where samples are located on the sample plate and press **OK**.

(**C**) Once selected, the screen turns yellow indicating where consumables should be loaded. (**D**) Open the AutoDG door and load consumables in the respective places. Load consumables from the back working toward the front. Make sure each time a consumable is added the light turns from yellow to green at that location. (**E**) Ensure the oil type used is oil for probes. (**F**) Close the AutoDG door and ensure all reagents are set in place by checking whether the AutoDG touch screen is green. (**G**) Press **START Droplet Generation** to generate droplets. (**H**) After droplet generation is complete, open the AutoDG and remove the droplet plate. (**I**) Seal the droplet plate using a pierceable foil heat seal.

Supplementary Figure 2: Steps on analysis of amplitude-based multiplex ddPCR assay results using an external software. (**A**) Install the external software on a computer. (**B**) Open the .qlp file by either right clicking on it and choosing **Open** with the installed external software or opening the external software and clicking on the **Browse** option to locate the file in your folder. Alternatively, one can drag the .qlp file and drop it the open external software to open it. (**C**) Once open, on the right side of the **Plate Editor** tab, choose probe mix triplex from the drop-down menu and assign target information accordingly, and click on **Apply**. (**D**) On the left side of the 2D amplitude tab, use the **Graph Tools** to assign specific colors to different targets for detection and quantification. Once droplet cluster targets are identified, the quantification results can be seen on the **Well Data** window in the same 2D amplitude tab.

Supplementary Figure 3: Steps on analysis of amplitude based multiplex ddPCR assay results using an external software. (**A**) Install the external software on a computer. (**B**) Open the .qlp file by either right clicking on it and choosing **Open** with the installed external software, or opening the external software and clicking on the **Browse** option to locate the file in your folder. Alternatively, one can drag the .qlp file and drop it in the already open external software to open it. (**C**) Once open, on the right side of the **Plate Editor** tab, choose amplitude multiplex from the drop-down menu and assign target information accordingly, and click on **Apply**. (**D**) On the left side of the 2D amplitude tab, use the **Graph Tools** to assign specific colors to different targets for detection and quantification. Once droplet cluster targets are identified, the quantification results can be seen on the **Well Data** window in the same 2D amplitude tab.

DISCUSSION:

Few resources are available on how to develop RT-ddPCR assays for SARS-CoV-2 detection. Though not used in this article, standard samples with known copies may be used to develop and optimize assays. In this work however, SARS-CoV-2 samples grown in Vero-E6 cells were spiked in a background of human genomic RNA and used as standard samples to develop the assays. Proper primer and probe sequences are essential when developing assays. Since most preliminary work on SARS-CoV-2 RT-ddPCR used the China CDC primer and probes targeting the ORF1ab and N gene, they had found them fit to be included in this work[7–11]. The analytical specificity and sensitivity of these primers have also been compared using ddPCR in the previous work[7]. The RBD2 primers and probe[13] were developed in-house and found fit for RT-ddPCR. Since the assays may be used for different applications, including diagnosis, the Ribonuclease P protein subunit p30 (RPP30) specific human gene was included in all multiplex assays. These gene can be used as a human endogenous control for diagnostic experiments. However, in the case of

environmental sampling or other research that do not need the human reference gene, one may alternate this target with another SARS-CoV-2 target.

In all assays, it is important to include controls to validate assays and experimental data. These controls may include: NTC (nuclease free water as sample) to help in setting thresholds and locating negative droplet cluster; positive control (sample with all SARS-CoV-2 targets, including human reference gene) to assess reagent failure, primer and probe integrity, and substantial reverse transcription detection/location of positive droplet targets; and extraction controls (pooled human samples from healthy volunteers or total nucleic acid extracted from a noninfectious cultured human cell) to detect extraction step failure or success.

Most ddPCR systems, including the QX200 system have a narrow dynamic range from 1 to 120,000 copies/20 µL reaction. When detecting unknow samples, the target concentration in the starting sample is often unknown and this may pose challenges when quantifying highly concentrated samples. To overcome this, it is recommended that when quantifying samples suspected to contain high amounts of target molecules (such as cell culture), one should plan to reduce the starting sample accordingly. In the case where the target copy number/genome is unknown, one should determine the optimal starting amount through a series of four tenfold serial dilution of each sample at the expected digital range. By assaying these four points, it is ensured that one of the data points is within the optimal digital range.

Using high primer and low probe concentration is a perquisite of most simplex and duplex ddPCR experiments. This difference in concentration increases the amplitude separation distance between the positive and negative droplet clusters, hence making it easy to analyze data. However, when developing higher order multiplex assays, changes in these concentrations may lead to changes in the position of positive droplet targets as shown in **Figures 3 and 4**. As a result, one assay optimization option apart from annealing temperature would be to change target primer or probe concentration to distinguish droplets. This phenomenon has been used and explained before[14–16].

Despite the assay performance, this work is a two-step RT-ddPCR workflow. The extra reverse transcription step before ddPCR, gives room for contamination of samples. However, if careful and proper sample handling techniques are used, this will be a non-issue. Positively, DNA is known to be more stable than RNA. The conversion of RNA to cDNA may extend the sample's shelf life during storage as compared to RNA. A two-step RT-ddPCR experiment is also cheaper than a one-step RT-ddPCR experiment.

Compared to RT-qPCR, RT-ddPCR is expensive. Hence, considerations should be taken when performing RT-ddPCR. For example, during diagnosis, one may use RT-ddPCR in the case where their samples have low abundant targets. However, there is a possibility that the costs of dPCR instruments and reagents will drop soon, and the technique will be adapted in many laboratories, as it happened in the past with regular PCR and qPCR. Hence, it is important to set protocols such as this for current and future users of dPCR. In conclusion, the developed assays give room for prospective users to vary targets based on their applications. Multiplexing will ensure that one

can efficiently detect many targets within a single sample in a single reaction. So far, this may be the first protocol that gives a full detail on how to use the AutoDG system, including the external software in SARS-CoV-2 detection. More work on assay optimization still needs to be done to achieve better separation in the quadruplex assay. The use of standards will also help improve the developed assays.

ACKNOWLEDGMENTS:

This research was funded by Megaproject of Infectious Disease Control from Ministry of Health of China, grant number 2017ZX10302301-005 and Sino-Africa Joint Research Center, grant number SAJC201605.

DISCLOSURES:

The authors have no conflicts to disclose.

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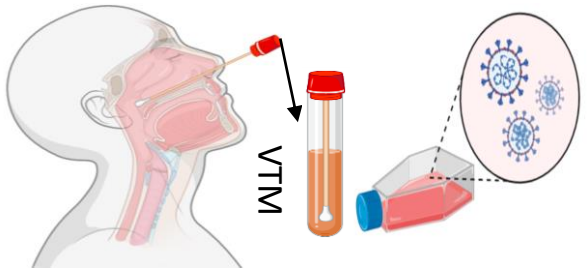
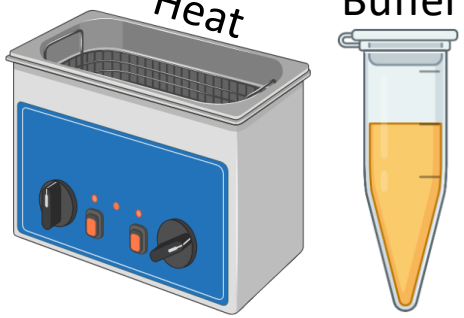
Figure 1

A)

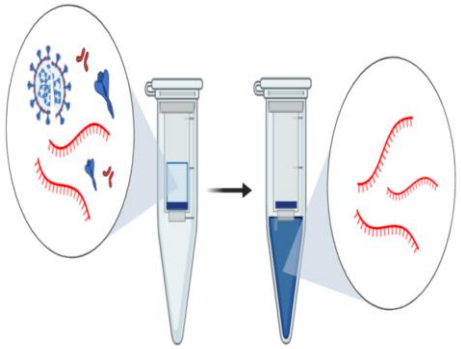
Collect samples and transport to BSL-2



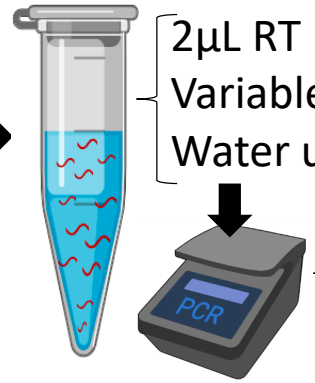
Lyse or inactivate samples



Extract RNA

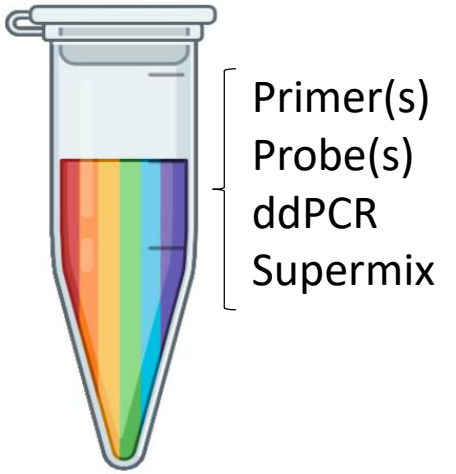


Generate cDNA

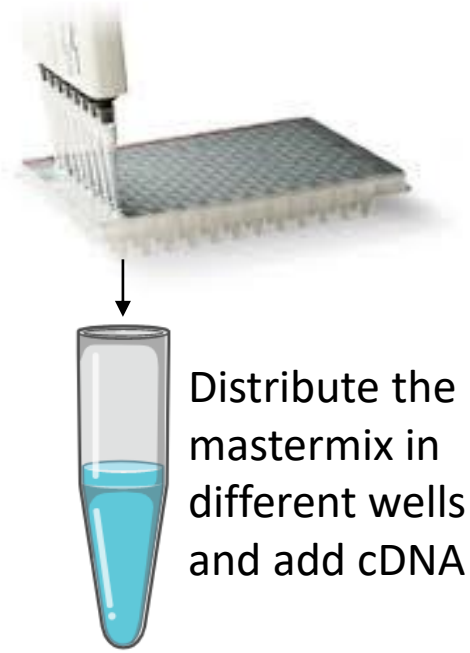


B)

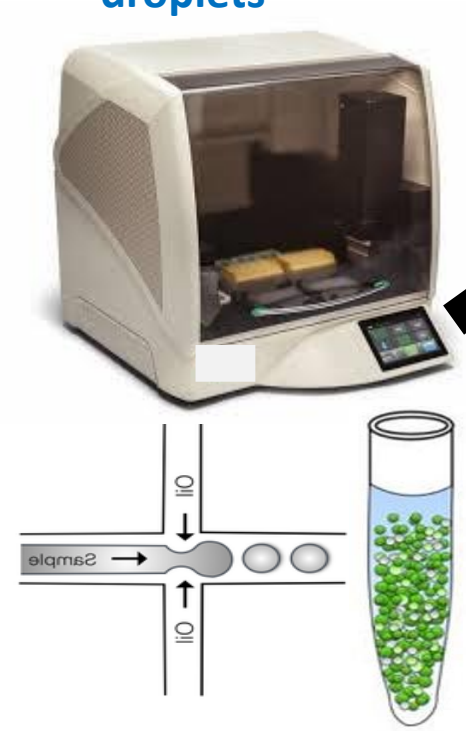
Prepare ddPCR ready Mastermix



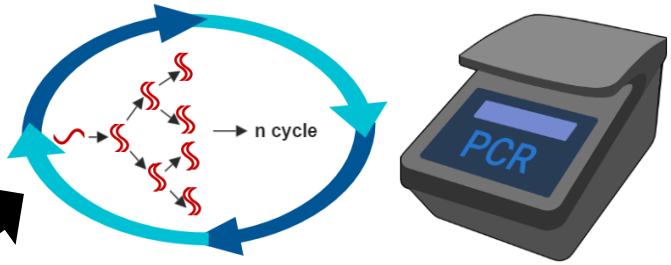
Prepare ddPCR reaction mix



Generate droplets



Amplify droplet targets by PCR



Read amplified droplet targets

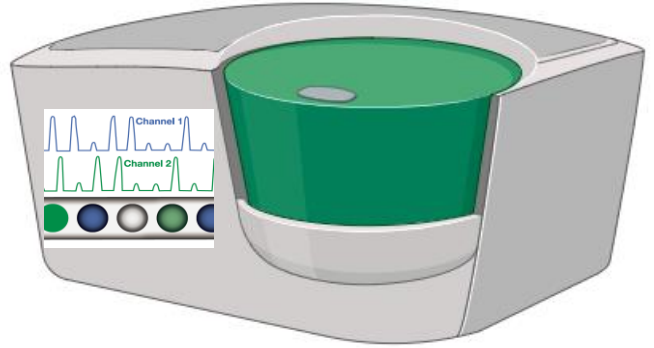
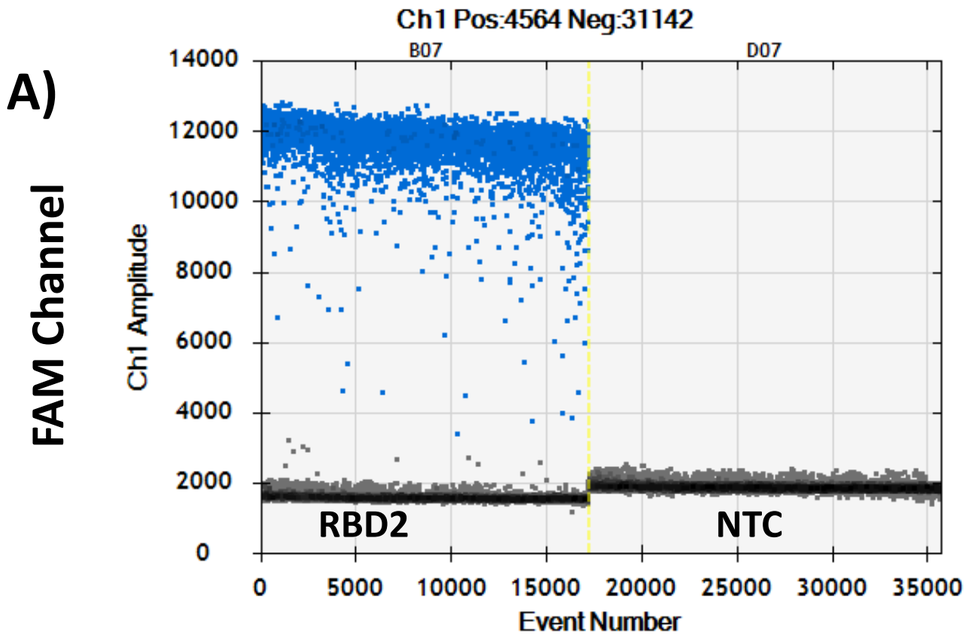
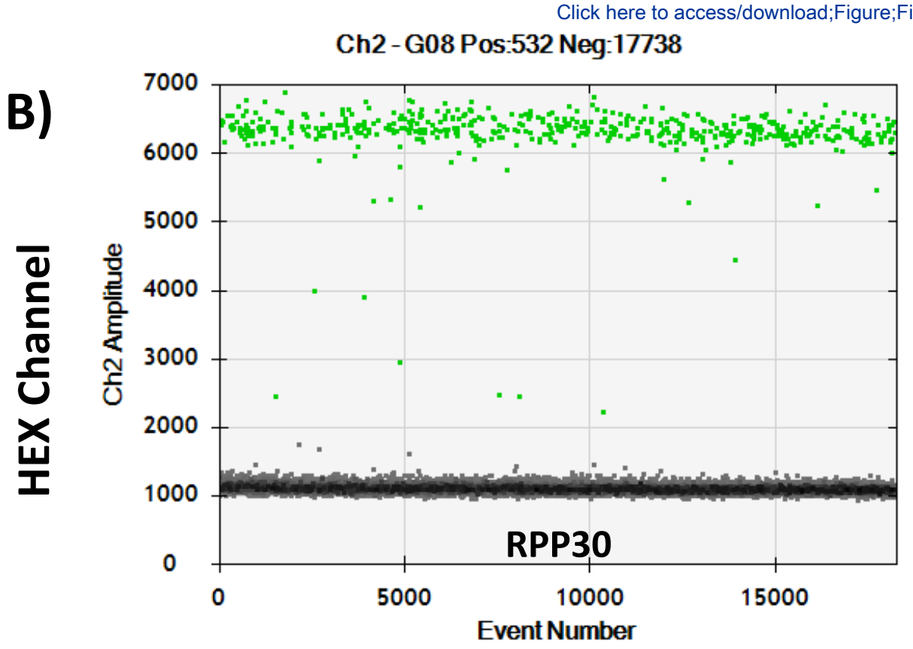
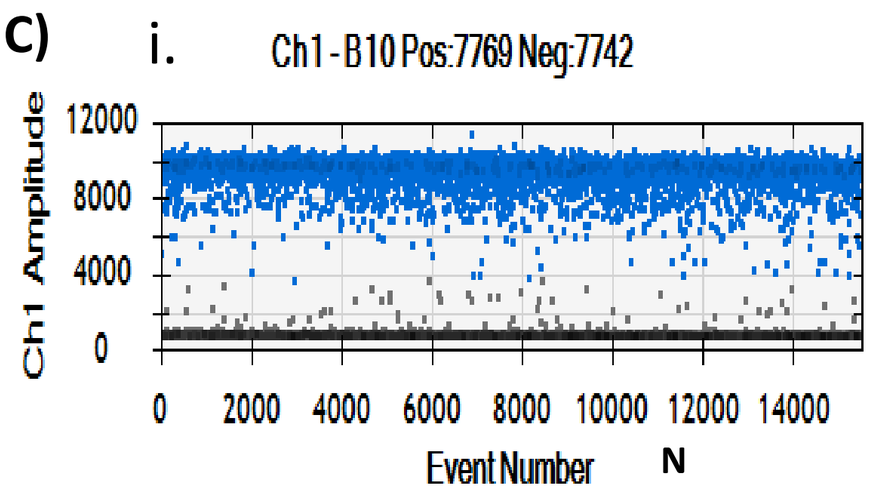


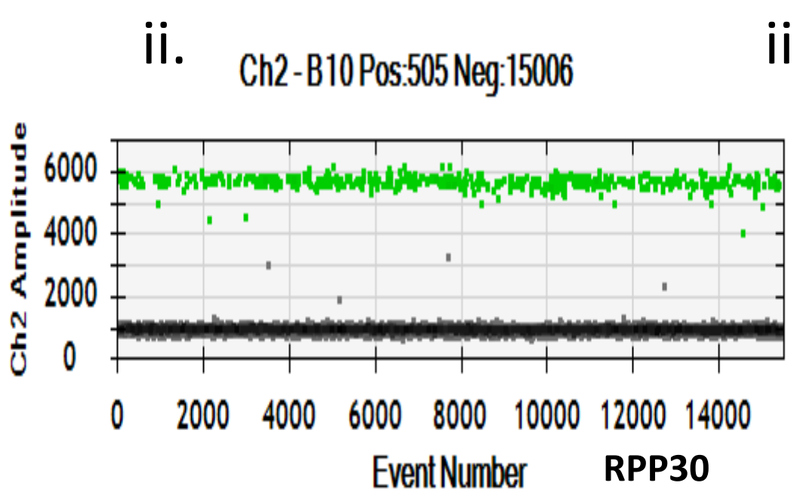
Figure 2

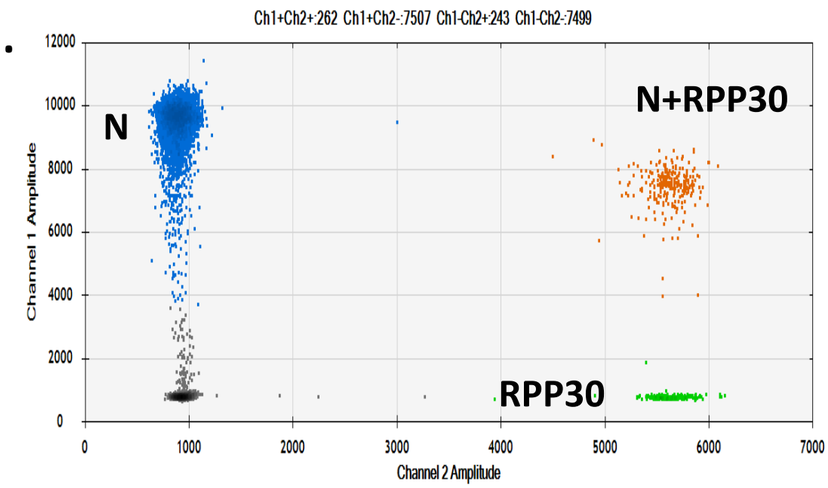
Click here to access/download;Figure;Figure 2 simplex and duplex assay.pdf

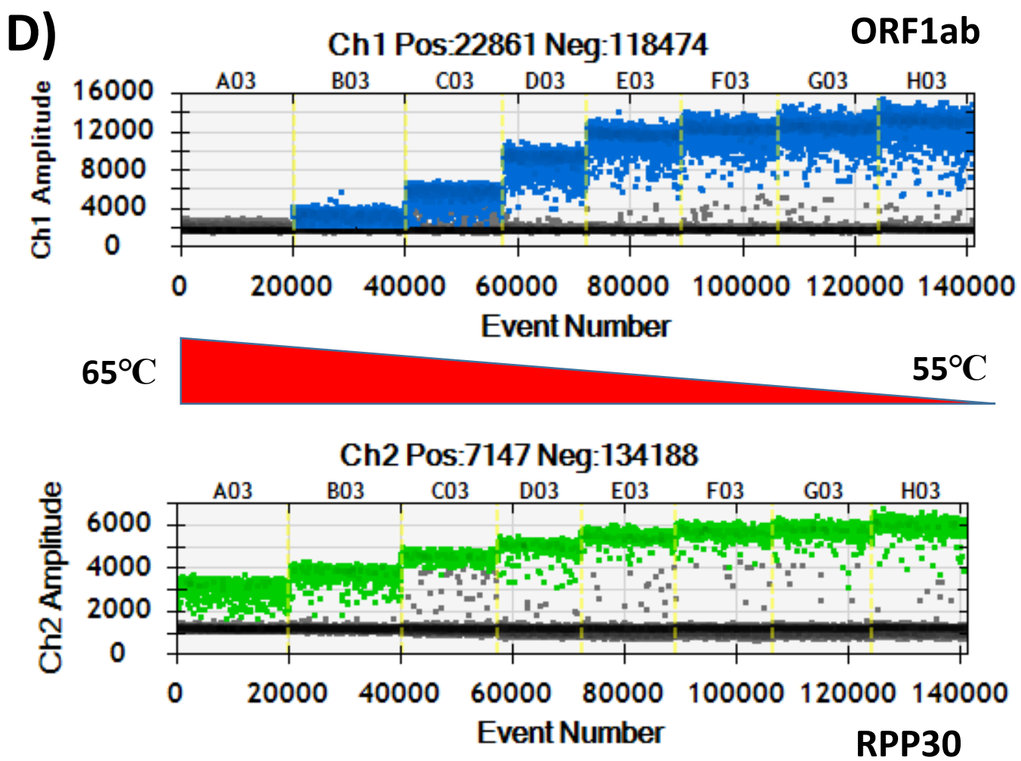












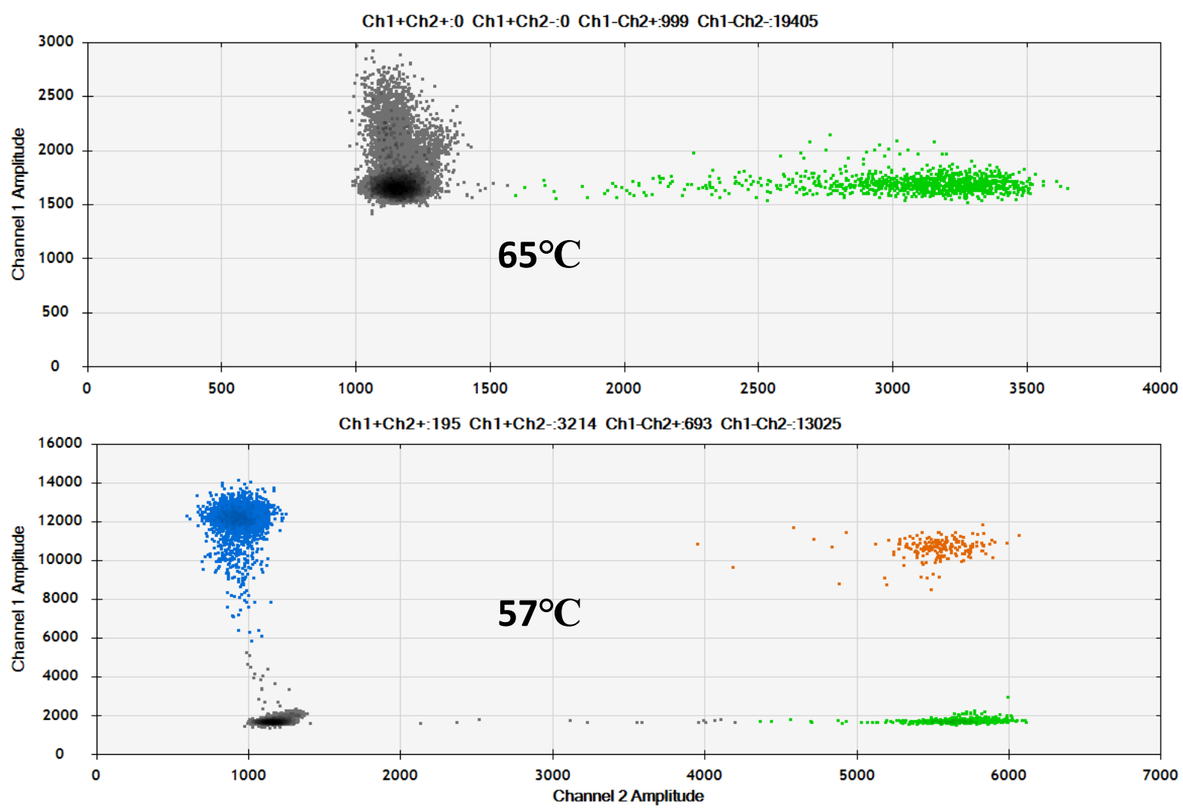
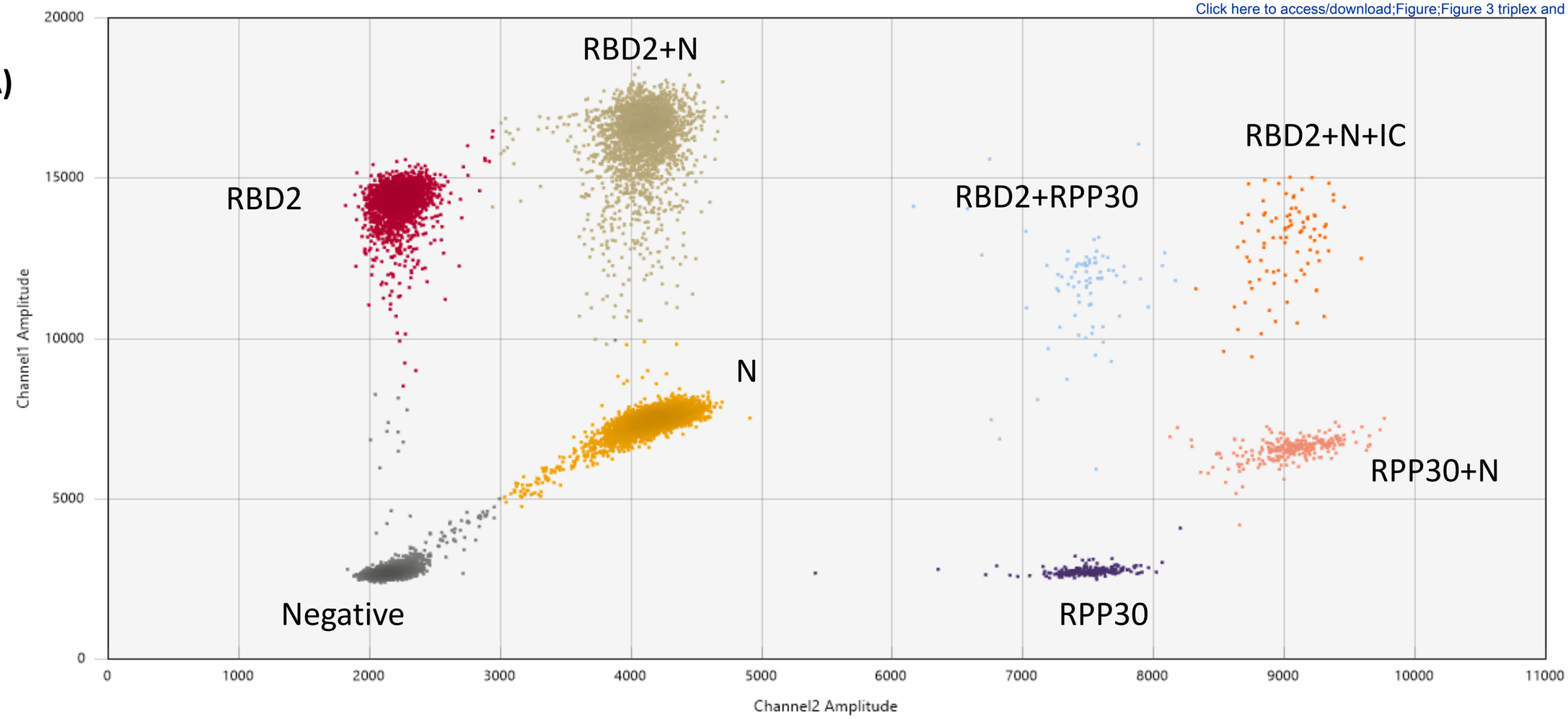


Figure 3

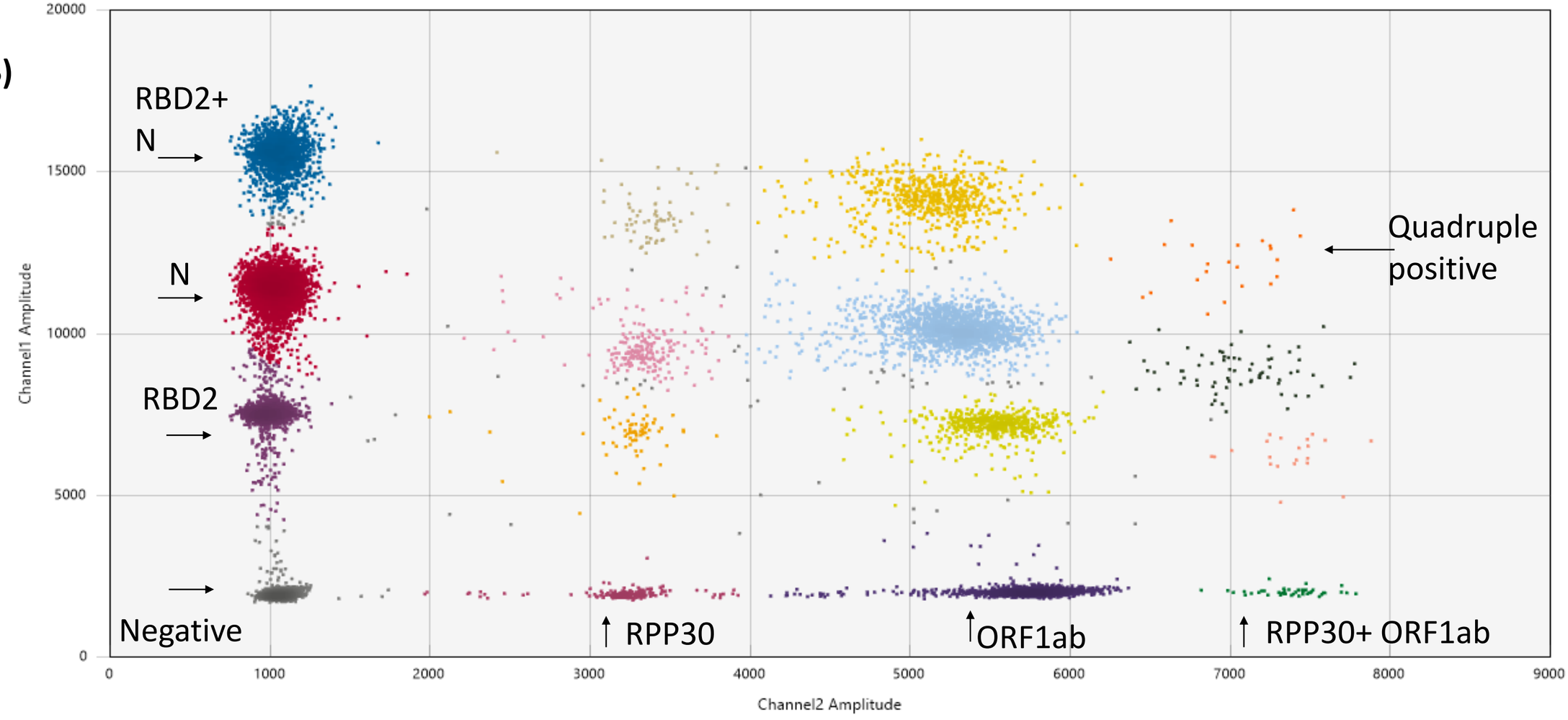
Click here to access/download;Figure;Figure 3 triplex and fourplex assay.pdf

FAM

A)



B)



HEX

Table 1

Primer and probe sequence used in developing various assays

Target		Sequence 5’ to 3’	Probe dye(s)
ORF1ab	Forward	CCCTGTGGGTTTTACACTTAA	5’- FAM and BHQ1-3’
	Reverse	ACGATTGTGCATCAGCTGA	5’- HEX and BHQ1-3’
	Probe	CCGTCTGCGGTATGTGGAAAGGTTATGG	
N	Forward	GGGGAACTTCTCCTGCTAGAAT	5’- FAM and BHQ1-3’
	Reverse	CAGACATTTTGCTCTCAAGCTG	5’- HEX and BHQ1-3’
	Probe	TTGCTGCTGCTTGACAGATT	
RPP30	Forward	AGTGCATGCTTATCTCTGACAG	
	Reverse	GCAGGGCTATAGACAAGTTCA	5’- HEX and BHQ1-3’
	Probe	TTTCCTGTGAAGGCG ATTGACCGA	
RBD2	Forward	CTCAAGTGTCTGTGGATCACG	
	Reverse	CCTGTGCCTGTTAAACCATTG	5’- FAM and BHQ1-3’
	Probe	ACAGCATCAGTAGTGTCAGCAATGTCTC	

Product length (bp)	Ref
119	[16]
99	[16]
87	[8]
121	[17]

Table 2

Click here to access/download;Table;Table 2. Final primer and probe concentration per assay.xlsx

Target	Primer/probe	Final concentration of prime	
		Simplex[b]	Duplex[c]
ORF1ab	ORF1ab F		
	ORF1ab R		
	ORF1ab FAM		
	ORF1ab HEX		
N	N F		800
	N R		800
	N FAM		250
	N HEX		
RPP30	RPP30 F	800	800
	RPP30 R	800	800
	RPP30 FAM		
	RPP30 HEX	250	250
RBD2	RBD2 F	800	
	RBD2 R	800	
	RBD2 FAM	250	
	RBD2 HEX		

[a] In all assays, the targets can be interchanged based on the users preference. The used targets are for der

[b] Only one target can be detectected at a time in the simplex assay in either the FAM or HEX channel. RBD

[c] Two target can be detectected at a time in the duplex assay in the FAM and HEX channel.

[d] Three targets can be detected using both channels i.e target 1 will be detected in FAM (1× probe concer

[e] Two targets are detected in each channel (FAM and HEX) with 1× and O.5× probe concentration in each

er-probe per assay in nM [a]		
	Triplex probe mix[d]	Fourplex amplitude[e]
		800
		800
		250
800		800
800		800
125		250
125		
800		800
800		800
250		125
800		800
800		800
250		125

monstration purposes of this experiment.
2 is used to demonstrate FAM channel results w

ntration), target two in HEX (1× probe concentra
channel.

Material list

Click here to access/download;Table of Materials;JoVE_Materials.xls

Name of Material/ Equipment	Company	Catalog Number
32-channel fully automatic nucleic acid extractor Purifier 32	Genfine Biotech	FHT101-32
AutoDG Oil for Probes	BioRad	12003017
ddPCR 96-Well Plates	BioRad	12003185
ddPCR Supermix for Probes (No dUTP)	BioRad	1863024
DG32 AutoDG Cartridges	BioRad	1864108
Electronic thermostatic water bath pot	Beijing Changfeng Instrument and Meter	XMTD-8000
FineMag Rapid Bead Virus DNA/RNA Extraction Kit	Genfine Biotech	FMY502T5
Pierceable Foil Heat Seals	BioRad	1814040
Pipet Tips for the AutoDG	BioRad	1864120
Pipet Tip Waste Bins for the AutoDG	BioRad	1864125
PrimeScript RT Master Mix (Perfect Real Time)	TaKaRa	RR036A
PX1 PCR Plate Sealer	BioRad	1814000
QuantaSoft 1.7 Software	BioRad	10026368
QuantaSoft Analysis Pro 1.0	BioRad	N/A
QX200 Automated Droplet Gererator (AutoDG)	BioRad	1864101
QX200 Droplet Reader	BioRad	1864003
T100 Thermal Cycler	BioRad	1861096

Comments/Description

Automated extractor for RNA
QX200 AutoDG consumable

Making ddPCR assay mastermix
QX200 AutoDG consumable

Heat inactivation of samples

Magnetic bead extraction of inactivated RNA samples

QX200 AutoDG consumable
QX200 AutoDG consumable

cDNA generation
Seal the droplet plate from AutoDG
Data acquisition and analysis
Data analysis

QX200 AutoDG consumable
Droplet reading and data acquisition
Droplet target amplification (PCR) and cDNA generation

Dear Editor

On behalf of all the authors, we thank you for the comments and review suggestions. We have revised the manuscript to address questions raised by the reviewers and editorial team. Of note, the manuscript contribution was more of a protocol based article and not a discursive article as most of the work has been discussed in our proof of concept paper published recently https://www.tandfonline.com/doi/full/10.1080/14737159.2021.1865807. We hope that based on this revision and corrections, the current manuscript will be fit for publication. Bellow is a point by point response to the concerns raised by reviewers and editorial team.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: All the authors have gone through the article and proofread the manuscript keenly. We hope that the revised manuscript will be fit for publication. However, we also welcome any further suggestions and corrections as some details may be missed owing to the bulk nature of the comments and review questions.

2. Please provide an institutional email address for each author.

Response: Most of the authors are students and are not given institutional addresses. Hence, this may not be necessary at the moment.

3. Please revise the following lines to avoid previously published work: 40-41.

Response: These lines have been revised accordingly.

4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Response: The text has been revised to avoid any personal pronouns.

5. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials: e.g., GenFine's FineMag Rapid Magnetic Bead Extraction Kit, TakaRa PrimeScript RT Master Mix, Biorad QX200 Automated Droplet Generator (AutoDG), QuantaSoft, etc. We must maintain our scientific integrity and prevent the subsequent video from becoming a commercial advertisement.

Response: We apologize for this oversight. Using guidance from previous published literature by JOVE, we have removed the commercial names in the manuscript.

6. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.

Response: An ethics statement has been added as suggested.

7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.

Response: The numbering of the protocol has been aadjusted.

8. Line 91: Please include the details of the amount/volume of samples taken.

Response: A volume and accompanying note has been included as suggested.

9. For time units, please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral, throughout the protocol. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks

Response: The text has been revised as suggested.

10. Line 96: Please mention how are the samples stored. Is there any specific sample container used?

Response: No specific storage container is used. A note has been added to explain this. Samples arrive in different containers and samples may be inactivated in VTM tubes if clinical or in 1.5 mL tubes from research.

11. Line 144-146: Please specify the volume of the samples required.

Response: The volumes of sample to be extracted is noted as 200 µL is point 3 and the final elute is indicated as 100µL in the same protocol. Corrections have also been made with regards to the kit protocol.

12. Please include a one line space between each protocol step and highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.

Response: A one-line spacing has been added between each protocol step. Additionally, text to be included in the video has been highlighted in yellow.

13. Please move the Figure and Table Legends to the end of the Representative Results Section.

Response: The Figure and Table Legends have been moved as suggested.

14. Please ensure that the corresponding reference numbers appear as numbered superscripts after the appropriate statement(s) for in-text formatting. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first

author then et al. Title case and italicize journal titles and book titles. Do not use any abbreviations

Response: The EndNote citation style for JoVE has been used to proof the reference list.

15. Please Rename the Supplementary figures as “Supplementary Figure 1”, “Supplementary Figure 2”, and “Supplementary Figure 3”,

Response: The changes have been made as suggested.

16. Figure 1/ Supplementary Figure 1: Please remove the commercial names from all the panels of the figure (GenFine, BoRad).

Response: The two figures have been modified as suggested.

17. Supplementary Figure 2: Please remove the commercial names from the all the panels of the figure (QuantaSoft).

Response: The changes have been made as suggested.

18. Please sort the Table of Materials in alphabetical order. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

Response: The changes have been made as suggested.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
This study describe how to do a rt-ddPCR for SAR-CoV-2, but it can be virtually used for any other RNA viruses in our virosphere.

Major Concerns:
There is no major concerns

Minor Concerns:
English has to be edited. E.g. ln 22 the steps are elaborate and points to improve the assays and experiment have been noted throughout the article.

Response: We thank the reviewer for noting this. We have restructured the sentence to mean that notes have been provided on how to improve the assays and experiment.

ln 54 from the chamber based to droplet based digital PCR???

Response: We thank the reviewer for this note. We have revised this part to explain the advancement of dPCR where reaction chambers were separated by the wall of a well into

reactions being conducted in the same well using water-in-oil emulsions i.e. droplet based dPCR. A citation has also been added for reference.

It needs more discussion on cost. qPCR is way more cheaper than dPCR or ddPCR per sample. There is only one succinct sentence on it. Maybe authors can discuss that in a few years from now dPCR machines will be cheaper, and the technique will be more extended in other labs and reagents cost will probably drop, as it happened in the past with regular PCR and qPCR.

Response: We agree and thank the reviewer for this suggestion. In our past articles we also noted this point. We have added the reviewers' suggestion in the discussion part of the manuscript as follows "However, there is a possibility that the costs of dPCR instruments and reagents will drop in the near future, and the technique will be adapted in many laboratories, as it happened in the past with regular PCR and qPCR. Hence, it is important to set protocols like this for current and future users of dPCR."

Further and expanded discussion in discussion section is needed to explain how to get a proper dilution of samples with unknown amount template. Authors know that it is important to have the template diluted to an acceptable range of number of copies within Poisson distribution. A priori, this is not known for most of biological samples. A recommendation on how to proceed should be given to readers.

Response: This is an important note by the reviewer. We have added this paragraph "Most ddPCR systems including the QX200 system have a narrow dynamic range from 1 to 120,000 copies/20 µL reaction. When detecting unknow samples, the target concentration in the starting sample is often unknow and this may pose challenges when quantify highly concentrated samples. To overcome this, it is recommended that when quantifying samples suspected to contain high amounts of target molecules (like cell culture), one should plan to reduce the starting sample accordingly. In the case where the target copy number/genome is unknown; one should determine the optimal starting amount through a series of four tenfold serial dilution of each sample at the expected digital range. By assaying these four points, it is ensured that one of the data points is within the optimal digital range." to address this note.

Poison is wrong written. it should be Poisson

Response: This change has been made as suggested.

It should be discussed other types of digital PCR approaches, such as the one based on chip well digital PCR assays. See for instance the recent viral application published by McMullen and Martinez-Garcia in Environmental Microbiology Reports ("Absolute quantification of infecting viral particles by chip-based digital polymerase chain reaction"). Thus, I miss a paragraph discussing and comparing other existing digital PCR assays.

Response: We appreciate the reviewers' comments and suggestion. However, since this article is mostly protocol based and not a discursive article, we saw and still see it not fit to compare

these approaches but rather state the one used presently. However, we do agree there is merit in doing so and we are currently working on a review article on the same.

Reviewer #2:

Manuscript Summary:

- The authors describe a detailed protocol for developing a two step protocol for reverse transcription and ddPCR detection of SARS-CoV-2. The protocol is largely a replication of the BioRad ddPCR protocol and I am not sure about its innovation. In addition, the protocols as discussed, replicated methods for SARS-CoV-2 qualitative detection without a discussion on how to use the assay for quantification. Using multiple genes will give three separate numbers of copies per target, so an explanation of how can a quantity be concluded is essential. The intense work-flow of ddPCR challenge its clinical implementation. SARS-CoV-2 diagnostics based on ddPCR didn't show better analytical sensitivity if compared to many available RT-PCR methods. Some clarifications and suggestions are below.

Major Concerns:

- The authors are using the BioRad droplet generator and analysis hard and software. BioRad has an FDA EUA assay for SARS-CoV-2 detection using the same methodology but as a one-step multiplex assay. Please discuss what the advantage of a one-step versus two steps and discuss the differences between the developed assay versus commercially available assays.

Response: We agree and note that BioRad has their own FDA EUA approved kit using the triplex probe mix assay. However, this kit may not be easily accessible to most labs. Hence, this work shows steps on how to develop similar assays that can be used for multiple applications. Additionally, this work differs from that as it was aimed at showing elaborate steps of not only designing the triplex assay but also other assays. The users of this work can use their own primers to design similar assays, something that is not explained in the commercial assays. Furthermore, this work can be adapted for different pathogens in our globe and may serve as a reference point for future applications. An extra paragraph has also been added in the representative results section on how to calculate the concentration.

As for the advantages of the two-step protocol versus one step; during the epidemic in Wuhan, we did not have the one-step kit at hand and only the supermix for probes as our previous work was mostly DNA based. Some of the original works on ddPCR to detect SARS-CoV-2 also used a two-step approach though not multiplexed (T Suo et.al https://doi.org/10.1080/22221751.2020.1772678) and these works showed that ddPCR was more sensitive than RT-PCR. Some of the advantages have been highlighted in the discussion whereby we noted that a two-step protocol is cheaper and the generated cDNA can be stored for a longer period. Additionally, we are currently working to compare the merits and demerits of a two-step RT-ddPCR protocol compared to that of a one-step RT-ddPCR protocol.

Reviewer #3:

Manuscript Summary:

Nyaruaba and colleagues presented an interesting and detailed ddPCR protocol for the

quantification of SARS-CoV-2. They described workflow, development of simplex, duplex, triplex and fourplex probes and analysis of the results.
Overall, the manuscript is well written. Here are some comments that could improve the quality of the manuscript:

Minor Concerns:
1) Introduction (line 56): correct "poison" to "poisson"

Response: We thank the reviewer for this comment. The change has been made as suggested.

2) Introduction (line 59-60 and 62-64): In support of the following sentences "SARS-CoV-2 is no exception to these applications" and "However, RT-d(d)PCR has been shown to be more accurate in detecting low abundant SARS-CoV-2 targets from both environmental and clinical samples when compared to RT-qPCR" is a recently published case report: PMID: 33238410 PMCID: PMC7700357 DOI: 10.3390 / life10110302

Response: This resource has been added to support the mentioned texts.

3) Introduction (line 62): change "RT-d(d)PCR" to "RT-ddPCR"

Response: We thank the reviewer for this comment. The change has been made as suggested.

4) Protocol (line 88): Define PPE

Response: The definition has been provided as suggested.

5) Protocol (line 96): "Store samples in 4 ℃ if not processed immediately." Define how long samples can be stored at +4°C.

Response: We have modified the sentence o indicate the sample can be stored in 4 ℃ for up to 24 h if not processed immediately.

6) Protocol (line 195-197): "NOTE: For the sample plate… 22 µL nuclease free water each". The instructions on the biorad droplet generator recommend filling the wells of "individual cartridges" with buffer control. Why not when using the plates?

Response: We agree with the reviewer that this is the proper recommendation when using the droplet generator system whether manual or when using the AutoDG system. However, from our experience, it makes no difference when one uses nuclease free water to fill the column as opposed to the buffer. At times one doesn't have the buffer hence they can substitute it with nuclease free water. We have revised the text to also include the buffer control thanks to the reviewer's observation.

7) Protocol (line 198): "…add 2.2 µL cDNA sample per well with Mastermix". Usually, ddPCR

protocols recommend adding 5 µL of sample (DNA / RNA), why do you recommend 2.2 µL of cDNA?

Response: We thank the reviewer for this observation. In our system we often found that after generating cDNA, 2.2 µL of the cDNA was enough for detection. Using higher amounts may result to many positive droplets if the initial sample had many copies of SARS-CoV-2. However, we agree that adjusting the volume to higher amounts e.g. 5.5 µL (due to 22 µL final vol) will be essential when detecting samples with low abundant SARS-CoV-2 targets. We have included this statement “Additionally, in the cases of lowly concentrated RNA samples, the sample volume can be adjusted up to 5.5 µL.” to support this fact.

8) Protocol (line 214): correct "catridges" to "Cartridges"

Response: We thank the reviewer for this observation. The correction been made as suggested.

9) References: reference 1 is the same as reference 6. To be corrected

Response: We thank the reviewer for this observation. The correction been made as suggested.

10) Table 1: it would be interesting to add a column showing the size of the analysed fragment

Response: The product size of the amplified fragment is added to table 1.

11) Table 2: Improve the structure and formatting of the table 2 and notes

Response: The notes and table have been left the same awaiting editorial processing instructions.

12) Table 2: correct note d

Response: Note [d] has been corrected and modified to be clearer.

13) Figure 3: in Figure 3B, the abbreviation IC indicates the quantification of the "RPP30" gene? if so, replace in the IC graph with RPP30

Response: Indeed, IC here refers to the quantification of the "RPP30" gene. The correction been made in figure 3 as suggested.

14) Figure S2: improve the resolution of figure s2

Response: The resolution of figure s2 has been improved as suggested.

15) Legend Figure S2 (line 488): remove double ")" in letter C

Response: This correction has been made as suggested.

16) Legend Figure S3: correct "Quanatasoft" to "QuantaSoft"

Response: We thank the reviewer for this observation. This correction has been made here and throughout the manuscript.

Reviewer #4:
This manuscript has described a two-step reverse transcription droplet digital PCR protocols for SARS-CoV-2 quantification. This work also developed simplex, duplex, triplex probe mix, and fourplex assays using primers and probes targeting different sites of the SARS-CoV-2 genome (N, ORF1ab, RPP30, and RBD2). It provides a detailed protocol including sample handling, ddPCR operation and data analysis. It requires some minor modification before it can be accepted for publication. Here are some comments to improve the protocol:

1) "1. Sample inactivation":
1-1) Please specify sample types: NP swab? saliva? All kinds of samples can be deactivated by the same heating protocol? Provide citation here to show that 65C 30min heating is good enough to heat-inactivate the samples.

Response: We thank the reviewer for the above questions. In our work, we have used this protocol for all kinds of samples. Our in-house elevations revealed that this temperature had minimal effects on RNA (data not published yet). In prior literature e.g. “Inactivation of coronaviruses by heat- doi: 10.1016/j.jhin.2020.03.025” it has been shown that heating at the same temperature at equal heat for about 15 mins is sufficient to inactivate the sample. However, in our work we use 30 mins. Additionally, for inactivation, we have also noted that one can use their lab preferred sample inactivation method in note 1.1.1.

1-2) The concentration and volume of the sample used in this paper should be specified.

Response: The sample volume and concentration of sample cannot be specified as when collecting samples, the sample volume is often not constant as samples come from various sources. However, in this article the SARS-CoV-2 samples used were mostly 1ml of which only 200µL was needed for extraction. As for the concentration, we noted that due to the absence of a SARS-CoV-2 standard sample, the starting amount or concentration was unknown as seen in the beginning of the discussion section. Additionally, we have also highlighted how to deal with unknown sample concentrations in line 380-387 of the discussion section.

1-3) "let them stand for 10 minutes to allow potential aerosols to settle." Samples are kept at room temperature or on ice?

Response: Inside the BSC, the sample are let to stand at room temperature but if not processed immediately they are stored at 4 °C for up to 24 h. We have added room temperature at this section to address this. In our tests also, when having multiple samples, no much difference was observed after leaving inactivated samples at room temperature for 1~2 h before extraction.

1-4) "Store samples in 4 °C if not processed immediately." How long do the samples last in 4C, especially for those low copy number samples?

Response: During most of our work, after inactivation, samples are processed within the same day (24 h) even if they have low copy number. We have included this time in the section.

2) "2. RNA extraction":
2-1) It's unclear how much original patient samples are used for RNA extraction and how much elutes are yielded.

Response: In point 3 of the RNA extraction step, we indicate 200 µL sample (not only for patient samples as the sample may be from the environment or other research work) and in step 7 of the same protocol, we indicate that ~100 µL elute is yielded after extraction for subsequent experiments.

2-2) "gently shake the orifice plate to concentrate reagent and magnetic beads at the plate's bottom." Why does shaking help concentrate the reagent and beads?

Response: After mixing reagents upside-down, the reagent and beads will be on the walls of the plate and on the plate foil. Hence, gently shaking the plate will help bring these contents at the bottom of the plate. A note has been included to explain this.

2-3) "Add 20 µL and 200 µL sample per well in columns 2 and 8 of the 96-well plate, respectively, and put the 96-well plate on the base of the 32-channel automatic nucleic acid extraction instrument." This step is hard to understand.

Response: We thank the reviewer for this note. We accidentally forgot to indicate the 20 µL belongs to Proteinase K. We have corrected it in the text. Additionally, the text has been broken down into two for ease of clarification as indicated in the kit manual.

3) "3. cDNA generation":
"or store at low temperature till further processing." Please specify at what temperature and for how long?

Response: We have restructured the sentence to indicate storage time at low temperatures.

4) "Common steps in all assays":
"To get the final volume reaction mix (22 µL), add 2.2 µL cDNA sample per well with Mastermix." Please explain why 2.2 ul cDNA is used. Why not using more samples to improve the detection sensitivity?

Response: We thank the reviewer for this observation. In our system we often found that after generating cDNA, 2.2 µL of the cDNA was enough for detection. Using higher amounts may result to many positive droplets if the initial sample had many copies of SARS-CoV-2. However, we agree that adjusting the volume to higher amounts e.g. 5.5 µL (due to 22 µL final vol) will be essential when detecting samples with low abundant SARS-CoV-2 targets. We have included

this statement “Additionally, in the cases of lowly concentrated RNA samples, the sample volume can be adjusted up to 5.5 µL.” to support this fact.

5) "Discussion":
5-1) "SARS-CoV-2 samples grown in Vero-E6 cells were spiked in a background of human genomic RNA": The concentration of the spiked in SARS-CoV-2 sample should be described.

Response: The concentration of the spiked sample was not described in the current literature as it was unknown. However, we took a sample with an equivalent Ct value of 24 to show how the results can be analyzed.

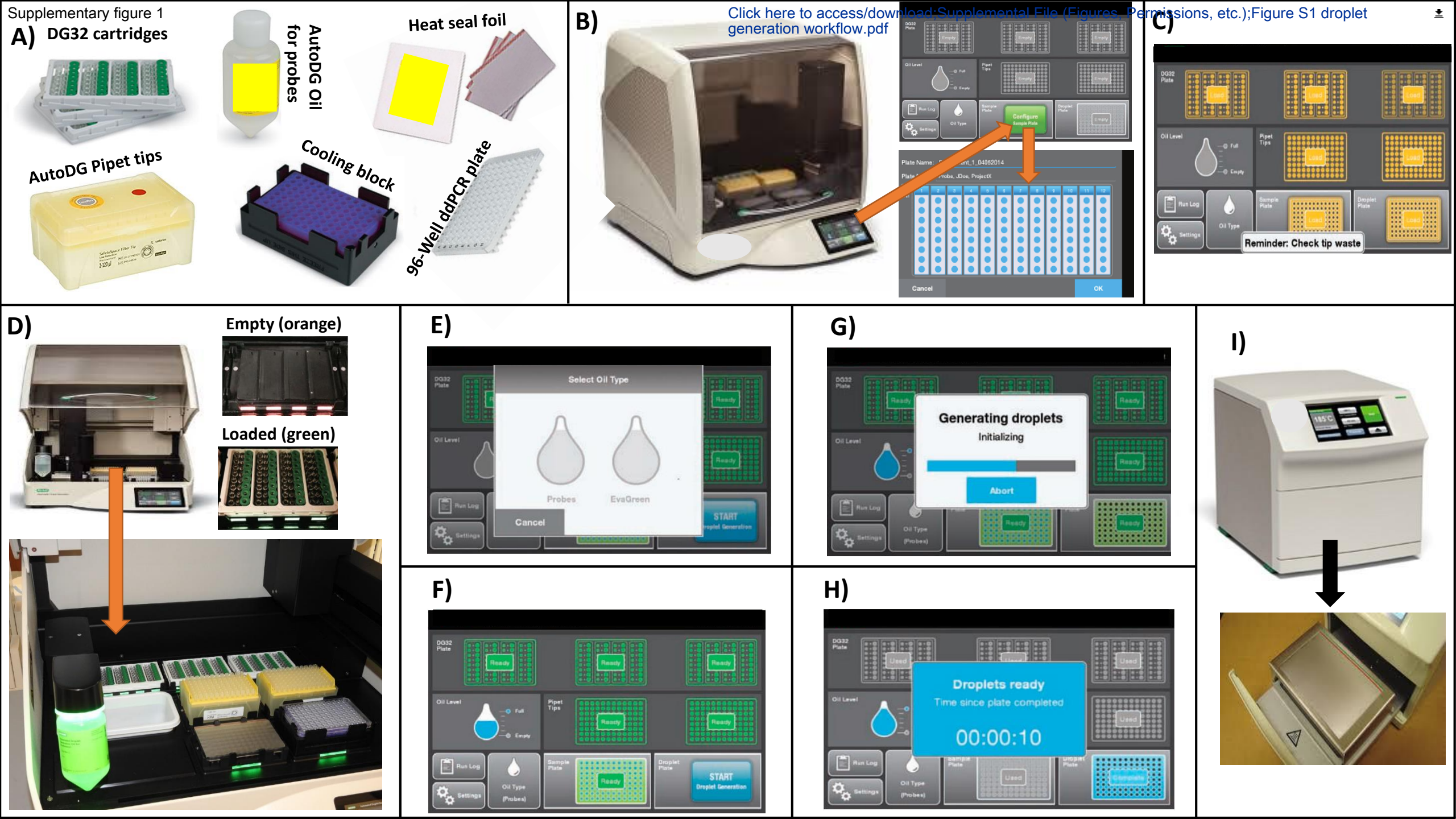
5-2) "A two-step RT-ddPCR experiment is also cheaper than a one-step RT-ddPCR experiment." Please explain why is it cheaper? A two-step workflow will also bring a lot more hands-on time than the one-step assay, which will significantly delay the tests when handling hundreds of samples and increases the labor costs.

Response: When it comes to cost, using BioRad No dUTP mix (two step), a kit for 500 reactions costs 546 dollars which is almost 6× cheaper when using the BioRad one-step advanced kit for probes that retails for 3,071 dollars for 500 reactions. The cDNA used in this work costs 1625 dollars for 800 reactions. Narrowing down to a single reaction will mean that for one sample; the one-step ddPCR test would cost ~6 dollars, and the two-step reaction would cost ~3 dollars. We agree that a two-step protocol adds an extra step which we also noted in our discussion, but, we find this not much of a concern especially if dealing with small number of samples. The one-step time for PCR is approximately 3h compared to the 1h 45min in the two-step protocol, hence, this may not significantly delay test results. We are also working on comparing the two protocols and more data will be made available after the comparisons.

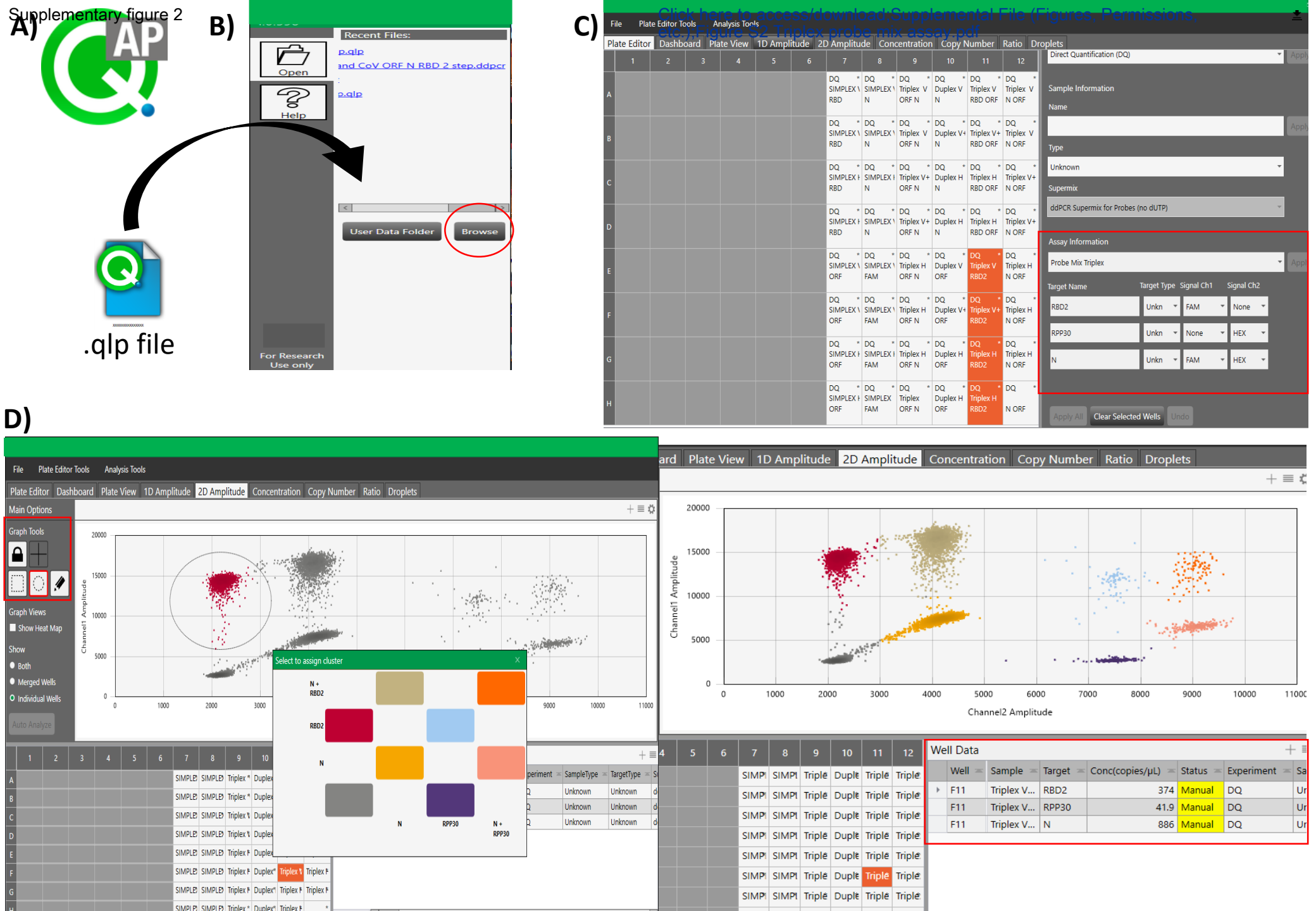
Sincerely

Hongping Wei

Supplementary figure 1



Supplementary figure 2



Supplementary figure 3