Liferiver®

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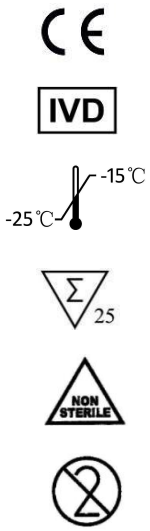
Ebola Virus (EBOV) Real Time RT-PCR Kit

Instructions for Use



For *In Vitro* Diagnostic Use Only

REF QR-0220-02

For use with Bio-Rad CFX 96; SLAN®-96; ABI Prism®7500; LightCycler®480 Instruments

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Table of Contents

Common Information

1 Intended Use

Ebola Virus (EBOV) Real Time RT-PCR Kit is an in vitro diagnostic test for the detection of all high pathogenic members of Ebolavirus: Zaire ebolavirus (ZEBOV), Sudan ebolavirus (SUDV), Taï Forest ebolavirus (TAFV) and Bundibugyo ebolavirus (BDBV) in blood, serum or plasma (non-heparin anticoagulant). It is intended to aid in the diagnosis of Ebola virus disease (EVD), formerly known as Ebola haemorrhagic fever, among symptomatic individuals. The results should be interpreted strictly according to the Interpretation of Results in this manual. Final judgment should be made with combination of other diagnostic methods. This test is based on real time RT-PCR technology utilizing reverse-transcriptase (RT) reaction to convert RNA into complementary DNA (cDNA). This kit is manually operated and to be used with the RNA extraction kits and PCR instruments listed below by trained laboratory personnel who are proficient in performing real-time RT-PCR assay.

2 Principle of Real Time RT-PCR

Real Time reverse transcription polymerase chain reaction (Real Time RT-PCR) is used when the starting material is RNA. In this method, RNA is first transcribed into complementary DNA (cDNA) by reverse transcriptase from total RNA. The cDNA is then used as the template for the Real Time PCR.

PCR is a process for amplifying target DNA sequence with thermophilic DNA polymerase. It involves three steps: melting (denaturing of the DNA duplex at a high temperature to yield single stranded DNA), annealing (primers anneal to the single stranded target sequence) and elongation (DNA polymerase extends the primers by adding dNTPs to the phosphate backbone). These steps complete one PCR cycle, and the cycle repeats until a sufficient DNA concentration is reached.

Real Time PCR is regular PCR with the advantage of detecting the amount of DNA formed after each cycle with a fluorescently-tagged oligonucleotide probe. The probeis complementary to the target sequence being amplified. A fluorophore attached to the 5’ end of the probe and a quencher dye attached to the 3’ end. The fluorescence emitted from fluorophore will be quenched by the quencher dye and so there is no fluorescence can be detected.During each Real Time PCR cycle the probe hybridizes to its target sequence, downstream from a PCR primer. As DNA polymerase extends the primer, it encounters and hydrolyzes the probe from the 5’ end, releasing the fluorophore from the probe. The fluorophore is excited and its emission is no longer quenched and there is an increase in fluorescence which can be detected by Rea-Time PCR instrument and generated quantitative data are collected and analyzed by specialized software.

3 Product Description

Ebola Virus (EBOV) is named after the Ebola River Valley in the Democratic Republic of the Congo (formerly Zaire), which is near the site of the first recognized outbreak in 1976 at a mission hospital run by Flemish nuns. It remained largely obscure until 1989 when several widely publicized outbreaks occurred among monkeys in the United States.

The virus interferes with the endothelial cells lining the interior surface of blood vessels and with coagulation. As the blood vessel walls become damaged and destroyed, the platelets are unable to coagulate, patients succumb to hypovolemic shock. Ebola is transmitted through bodily fluids, while conjunctiva exposure may also lead to transmission.

EBOV Real Time RT-PCR kit contains a specialized ready-to-use one step real time RT-PCR system for the detection of EBOV RNA, which includes reverse transcription (RT) for the transcription of EBOV RNA into cDNA and real time PCR for the amplification and detection cDNA from EBOV RNA.

By the end of 2013, 64 Ebola virus strains have been sequenced:

1) 46 Zaire ebolavirus sequences and GenBank accession numbers are HQ613403 , KF827427, JQ352763, EU224440, AF086833, AY354458, AY142960, AF499101, AF272001, L11365, J04337, HQ613402, JA489037, HC069241, HC069239, HC069235, HC069221, HC069217, HC874661, NC002549, HC874677, HC874681, HC874683, HC874665, HC874667 and HC069223;
2) 14 Sudan ebolavirus sequences and GenBank accession numbers are FJ968794, KC242783, EU338380, JN638998, KC545392, KC545391, KC545390, KC545389, AY729654, KC589025, AF173836, HC918462, HC918459 and HC874655;
3) 2 Taï Forest ebolavirus sequences and GenBank accession numbers are FJ217162 and JA489027;
4) 6 Bundibugyo ebolavirus sequences and GenBank accession numbers are FJ217161, KC545393, KC545394, NC_014373, KC545395 and JA489018.

All sequences above have been covered by the primers and probes in this kit.

The latest Ebola virus sequence update was on October 14, 2014 and 108 sequences were added. All of them were Zaire ebolavirus and GenBank accession numbers are KC242789, KC242788, KC242787, KC242786, KC242784, KC242790, KC242785, KF990214, KF990213, KC242801, KC242799, KC242794, KC242796, KC242792, KC242791, KC242793, KM655246, KC242800, KJ660348, KJ660347, KJ660346, KM233116, KM233115, KM233114, KM233113, KM233112, KM233110, KM233109, KM233107, KM233106, KM233105, KM233104, KM233103, KM233102, KM233101, KM233100, KM233099, KM233098, KM233097, KM233096, KM233095, KM233093, KM233092, KM233091, KM233089, KM233088, KM233085, KM233084, KM233081, KM233080, KM233077, KM233075, KM233074, KM233073, KM233072, KM233070, KM233069, KM233067, KM233066, KM233065, KM233064, KM233063, KM233062, KM233061, KM233057, KM233056, KM233055, KM233054, KM233053, KM233052, KM233051, KM233050, KM233049,

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All sequences above have been covered by the primers and probes in this kit.

A positive control, a negative control and an internal control (IC) are included in this kit to identify false negative results, false positive results, low extraction efficiency and possible PCR inhibitors. Pseudovirus vectors of four species were constructed as positive control, each of which included one partial Ebola virus sequence respectively. The insert sequences are 1-500 nt of Zaire ebolavirus (KJ660347), 1-500 nt of Sudan ebolavirus (EU338380), 1-500 nt of Taï Forest ebolavirus (JA489027) and 1-500 nt of Bundibugyo ebolavirus (FJ217161).

4 ⚠Warnings and Precaution

- Carefully read this instruction before starting the procedure.
- For in vitro diagnostic use only.
- This assay needs to be carried out by skilled personnel.
- Specimens should be regarded as potentially infectious materials and handled in accordance with the guidelines recommended by the World Health Organization (WHO) and Centers for Disease Control and Prevention (CDC) or local national guidelines.
- This assay needs to be run according to Good Laboratory Practice.
- Do not use the kit after its expiration date.
- Avoid repeated freeze-and-thaw of reagents, which may reduce the sensitivity of the test.
- Once the reagents have been thawed, vortex and centrifuge briefly the tubes before use.
- Prepare Master Mix on ice or in cooling block quickly.
- Use separated and segregated working areas for (i) sample preparation, (ii) reaction setup and (iii) amplification/detection activities. The workflow in the laboratory should proceed in unidirectional manner. Always wear disposable gloves in each area and change them before entering a different area.
- Pipets, vials and other working materials should not circulate among working units.
- Use always sterile pipette tips with filters.
- Wear separate coats and gloves in each area.
- Avoid aerosols.

5 Kit Contents

Ref.	Kit Contents	Volume
1	EBOV Super Mix	1 vial, 513μl
2	RT-PCR Enzyme Mix	1 vial, 27μl

3	Internal Control (IC)	1 vial, 30µl
4	EBOV Negative Control	1 vial, 200µl
5	EBOV Positive Control	1 vial, 200µl

Control materials

- EBOV Negative Control is saline that will serve as an external negative specimen during RNA extraction procedure.
- EBOV Positive Control is a pseudovirus containing partial EBOV RNA fragment that will serve as an external positive control during RNA extraction procedure. The EBOV RNA fragment in pseudovirus is designed to cover the target sequence to react with the Real Time RT-PCR reagents in this kit to indicate whether the Real Time RT-PCR worked.
- Internal Control (IC) is a pseudovirus containing non-target RNA fragment that will be added into the specimen before RNA extraction procedure as internal control to evaluate RNA extraction efficiency and identify possible PCR inhibitors. The RNA fragment in peudovirus will be amplified by the primers used to amplify target sequence, but it will be detected by another probe.

6 Storage

- All reagents should be stored at -20±5°C. Storage at +4°C is not recommended.
- All reagents should be used before expiration date indicated on kit.
- Repeated thaw-freeze cycles should be avoided more than 3 times, as this may reduce the sensitivity of the assay.

7 Specimen Requirements

7.1 Whole blood

Take 2ml venous blood with a sterile syringe. The blood is collected in a disposable sterile tube with anticoagulant (EDTA).

7.2 Plasma

Take 2ml venous blood with a sterile syringe. The blood is collected in a disposable sterile anticoagulant tube (EDTA). Separate plasma from blood by centrifugation at 3000rpm for 10 minutes.

7.3 Serum

Take 2ml venous blood with a sterile syringe. The blood is collected in a disposable sterile tube. After coagulation of blood, centrifuge the blood at 3000rpm for 10 minutes

Notes:

Suspected infectious blood, plasma or serum must be manipulated at a BSL-4 laboratory before virus inactivation. Four commercial kits are recommended for EBOV RNA extraction, and related inactivation is listed as follows.

Volume of specimen	Treatment	Kits	Cat.No.
200μl	Mix with 25μl QIAGEN Protease (QP), and then add 200 μl Buffer AL (containing 28 μg/ml of carrier RNA). （See 8.2.1 step1-3）	QIAamp DSP Virus Spin Kit (Qiagen)	61704
140μl	Mix with 560μl Buffer AVL–carrier RNA. （See 8.2.2 step1-2）	QIAamp Viral RNA Mini Kit (Qiagen)	52904/ 52906
140μl	Mix with 526μl Binding solution (See 8.2.3 step1-3)	RNA Isolation Kit (Paramagnetic Beads Column) (Liferiver)	ME-0010
200μl	Pipette into a row A well of Preparing Plate (containing 500μl binding buffer, 6μl Carrier RNA and 20μl RNA binding Beads) (See 8.2.4 step1-3)	Ribonucleic Acid (RNA) Isolation Kit (Preloaded for Auto-Extraction) (Liferiver)	ME-0014

It is necessary to use a biosafety cabinet to open blood collection tubes with specimens and extract nucleic acid with the commercial kits mentioned above at a BSL-3 laboratory or BSL-2 laboratory. All the inactivated specimens can be stored at 2-8 ℃ for 72 hours. The samples must be stored at -70℃ for long term storage. Additionally, multiple freeze/thaw should be avoided.

Inactivated specimens should still be considered as biohazard materials.

8 RNA Extraction Procedure for Specimens and Controls

The nucleic acid extraction reagents are not supplied in this kit and four commerical kits are recommended for EBOV RNA extraction:

1) QIAamp DSP Virus Spin Kit (Qiagen, Cat. No. 61704);
2) QIAamp Viral RNA Mini Kit (Qiagen, Cat. No. 52904 or 52906);
3) RNA Isolation Kit (Paramagnetic Beads Column) (Liferiver, Cat. No. ME-0010);
4) Ribonucleic Acid (RNA) Isolation Kit (Preloaded for Auto-Extraction) (Liferiver, Cat.No. ME-0014).

It is noted that the EBOV positive control and EBOV negative control in this kit should be extracted with the same protocol for specimens. The internal control in this kit should be added into the extraction mixture with 1 μl/test to monitor the whole process.

8.1 Experiment Preparation

8.1.1 Instruments and Materials Preparation

Instruments and materials required for extraction but not provided are listed as below:

- Refrigerator and Freezer ,2-8°C
- Vortexer
- Microcentrifuge† (with rotor for 1.5 ml and 2 ml tubes)
- RNase-free microtubes
- Heating block or water bath for lysis of specimens at 56°C
- Tube racks
- Class II biosafety cabinet (or glove box)
- Personal protective equipment: Powder-free gloves, lab coat, eye protection, etc.
- Personal protective equipment: Powder-free gloves, fit-tested masks such as N95 Respirators and Filtering Face Piece (FFP) 3, Powered Air Purifying Respirators (PAPR) if fit-testing fails, full face shields, and disposable impermeable gowns, etc.
- Pipets† and pipette tips (to prevent cross-contamination, we strongly recommend the use of pipet tips with aerosol barriers)
- Ethanol (96–100%)*
- QIAamp DSP Virus Spin Kit (Cat. No. 61704) or QIAamp Viral RNA Mini Kit (Qiagen, Cat. No. 52904 or 52906) or RNA Isolation Kit (Paramagnetic Beads Column) (Liferiver, Cat. No. ME-0010) or Ribonucleic Acid (RNA) Isolation Kit (Preloaded for Auto-Extraction) (Liferiver, Cat. No. ME-0014) associated with EX2400 Automated Nucleic Acid Extraction system (Liferiver, Cat. No. IE-0001)

Notes:

** Do not use denatured alcohol, which contains other substances such as methanol or Methylethylketone.*

† To ensure that specimens are properly processed according to the Kit procedures, we strongly recommend that instruments (e.g., pipettes and heating blocks) are calibrated according to the manufacturers' recommendations.

8.1.2 Specimens Preparation

Appropriate specimens are inactivated whole blood, plasma or serum specimens. Non-inactivated specimens should be handled at the highest biosafety level before extraction[1]. After collection and centrifugation, plasma or serum can be stored at 2–8°C for up to 6 hours. For long-term storage, freezing at -20°C or -80°C in aliquots is recommended.

[1] Theoretically those recommended extraction kits would be able to inactivate Ebola virus. However, the manufacturer has not verified if Ebola virus would be completely inactivated with those recommended extraction kits due to the inaccessibility of active Ebola virus specimens.

Frozen plasma or serum specimens must not be thawed more than once. Repeated freeze-thaw leads to denaturation and precipitation of proteins, resulting in reduced viral titers and therefore reduced yields of viral nucleic acids. In addition, cryoprecipitates might be formed during freeze-thaw. If cryoprecipitates are visible, they can be pelleted by centrifugation at approximately 6,800 x g for 3 min. The cleared supernatant should be removed and processed immediately without disturbing the pellet.

8.2 RNA Isolation

8.2.1 QIAamp DSP Virus Spin Kit

1) Pipette 25 µl QIAGEN Protease (QP) into a lysis tube (LT).
2) Add 200 µl of specimen, or EBOV positive control or EBOV negative control into the lysis tube (LT), add 1 µl of internal control into each LT.
3) Add 200 µl Buffer AL (containing 28 µg/ml of carrier RNA). Close the cap and mix by pulse-vortexing for ≥15 sec.
4) Incubate at 56°C ± 3°C for 15 min ± 1 min in a heating block.
5) Briefly centrifuge the lysis tube (LT) to remove drops from the inside of the lid.
6) Add 250 µl ethanol (96–100%) to the tube, close the lid, and mix thoroughly by pulse-vortexing for ≥15 sec. Incubate the lysate with the ethanol for 5 min ± 30 sec at room temperature (15–25°C).
7) Briefly centrifuge the tube to remove drops from the inside of the lid.
8) Carefully apply all of the lysate from step 7 onto the QIAamp MinElute column without wetting the rim. Close the cap and centrifuge at approximately 6000 x *g* for >1 min. Place the QIAamp MinElute column in a clean 2 ml wash tube (WT), and discard the wash tube containing the filtrate.
9) Carefully open the QIAamp MinElute column, and add 500 µl Buffer AW1 without wetting the rim. Close the cap and centrifuge at approximately 6000 x *g* for ≥1 min. Place the QIAamp MinElute column in a clean 2 ml wash tube (WT), and discard the wash tube containing the filtrate.
10) Carefully open the QIAamp MinElute column, and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at approximately 6000 x *g* for >1 min. Place the QIAamp MinElute column in a clean 2 ml wash tube, and discard the wash tube containing the filtrate.
11) Carefully open the QIAamp MinElute column and add 500 µl ethanol (96 –100%) without wetting the rim. Close the cap and centrifuge at approximately 6000 x *g* for >1 min. Discard the wash tube containing the filtrate.
12) Place the QIAamp MinElute column in a clean 2 ml wash tube (WT). Centrifuge at full speed (approximately 20,000 x *g*) for 3 min ± 30 sec to dry the membrane completely.
13) Place the QIAamp MinElute column into a new 2 ml wash tube (WT), open the lid, and incubate the assembly at 56°C ± 3°C for 3 min ± 30 sec to dry the membrane completely.
14) Place the QIAamp MinElute column in an elution tube (ET), and discard the

wash tube with the filtrate. Carefully open the lid of the QIAamp MinElute column, and apply 20–150 µl Buffer AVE to the center of the membrane. Close the lid and incubate at room temperature for 5 min.

15) Centrifuge at full speed (approximately 20,000 x *g*) for >1 min. RNA is now present in the eluate and ready to test. Store specimens and controls at 2-8°C until PCR master mixes are prepared.

16) The RNA solution should be tested by PCR within 6 hours of completing the extraction process. Redundant specimens should be stored at 2-8°C while testing is in progress. Long-term storage of RNA solutions (> 6 hours) should be at -20°C (preferably -80°C). Minimize (not to exceed 3) repeated freeze-thaw cycles.

Note: For more information, please refer to the manufacturer's instructions.

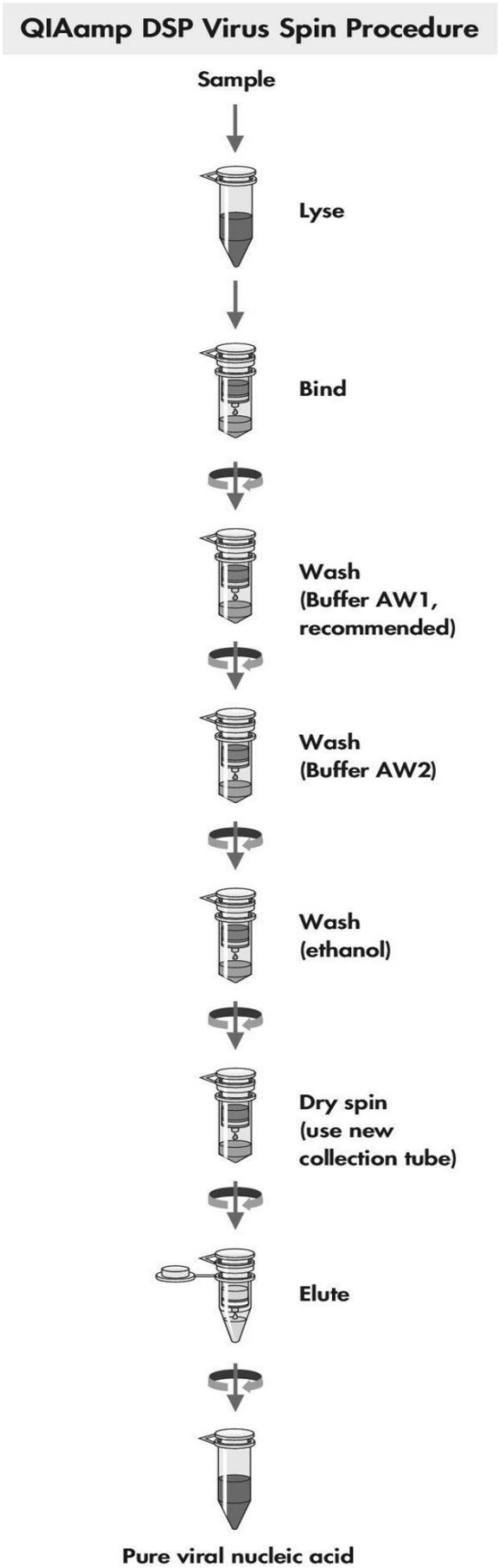


Figure 1. QIAamp DSF Virus Spin Procedure for Nucleic Acid Isolation. The illustration shows the simple steps (lyse, bind, wash, and elute) of the extraction protocol.

8.2.2 QIAGEN--QIAamp Virus RNA Mini Kit

Note: Qiagen Buffer AVL with carrier RNA is stable at room temperature for 3-4 hours; however, store reconstituted Buffer AVL/Carrier RNA at 2-8°C for longer periods. A precipitate will form and must be redissolved by warming at 80°C before use. DO NOT warm solution more than 6 times and DO NOT incubate for more than 5 min (see manufacturer's guidelines for more information). It is recommended to aliquot out the Qiagen Buffer AVL containing carrier RNA so as to prevent warming the reagent vial more than 6 times. If aliquoting in larger than single use volumes, then mark on the vial how many times the vial has undergone warming.

1) Pipette 560 µl of prepared Buffer AVL containing carrier RNA into a 1.5 ml microcentrifuge tube.
2) Add 140 µl of specimen, or positive control or negative control and 1 µl internal control to the Buffer AVL-carrier RNA in the microcentrifuge tube. Mix by by pulse-vortexing for 15 sec.
3) Incubate at room temperature (15–25°C) for 10 min.
4) Briefly centrifuge the tubes to remove drops from the inside of the lid.
5) Add 560 µl of absolute ethanol (96-100%) to each tube, and mix by pulse-vortexing for 15 sec. After mixing, briefly centrifuge the tubes to remove drops from inside the lid.
6) For each specimen and control, place a QIAamp spin column into a 2 ml collection tube (from the QIAamp Viral RNA Mini Kit). Be sure to label the top of the columns clearly.
7) Carefully transfer the mixture from step 5, including any precipitate, to the QIAamp spin column WITHOUT wetting the rim of the column.
8) Centrifuge 1-2 min at 6,000 x *g*. If the specimen has not cleared the filter after the first run, repeat centrifugation until the specimen has cleared the filter.
9) For each specimen and control, place the QIAamp spin column into a sec, clean 2 ml collection tube (from the QIAamp Mini Kit) and add 500 µl of Buffer AW1. Discard the tube containing the filtrate from the previous step.
10) Centrifuge 1-2 min at 6,000 x *g*. If the buffer has not cleared the filter after 1-2 min, repeat centrifugation until buffer has cleared the filter.
11) Place each QIAamp spin column into a third clean 2 ml collection tube (from the QIAamp Mini Kit). Carefully open the QIAamp spin column and add 500 µl of Buffer AW2.
12) Centrifuge at full speed (approx. 14,000 x *g*) for 3 min. Discard the tube containing the filtrate from the previous step.
13) To eliminate any possible Buffer AW2 carryover, place the QIAamp spin column into a new collection tube, discard the old collection tube, and centrifuge at full speed (approx. 14,000 x *g*) for 1 min.
14) Place the QIAamp Mini column in a clean, clearly labeled 1.5 ml RNase-free microcentrifuge tube (not provided). Discard the old collection tube containing the filtrate.
15) Carefully open the QIAamp Mini column and add 60 µl of Buffer AVE that has

been equilibrated to room temperature. Close the cap, and incubate at room temperature for 1 min.

16) Centrifuge at 6,000 x *g* for 1 min. RNA is now present in the eluate and ready to test. Store specimens and controls at 2-8°C until PCR master mixes are prepared.

17) Extracted specimens should be tested by PCR within 6 hours of completing the extraction process. Redundant specimens should be stored at 2-8°C while testing is in progress. Long-term storage of extracted specimens (> 6 hours) should be at -20°C (preferably -80°C). Minimize (not to exceed 3) repeated freeze-thaw cycles.

Note: More information please refer to the manufacturer's instruction.

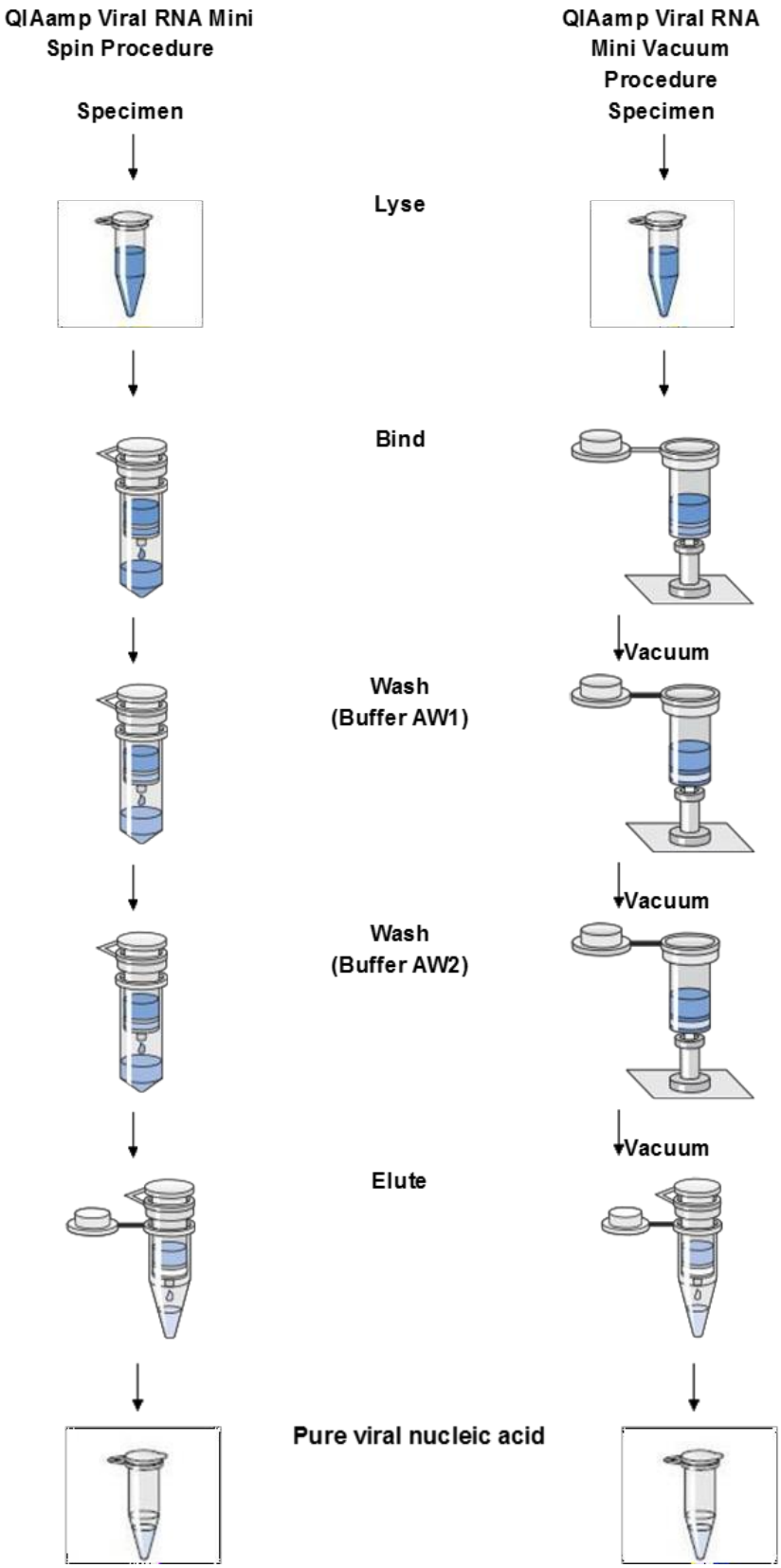


Figure 2. Viral RNA Isolation Procedure using QIAamp Viral RNA Mini Kit. The illustration shows the simple steps (lyse, bind, wash, and elute) of the spin protocol and the vacuum protocol.

8.2.3 Liferiver™ RNA Isolation Kit (Paramagnetic Beads Column)

1) Pipette 526 µl of prepared Binding Solution containing 20 µl of magnetic beads suspension and 6 µl of carrier RNA into each 1.5ml RNase-free tube.
2) Transfer 140 µl of specimen, or EBOV positive control or EBOV negative control and 1 µl of internal control to the Binding Solution in the RNase-free tube.
3) Mix by vortexing gently for 10 sec or by inverting the tube 5~10 times and incubate at room temperature for 3 min
4) Transfer the 667 µl liquid in step 3 to a Binding Column
5) Centrifuge the tube at 16000 × g for 60 sec. Discard the filtrate in the collection tube.
   Note: In some cases, leakage of beads through the membrane would be observed. It produces no significant effect on the extraction efficiency.
6) Add 500 µl of Washing Buffer A to each specimen and centrifuge the tube at 16000 × g for 40 sec. Discard the filtrate in the collection tube.
7) Repeat step 6.
8) Add 500 µl of Washing Buffer W to each specimen and centrifuge the tube at 16000 × g for 15 sec. Discard the liquid in the collection tube.
9) Repeat steps 8.
10) Centrifuge the binding columns at 16000 × g for 2min to thoroughly dry the membrane.
11) Add 50 µl of Elution Buffer (preheated to 65°C) to each column, and incubate for 2 min at room temperature.
12) Centrifuge the tube at 16000 × g for 2min, and store the purified RNA in the 1.5 ml RNase-free tube at –20 °C.

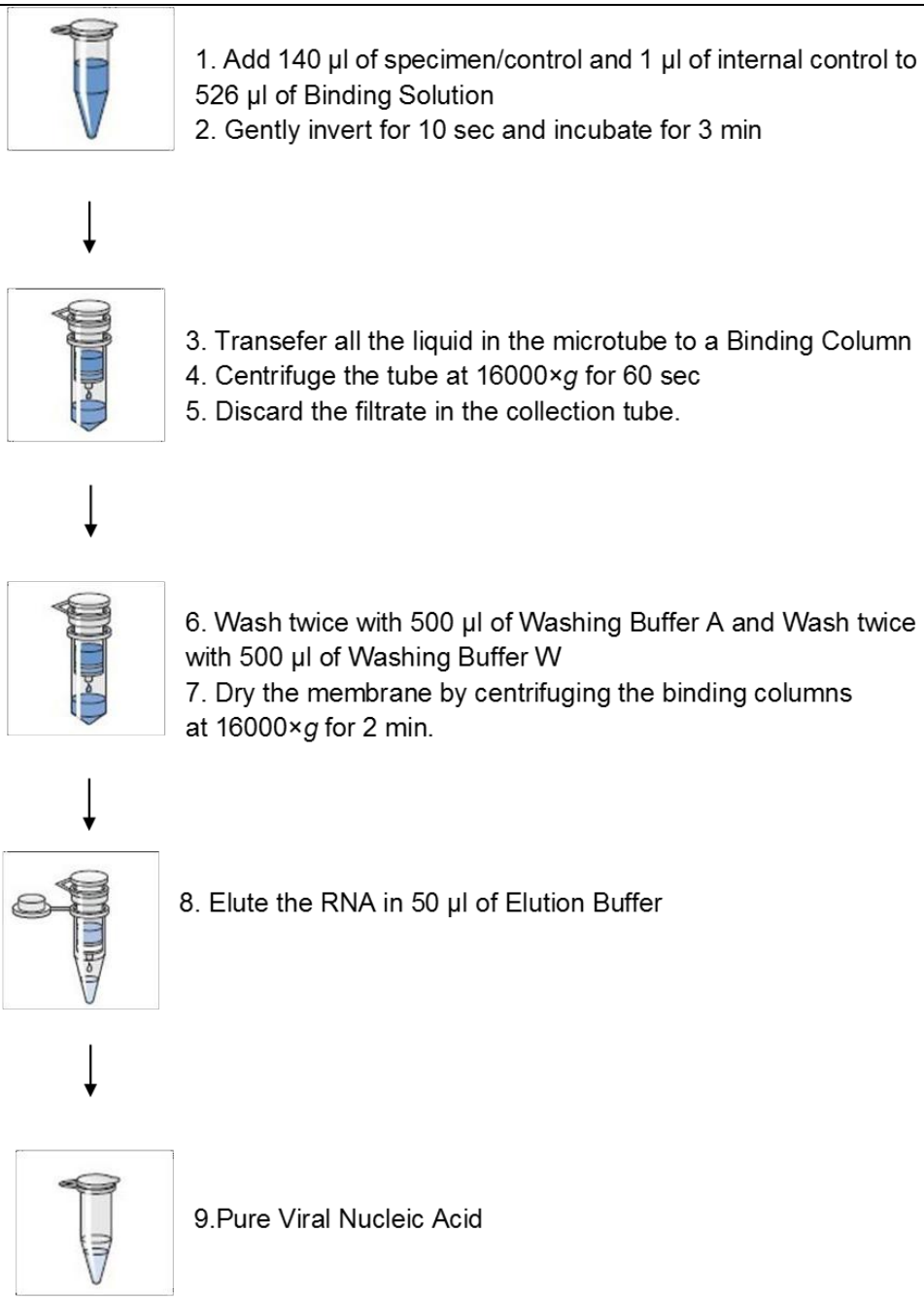


Figure 3. Viral RNA Isolation Procedure using Liferiver™ RNA Isolation Kit (Paramagnetic Beads Column). The illustration shows the simple steps (lyse, bind, wash, and elute) of the protocol.

8.2.4Liferiver™ RNA Isolation Kit (Preloaded for Auto-Extraction)

EBOV nucleic acid extraction can be fully automated using the Liferiver RNA isolation Kit (Preloaded for Auto-Extraction) on the EX2400 Automated Nucleic Acid Extraction system (Liferiver, Cat. No. IE-0001).

8.2.4.1 Kit Components and Storage Conditions

Ref.	Component	Amount	Storage	Volume/test
1	Preparing Plate ***a***	20 pieces	room temp	
2	Magnetic Cap	20 strips	room temp	
3	Elution Buffer	1ml×2	room temp	
4	Carrier RNA ***b***	5 tubes	room temp	6μl
		(add 350μl Elution Buffer to each tube before use)		

Note:

a *Do not make Preparing Plate frozen. Wells in Row A of preparing plate tend to be crystallized at low temperature, so dissolve it at 37*°C *before use.*

b *Once Elution Buffer has been added to Carrier RNA, store the Carrier RNA at 2*°C*-8*°C *for 6 months or at-20*°C *for 24 months.*

Properly stored kits are guaranteed for 24 months from the date manufactured.

8.2.4.2 RNA Isolation Procedure using the Liferiver™ RNA Isolation Kit (Preloaded for Auto-Extraction)

1) Pipette 1 μl EBOV internal control into each well in row A.
2) Take out one piece of preparing plate and tear the aluminum foil carefully.
3) Add 6μl Carrier RNA and 200 μl mix (specimen, EBOV positive control or EBOV negative control and EBOV internal control) into the well A1-A12 of the preparing plate.
4) Put the preparing plate on the transport platform carefully, insert the magnetic cap.
5) Choose “RNA Isolation” program, press “START” to run the test.
6) After the program finished, discard the magnetic cap, take out the preparing plate and transfer the liquid in well E1-E12 into RNase-free EP tubes. It can be used for immediate experiment or store at -20 °C for preservation.

9 PCR Setup

8.1 Determine the number of reactions (N) that equals to sample number (n) including positive and negative control plus 1 (N=n+1) due to pipetting error.Prepare EBOV Master Mix by mixing EBOV Super Mix and RT-PCR Enzyme Mix in a 1.5ml centrifuge tube. Required volumes of both reagentsare calculated according the formulation in the table below.

Reagent	Volume of Reagent (N× per reaction)
EBOV Super Mix	N×19μl
RT-PCR Enzyme Mix	N×1μl
Total Volume of EBOV Master Mix	N×20μl

***Number of the reactions (N) includes sample number, control number and one additional reaction due to pipetting error.**

8.2 Mix the reagent mixture completely by gently pipetting up and down and then spin briefly.

8.3 Dispense 20µl Master Mix into each PCR tube. Pipette 5µl sample including positive and negative control into each tube. Close the tubes immediately to avoid possible contamination and spin briefly.

Instrument Specific Instructions

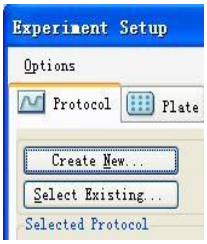
10 Operation Procedure on Bio-Rad CFX 96

10.1 Double-click the icon 

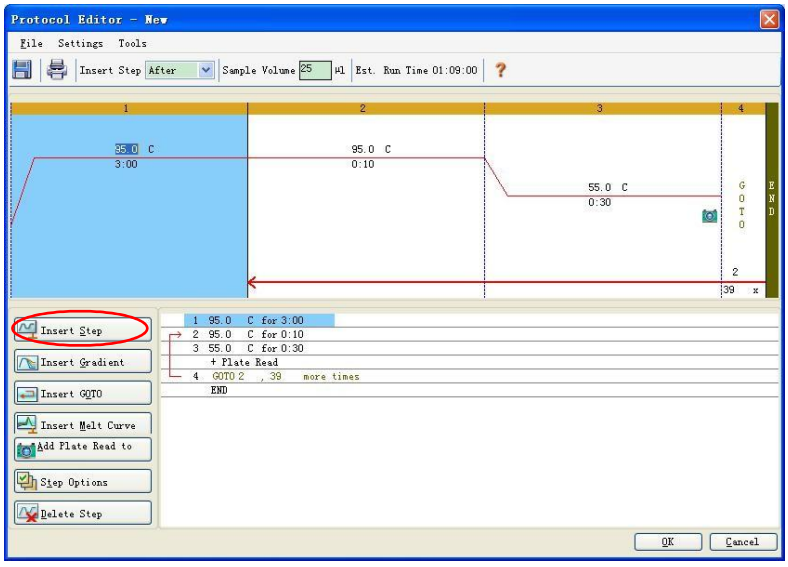
to open the Bio-Rad CFX software.

10.2 Create a new Experiment and choose the type of the instrument, click "OK".

10.3 Under "Protocol" interface, click the "Create New" button to create a new protocol or click the "Select Existing" button to invoke the existed protocol file.



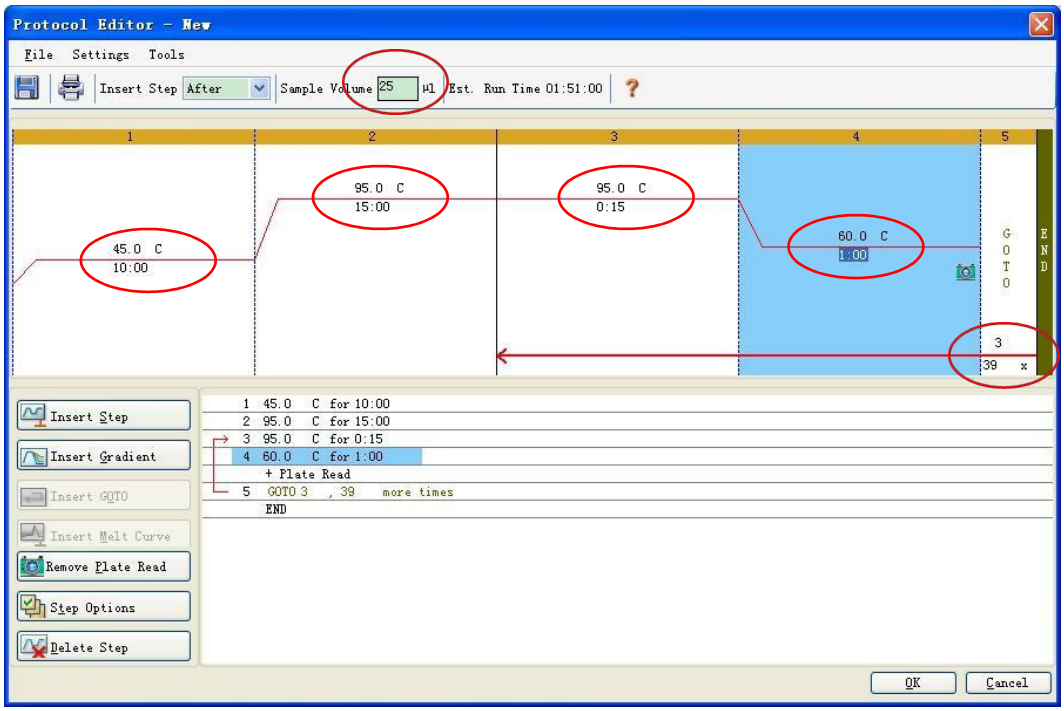
10.4 After click the "Create New" button, you can see the below interface. Insert a step first.



10.5 Parameters setting:

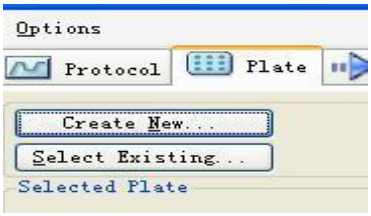
Step 1	45°C for 10min	1 cycle
Step 2	95°C for 15min	
Step 3	95°C for 15sec	40 cycles
Step 4	60°C for 1min *Fluorescence measured at 60°C	

Sample Volume: 25µl



10.6 Click the “Ok”. Save the protocol.

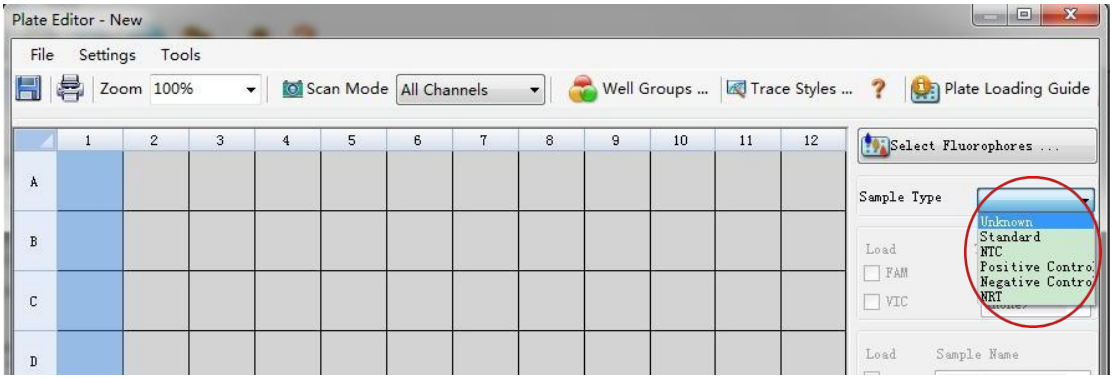
10.7 Under “Plate” interface, click the “Create New” button to create a new plate or click the “Select Existing” button to invoke the existed plate file.



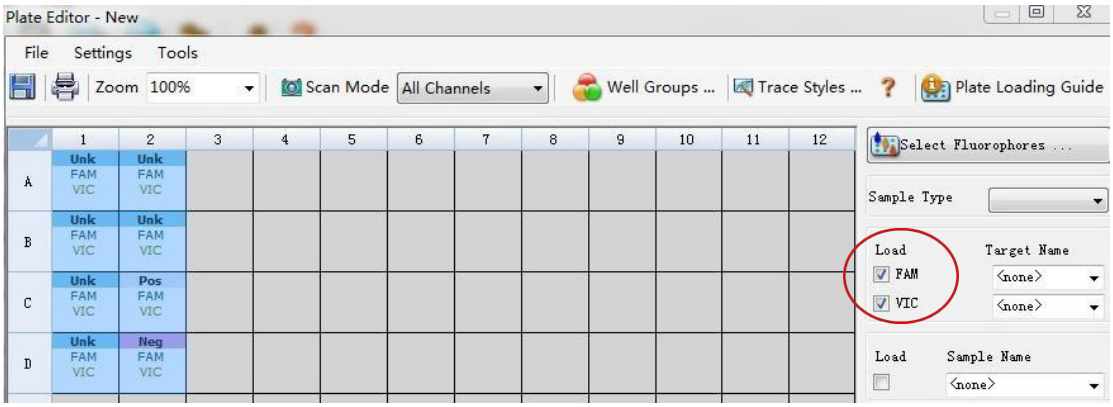
10.8 Select the fluorescence channel: Click “ 

” to choose “FAM” and “VIC” channel, click “OK”.

10.9 Choose the well and select sample type in the drop-down list box. Sample choose "Unknown", positive control choose "Positive Control", negative control choose "Negative Control".

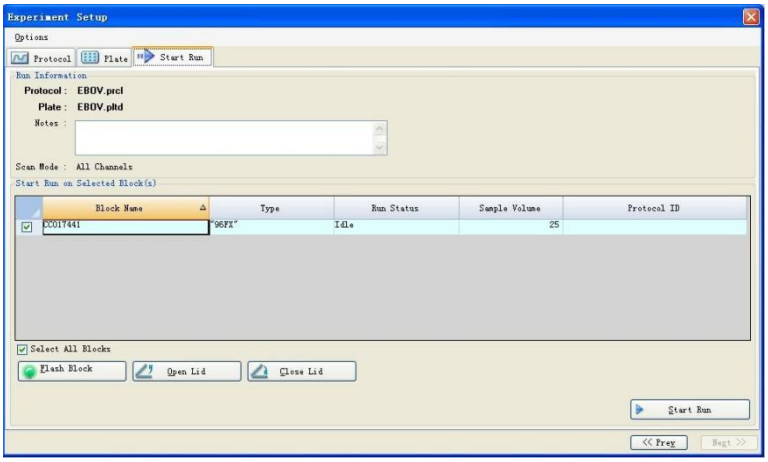


10.10 Choose all the wells in the experiment, click "FAM" and "VIC"

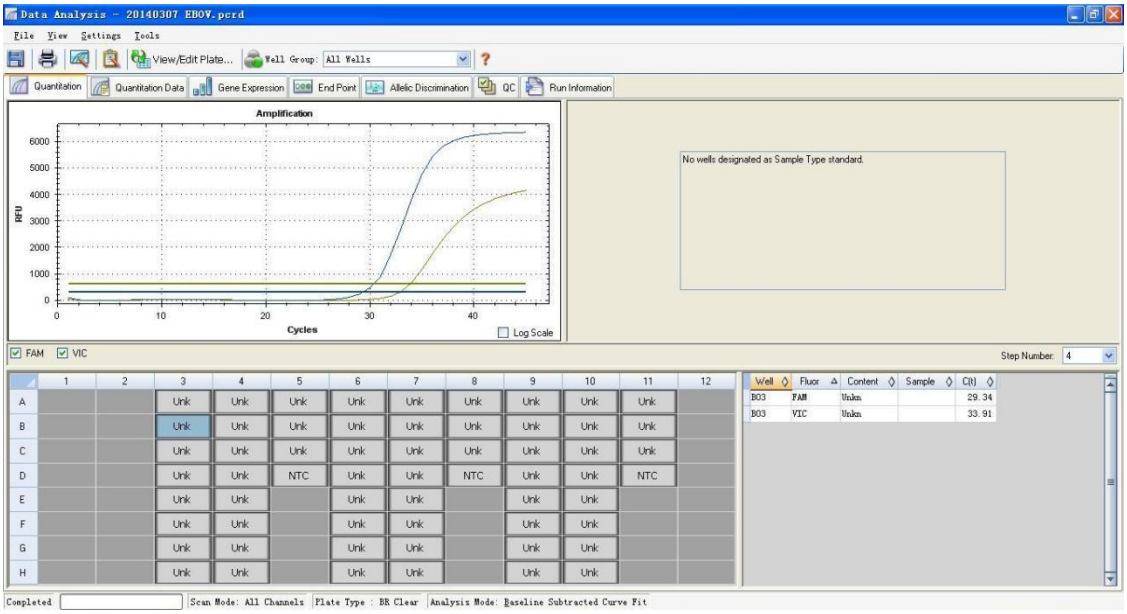


10.11 Click the "Ok". Save the plate.

10.12 Under "Start Run" interface, click "Open Lid", then add the plate into the instrument, click "Close Lid". Click "Start run". Then save the run file. The experiment begins.



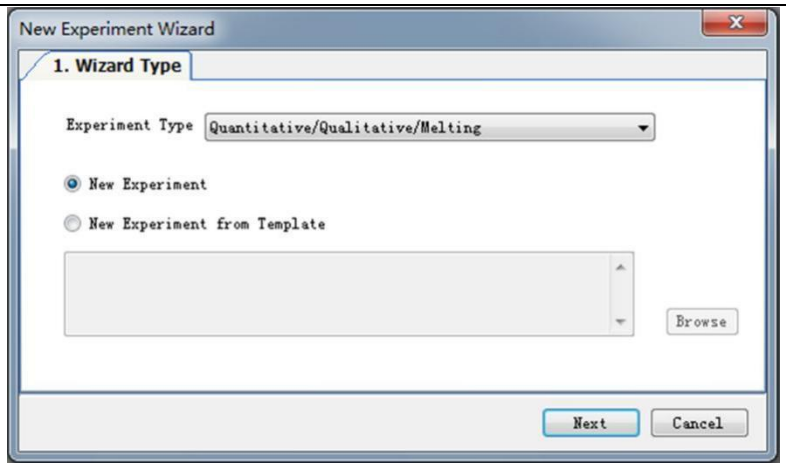
10.13 PCR Analysis: after the run is completed, click the well to read the Ct value on the lower right and amplification curve on the upper left.



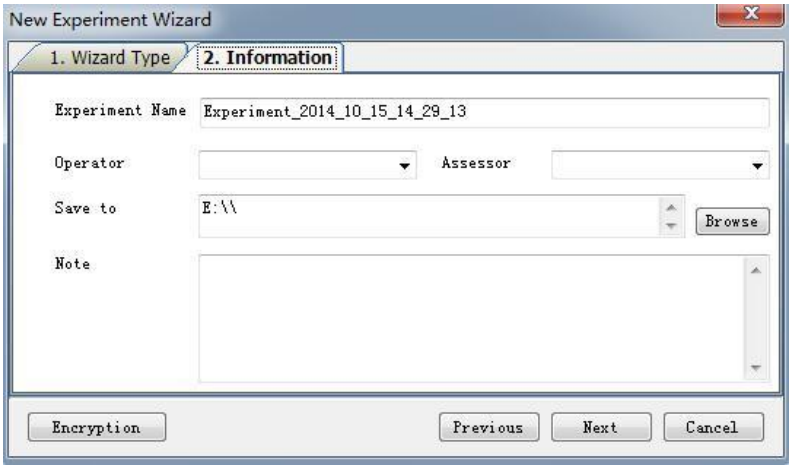
11 Operation Procedure on SLAN®-96

11.1 Enter the SLAN PCR program by double clicking on the SLAN system icon on the desktop.

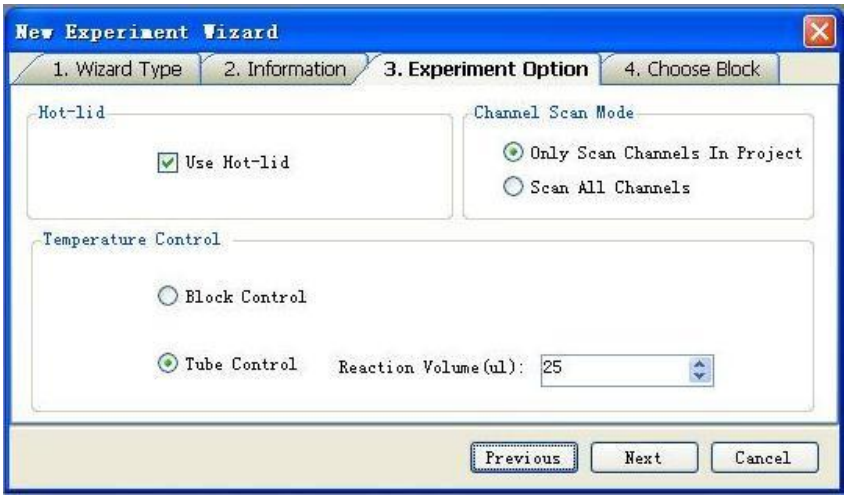
11.2 Select “Creat New Document” from the Quick start up menu. The “New Experiment Wizard” screen will appear.



11.3 Click “Next”, then enter the experiment name and save path in the new interface.

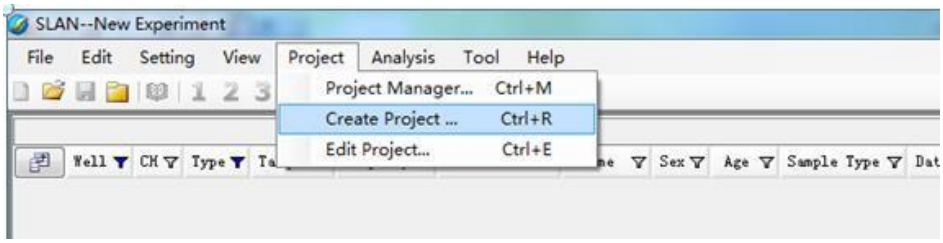


11.4 Click “Next”. Under temperature control, select “Tube Control”, enter the PCR reaction volume: 25μl.

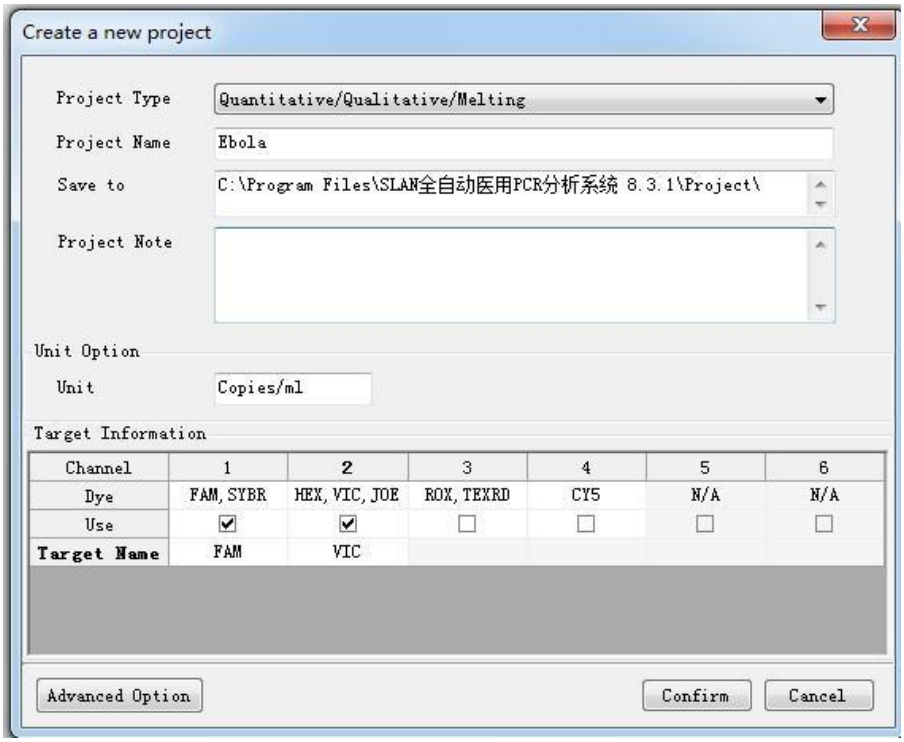


11.5 Click “Next”, choose block A and/or block B where the PCR tubes are.

11.6 Click “Confirm”. In the new screen, edit project parameters: click “Project” → “Create Project”.



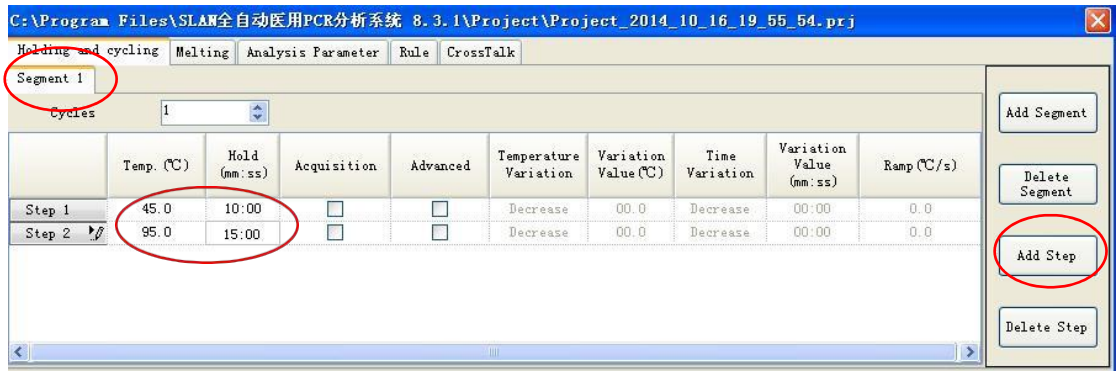
11.7 The below window will appear. Enter the project name and save path, select the channel (FAM & VIC).



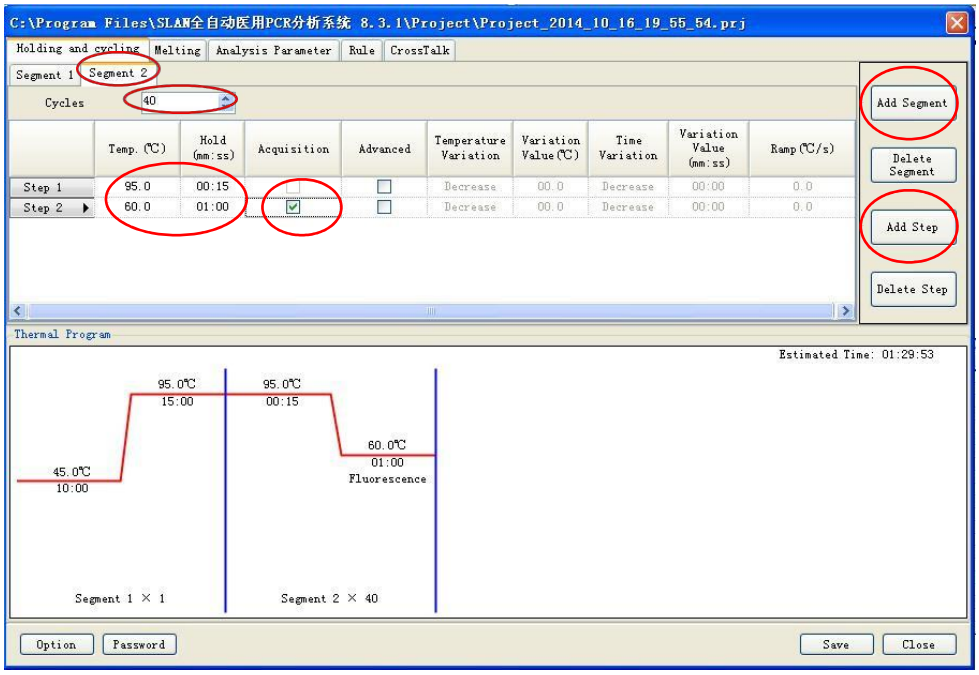
11.8 Click “Confirm”. Set parameters in the new window.

Segment 1	Step 1	45°C for 10min	1 cycle
	Step 2	95°C for 15min	
Segment 2	Step 1	95°C for 15sec	40 cycles
	Step 2	60°C for 1min *Fluorescence measured at 60°C	

11.8.1 Segment 1: click “Add Step”, set the parameters.

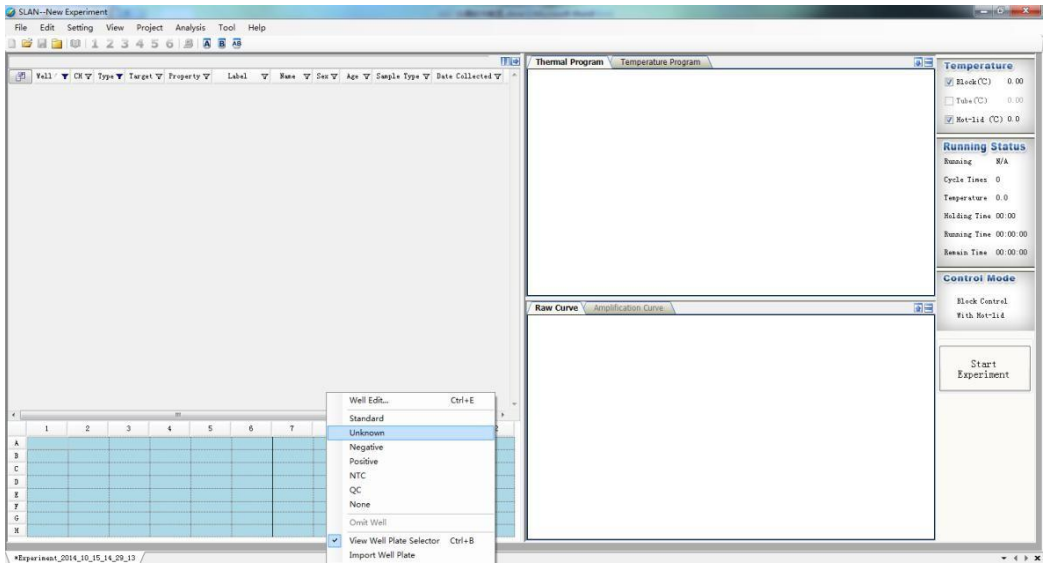


11.8.2 Click “Add Segment”. Under Segment 2: click “Add Step”, set the parameters.

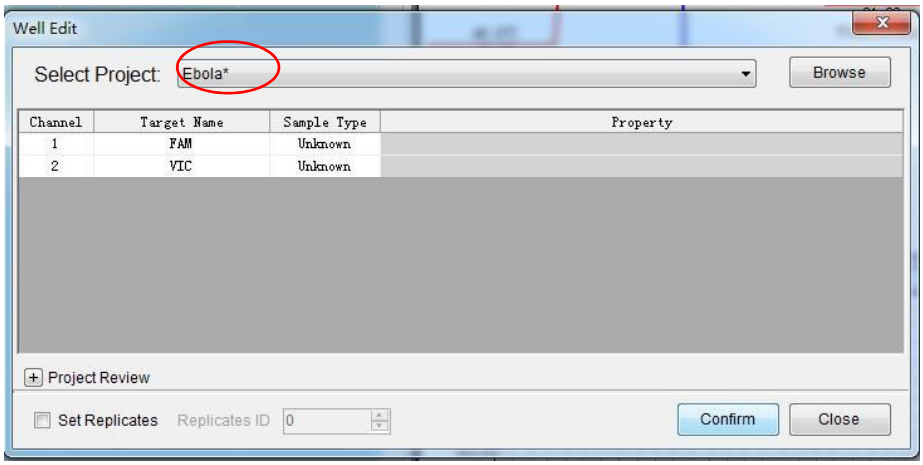


11.9 Click “Save”, then click “Close” to exit.

11.10 Well Edit: select the well where the tubes are, right-click. If are samples, choose “Unknown”, if are negative control or positive control, choose “Negative” or “Positive”.



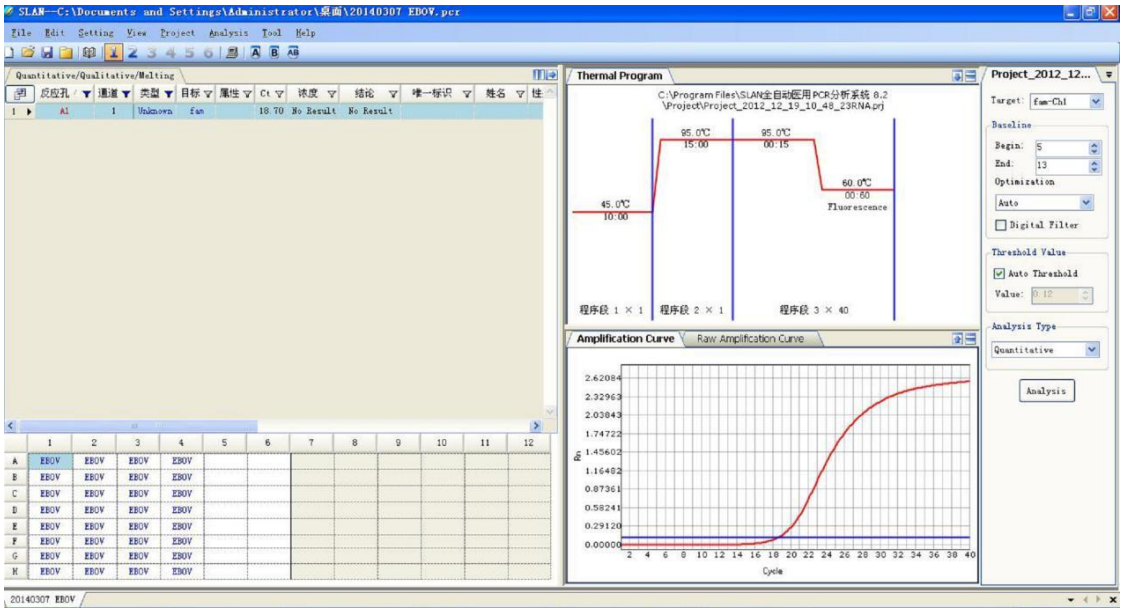
11.11 Take a sample well as an example. Click “Unknown”, the below screen will appear. Select the project setup in 11.7, click “Confirm”.



11.12 Click block A or block B or block A&B to unlock the hot lid needed. Then open the machine lid to place the sample in.

11.13 Start run by clicking “Start Experiment” button.

11.14 PCR Analysis: after the run is completed, click the well to read the result. View and adjust the raw data on the right. Selecting “Baseline” and “Thresheld Values”, then click ‘Analysis’. The Ct value is on the left and the amplification curve on the lower right.

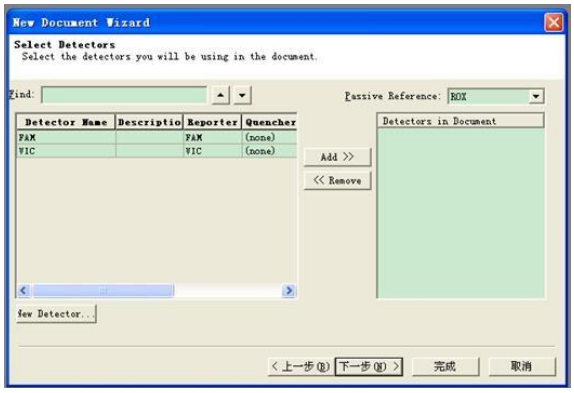


12 Operation Procedure on ABI Prism®7500

12.1 Double click on the ABI system icon on the desktop to enter the program.

12.2 Select “Create New Document” from the Quick start up menu. The “New Document Wizard” screen will appear. Change the plate name.

12.3 Click “Next”, a new screen will appear as below.



12.4 If there is no “FAM” and “VIC”, click the “New Detector” 

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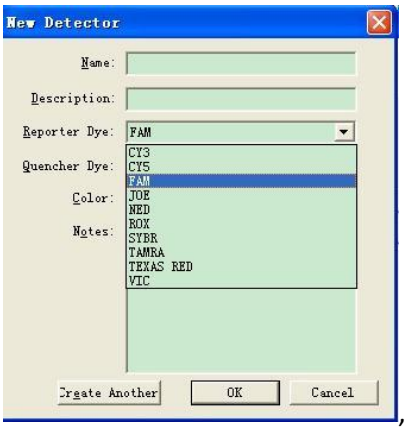
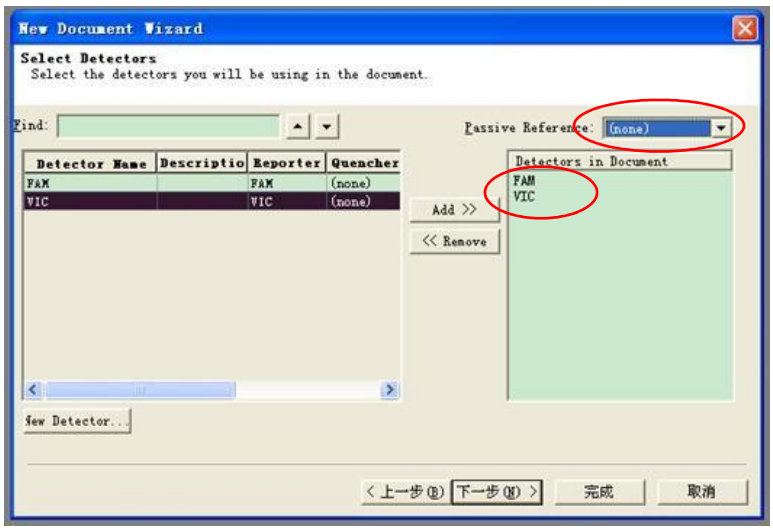
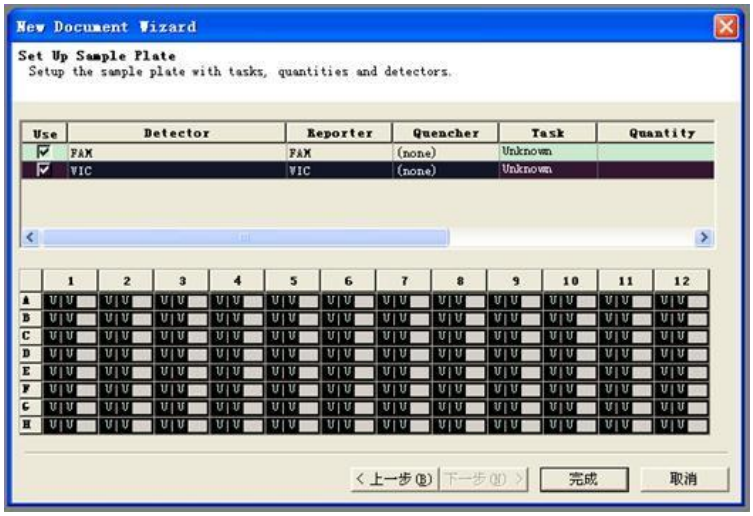


, enter the name.

12.5 Select the Detector (FAM & VIC), change “Rox” to “None” in the “Passive Reference” drop-down menu.



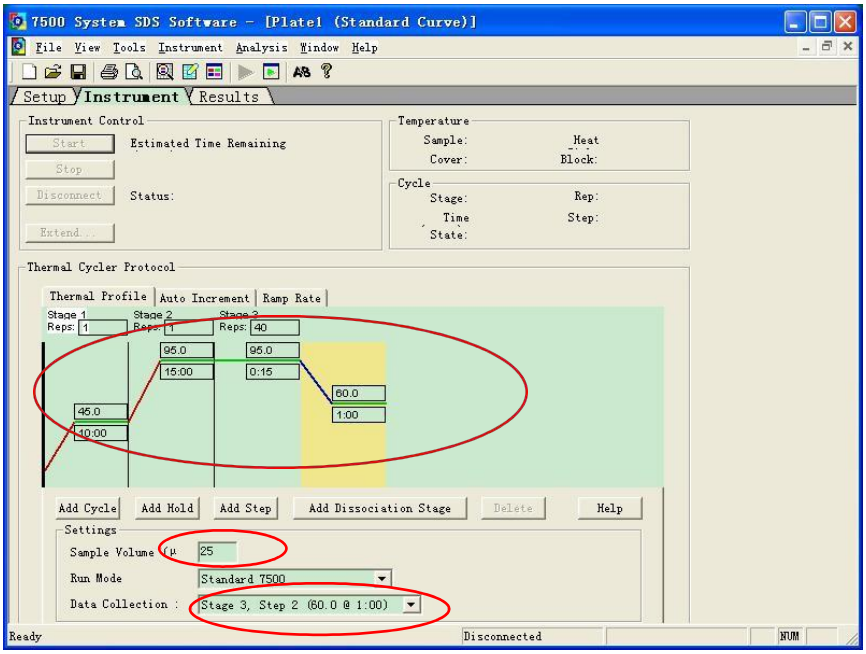
12.6 Click “Next”, select the well containing the samples and controls, and then click the Detector.



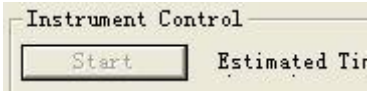
12.7 Click “Finish”.

12.8 Select the “Instrument” tab. Set up the parameters as follows:

- Stage 1: 45℃ for 10 min, 1 cycle;
- Stage 2: 95℃ for 15 min, 1 cycle;
- Stage 3: 95℃ for 15 sec, 60℃ for 60 sec, 40 cycles.
- Sample Volume: 25 (μl)
- Data Collection at Stage 3, Step 2 (60.0 @ 1:00)

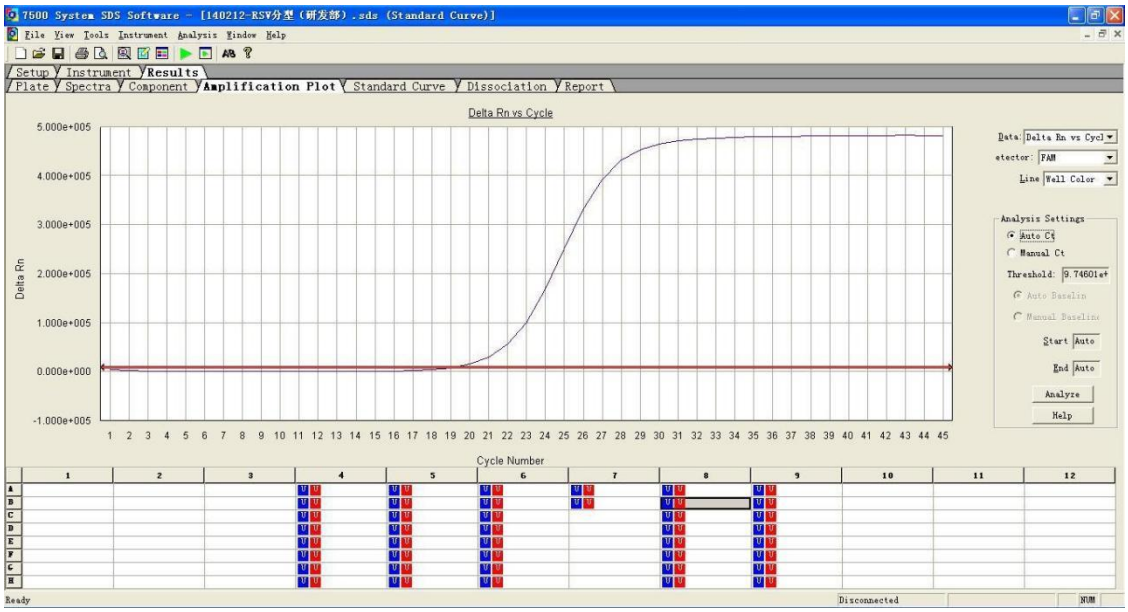


12.9  Saving the document, after the saving to run the evaluation, click the “Start”.

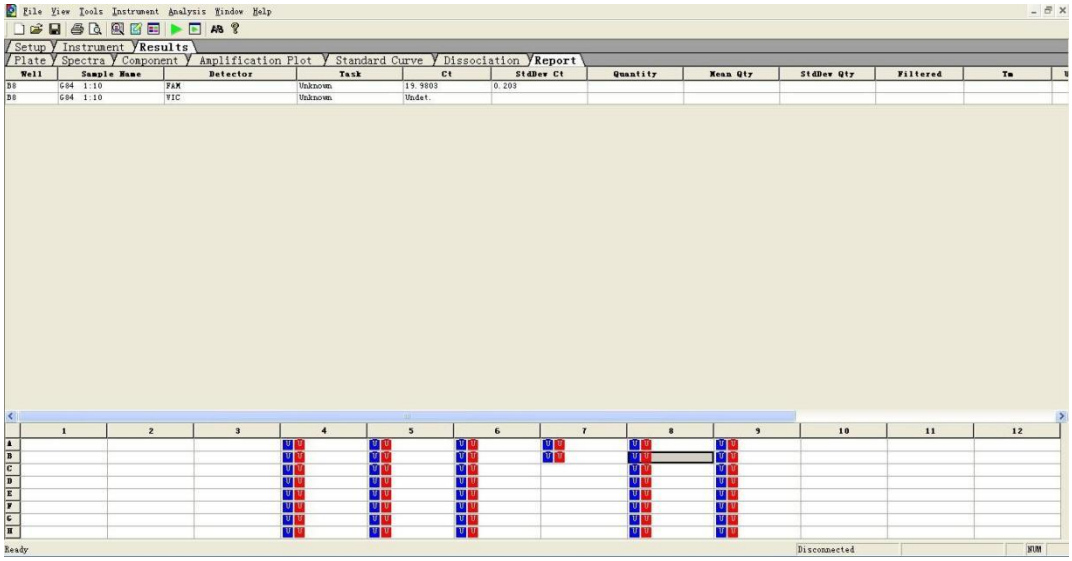


12.10 PCR Analysis: after the run is completed, click the “Results”. Click the “Amplification Plot” tab and view and adjust the raw data.

- In the “Data” window, “Delta Rn vs Cycle” should be selected.
- In the “Detector” window, “FAM” and “VIC” should be selected.
- The “Start (cycle)” window should read “6.” The “End (cycle)” should be 15.
- Lastly, be sure to click the “Analyze” icon to update the analysis.



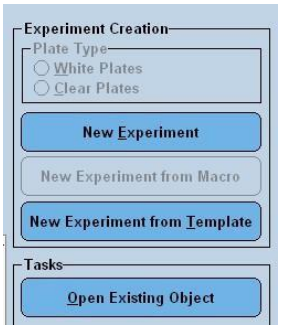
12.11 Click the “Report” icon above the graph to display the cycle threshold (Ct) values.



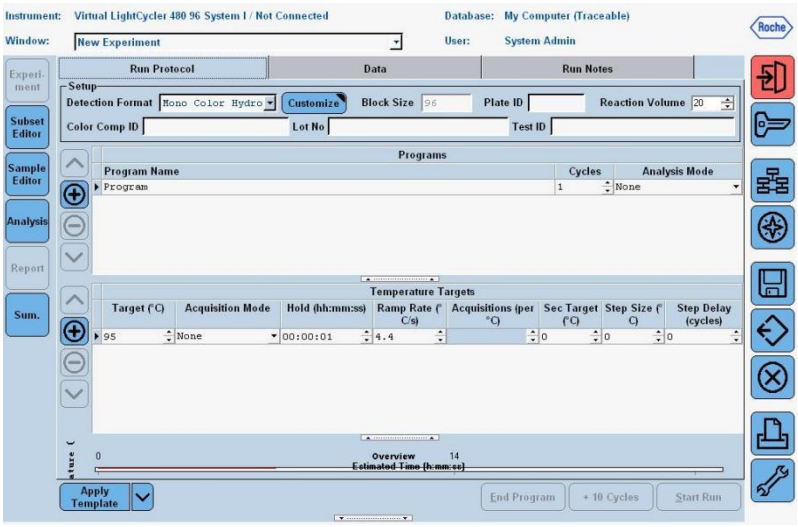
13 Operation Procedure on LightCycler®480

13.1 Open the software of LightCycler 480.

13.2 Click “New Experiment” on the right side.

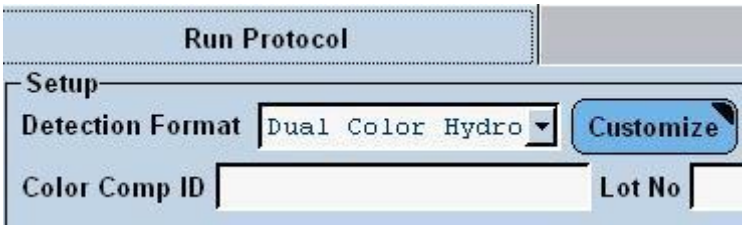


Show the following interface:

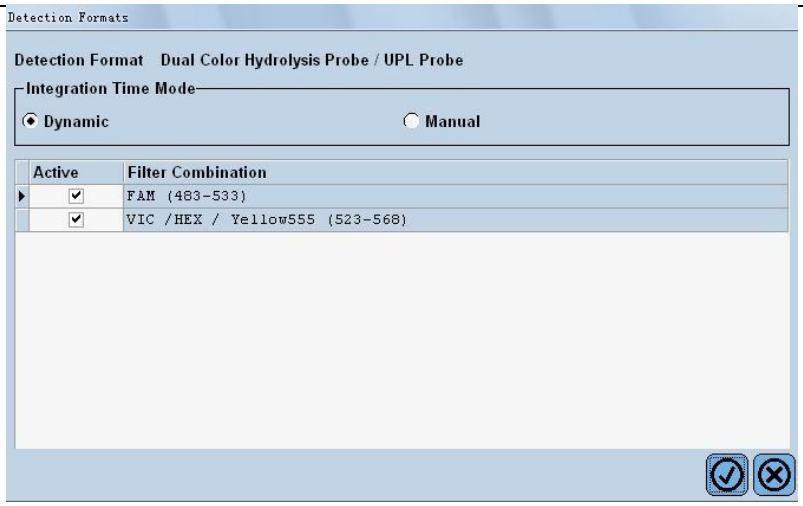


13.3 Choose the detection channel:

13.3.1 Under “Detection Format” option, choose “Dual Color Hydrolysis Probe/UPL Probe”.

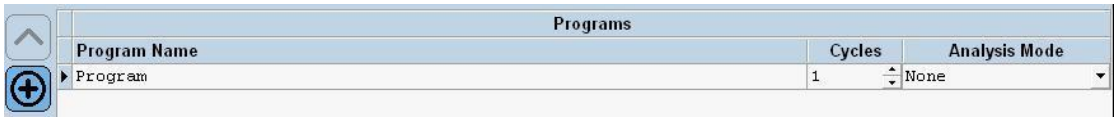


13.3.2 Click “Customize”, and choose “FAM” and “VIC/HEX/Yellow”, then click OK  .

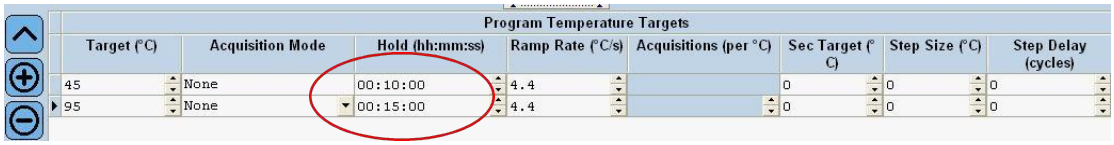


13.4 Change the “Reaction Volume”: 25ul

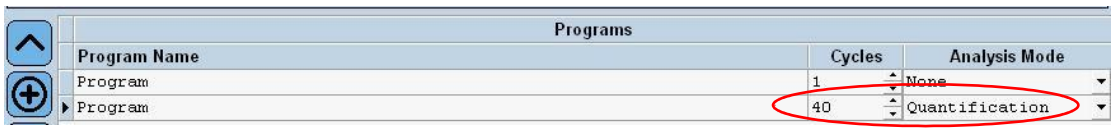
13.5 Under “Programs” tab, select “Program”, 1 Cycles, you can also change the Program Name.



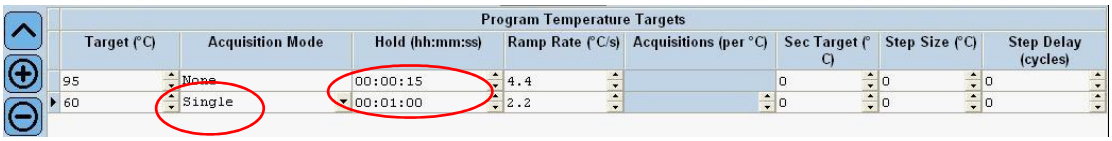
13.6 Under “Program Temperature Targets” tab, add one step  , then set the temperature and time parameter: 45℃, 10 min; 95℃, 15 min.



13.7 Go back to “Programs” tab, add a new program  , 40 Cycles and choose “Quantification” under the Analysis Mode option.

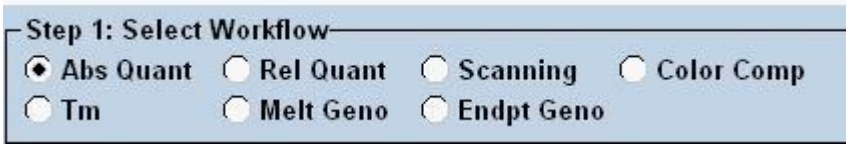


13.8 Under this new added program, go to the “Program Temperature Targets” tab, and then set the temperature and time parameter: 95℃, 15sec; 60℃, 60sec, and the “Acquisition Mode” should be chosen Single.

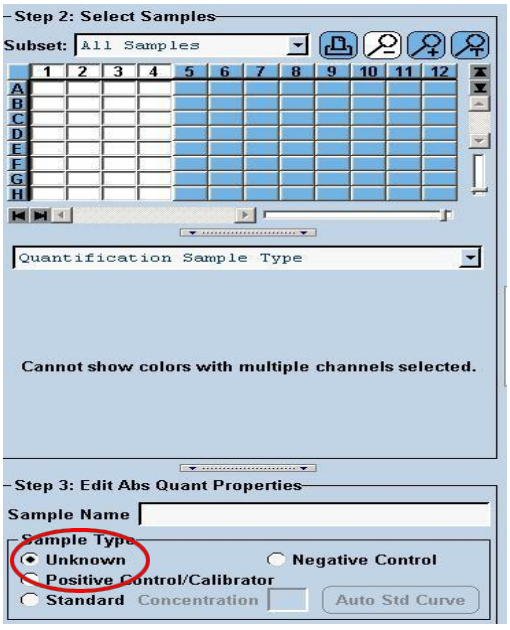


13.9 Then choose the “Sample Editor” on the left side.

13.10 Choose “Abs Quant”.



13.11 Select the wells containing the samples, then enter the sample name and choose the sample type “Unknown”. If wells contain positive control or negative control, choose the sample type “Positive Control” or “Negative Control”.



13.12 Open the machine and place the samples in. Then go back to “Experiment” 

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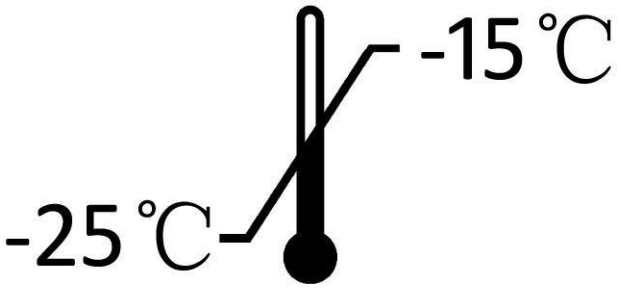
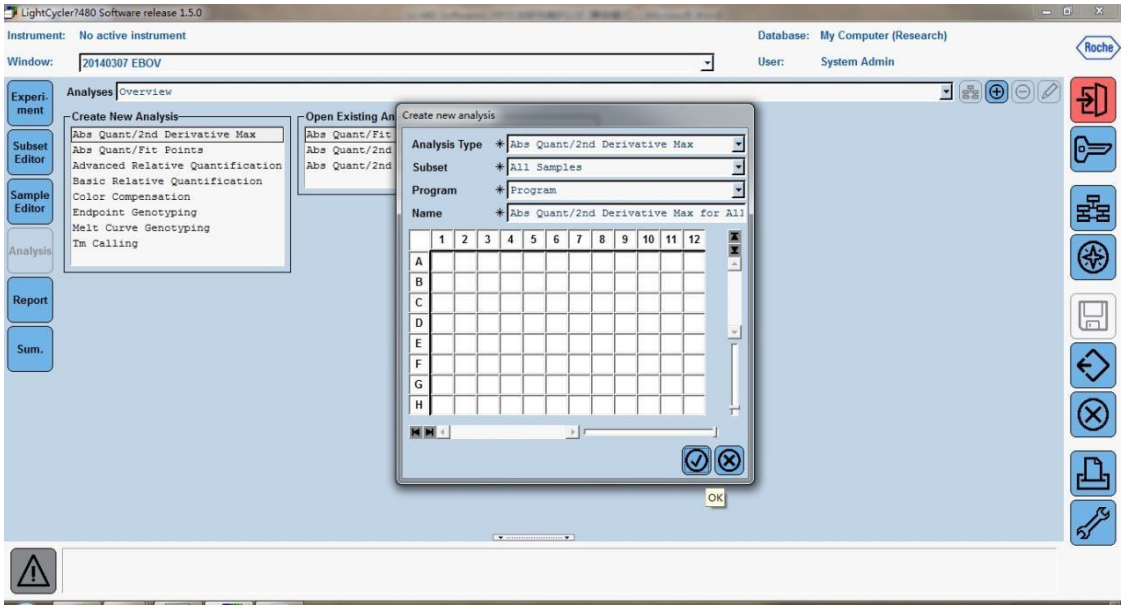
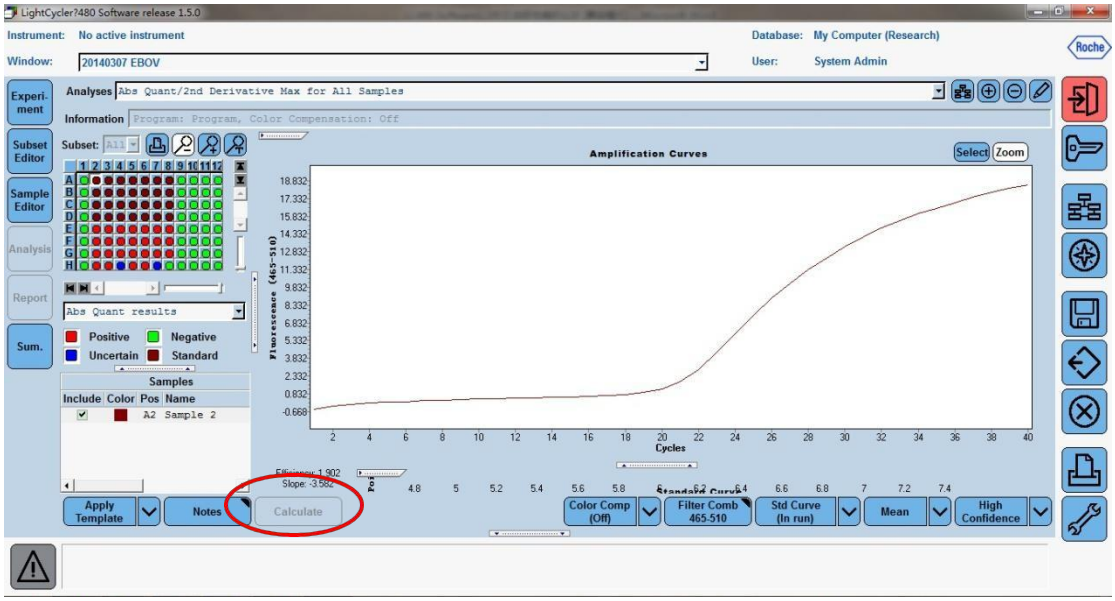
and click “Start Run”





Click “Calculate” button on the lower left.



Interpretation of Results

14 Interpretation of Controls and Clinical Specimens

14.1 Controls

Test Run is Valid when all Controls Meet the Following Stated Standards.

Negative control reaction must be negative without an amplification curve at FAM channel and Ct value between 25 and 38 at VIC channel. If failed, the test run is invalid and potential sources of contamination should be identified and corrected (see Table 1).

Positive control reaction should be positive at FAM channel, Ct value must be ≤35. If failed, obtain new reagents and re-test the run (see Table 1).

Table 1 Interpretation of controls

Controls	Ct value	
	FAM	VIC
Negative control	UNDET, No Ct, N/A	25~38
Positive control	≤35	--

14.2 Specimen

15.1.1 When all controls are performed correctly, the run is valid.

15.1.2 A specimen is considered positive for Ebola virus RNA if the specimen has a positive amplification result at FAM channel and Ct value ≤38 (see Table 2)[2].

15.1.3 When Ct value is between 38 and 40 at FAM channel and Ct value is between 25 and 38 at VIC channel, the sample should be re-tested. If it is still the same result, the specimen is reported as below the detection limit or negative for Ebola virus (see Table 2).

15.1.4 A specimen is considered negative for Ebola virus if it does not have an amplification curve at FAM channel and Ct value between 25 and 38 at VIC channel (see Table 2).

15.1.5 If it has no positive amplification result at FAM and VIC channel, it is considered as no diagnosis because of some inhibition on the procedure. Repeat the whole run and re-analyze (see Table 2).

Table 2 Interpretation of results

	Ct value		Result Analysis
	FAM	VIC	
1#	UNDET, No Ct, N/A	25~38	Below the detection limit or negative

[2] Assay results are for the presumptive identification of Ebola virus and intended for use as part of a multi-test algorithm to detect the presence of Ebola virus RNA. Laboratories are required to report results to the appropriate public health authorities. The definitive identification of Ebola virus requires additional testing to be performed by designated laboratories. The diagnosis of Ebola must be made based on history, signs, symptoms, exposure likelihood, and other laboratory evidence, in addition to the detection of Ebola virus RNA

2#	≤38	--	Positive;
3#	38~40	25~38	Re-test; if it is still 38~40, report as 1#
4#	UNDET, No Ct, N/A	UNDET, No Ct, N/A	PCR inhibition; no diagnosis can be concluded

15 Limitations

- All results should be interpreted by a trained professional in conjunction with the patient's history and clinical signs and symptoms.
- Interpretation of results must account for the possibility of false-negative and false-positive results.
- Negative results do not preclude infection with Ebola virus and should not be the sole basis of a patient treatment/management decision.
- False positive results may occur from cross-contamination by target organisms, their nucleic acids or amplified product.
- Failure to follow the assay procedures may lead to false negative results.
- Improper collection, storage, or transport of specimens may lead to false negative results.
- Inhibitors present in the samples may lead to false negative results.

Analytical Performance Characteristics

Accuracy of measurement

A series of precision studies have demonstrated high accuracy of the kit. Precision tests were performed with three lots of kits and three different concentrations of EBOV pseudovirus plasmid samples. Each test was repeated for 10 times. All CV% values are <3%. The test results are summarized in the following table:

Sample	Sample concentration (copies/ml)	CV%			CV% (between 3 lots)
		Lot# 1	Lot# 2	Lot# 3	
P0	0	1.25%	1.13%	2.14%	1.55%
P1	1×10^4	1.54%	2.25%	2.06%	1.97%
P2	1×10^7	1.22%	1.57%	1.55%	1.50%

Analytic sensitivity

Four EBOV pseudovirus plasmids were examined in the sensitivity tests. These pseudoviruses include EBOV-Z-1, EBOV-S-1, EBOV-B-1, and EBOV-C-1. The plasmid samples were diluted lineally into various concentrations. The tests were performed with three lots of kits. Each test was repeated for 3 times. Then confirming tests for the positive samples with lowest EBOV concentration were further repeated for 20 times. The results suggested that the minimum detection for all four Ebola pseudovirus is 1×10^3 copies/ml.

Analytic specificity

22 microorganisms and 3 human genomic DNA samples were examined with EBOV Real Time RT-PCR Kit in the specificity tests. The tests were performed with three lots of kits. Our results showed that the kit only detected Ebola virus; whereas negative EBOV detection were observed using other 22 microorganisms and human genomic DNA samples, suggesting high specificity of the kit for Ebola virus detection. The test results with 22 microorganisms and human genomic DNA samples are summarized in the following table:

Sample	Lot # 1		Lot # 2		Lot # 3	
	EBOV test	Internal control	EBOV test	Internal control	EBOV test	Internal control
HCV	-	+	-	+	-	+
Influenza A virus	-	+	-	+	-	+
Influenza B virus	-	+	-	+	-	+
Enterovirus 71	-	+	-	+	-	+
Coxsackie virus A16	-	+	-	+	-	+
RS virus	-	+	-	+	-	+
Parainfluenza virus	-	+	-	+	-	+

EB virus	-	+	-	+	-	+
HBV	-	+	-	+	-	+
Human corona virus	-	+	-	+	-	+
Japanese encephalitis virus	-	+	-	+	-	+
Dengue virus	-	+	-	+	-	+
Streptococcus pneumonia	-	+	-	+	-	+
Human metapneumovirus	-	+	-	+	-	+
Hemophilus influnzae	-	+	-	+	-	+
Adenovirus	-	+	-	+	-	+
CMV	-	+	-	+	-	+
Epidemic parotitis virus	-	+	-	+	-	+
Staphylococcus aureus	-	+	-	+	-	+
Measles virus	-	+	-	+	-	+
Rhinovirus	-	+	-	+	-	+
Streptococcus pyogens	-	+	-	+	-	+
Human genomic DNA-1	-	+	-	+	-	+
Human genomic DNA-2	-	+	-	+	-	+
Human genomic DNA-3	-	+	-	+	-	+

Traceability of calibrators and control material values

We have generated four EBOV pseudoviruses as positive references through vector cloning/transfection to host cells/selection of positive host cells/collection of pseudoviruses from culture media of the positive host cells. The Ct values of four EBOV pseudoviruses are summarized in the following table:

	EBOVZ	EBOVS	EBOVC	EBOVB
Ct value	16.56	16.93	17.21	16.36

We also generated five EBOV negative references from clinical samples.

Additionally, we have generated EBOV positive control, negative control, and internal control. Both positive and internal controls are pseudoviruses that were generated by the similar methods as described above. Negative control is a 0.9% saline solution that was aliquoted and stored at -20°C. Both positive and internal control were diluted before they were aliquoted and stored at -20°C. The Ct values of the positive and internal control are summarized in the following table:

	Positive control	Internal control
Ct value	16.85	16.71
Ct value after dilution	21.12	30.26