WHO Emergency Use Assessment and Listing for Zika IVDs
PUBLIC REPORT

Product: Liferiver Zika Virus (ZIKV) Real Time RT-PCR Kit
WHO EUAL reference number: EAZ 0004-003-00

Emergency Use Assessment and Listing of In Vitro Diagnostics Procedure

WHO has developed an Emergency Use Assessment and Listing (EUAL) procedure to expedite the availability of in vitro diagnostics (IVDs) needed in public health emergency situations. This EUAL procedure will generate WHO recommendations in order to provide advice to procurement agencies and Member States on the acceptability of a specific IVD in the context of a public health emergency, based on a minimum set of available quality, safety, and performance data and an agreed plan for further evaluation.

The EUAL procedure is comprised of three components that aim to assess the safety, quality and performance of the IVD:

- a review of the manufacturer's quality management system documentation;
- a review of the documentary evidence of safety and performance; and
- an independent performance evaluation

Liferiver Zika Virus (ZIKV) Real Time RT-PCR Kit with product code **ER-0360-02** manufactured by **Shanghai ZJ Bio-Tech Co., Ltd,** Building #26, 588 Xinjunhuan Road, Shanghai 201114, China, rest of world regulatory version, was listed as eligible for WHO procurement on 06 March 2018.

Intended Use

The Liferiver Zika Virus (ZIKV) Real Time RT-PCR Kit is an in vitro diagnostic medical device, based on real time RT-PCR technology utilizing reverse-transcriptase (RT) reaction to convert RNA into complementary DNA (cDNA). It is intended for the qualitative detection of Zika virus RNA. Serum, saliva and urine specimens are validated for use. The function of the assay is for the diagnosis or aid for diagnosis. Reactive results should be confirmed by details of confirmatory step which have been introduced in the Interpretation of Results. The assay is used to detect, define or differentiate a condition or risk factor of interest. The assay is manually operated and to be used with the RNA extraction kits and PCR instruments listed below. The assay is for use by a laboratory professional trained to use real time PCR in a laboratory setting. The use of RT-PCR method shortens the testing window, which is conducive to earlier identification and treatment of infection.

Test kit contents:

Reagents	Quantity Sufficient for 25 Tests	Colour Coding
ZIKV Super Mix	1 vial, 513µl	transparent
RT-PCR Enzyme Mix	1 vial, 27µl	green
ZIKV Internal Control	1 vial, 30µl	orange
ZIKV Negative Control	1 vial, 200µl	green
ZIKV Positive Control	1 vial, 200µl	white
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Items required but not provided:

Material	Product name/description
RNA extraction kits	Options: • QIAamp Viral RNA Mini Kit (Qiagen, Cat. No. 52904 or 52906) • RNA Isolation Kit (Paramagnetic Beads Column) (Liferiver, Cat. No. ME-0010) • 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)
Real-Time PCR instrumentation	Options: • Bio-Rad CFX 96 • SLAN-96 • ABI Prism 7500
Other equipment requirements	• Microcentrifuge (with rotor for 1.5ml and 2ml tubes) • Freezer • Refrigerator • Class II biosafety cabinet • Heating block or water bath for lysis of specimens at 56°C • Vortex mixer • Pipettes
Consumables	• Pipette tips with aerosol barrier • RNase-free microtubes • Tube racks • Ethanol (96–100%) • Powder-free gloves • Lab coat • Eye protection

Storage: The test kit should be stored at minus 15 to 25°C.

Shelf-life upon manufacture: 18 months.

Summary of the WHO EUAL assessment for Liferiver Zika Virus (ZIKV) Real Time RT-PCR Kit

Shanghai ZJ Bio-Tech Co. submitted an expression of interest for WHO emergency quality assessment of Liferiver Zika Virus (ZIKV) Real Time RT-PCR Kit on 04 March 2016.

Review of quality management documentation

To establish the eligibility for WHO procurement, Shanghai ZJ Bio-Tech Co. was asked to provide up-to-date information about the status of their quality management system.

Based on the review of the submitted quality management system documentation, it was established that sufficient information was provided by Shanghai ZJ Bio-Tech Co. to fulfil the requirements described in the "*Invitation to manufacturers of in vitro diagnostics for Zika virus to submit an application for emergency use assessment and listing by WHO*"[1].

Quality management documentation for Emergency Use Assessment and Listing conclusion: **Acceptable**

Product dossier assessment

Shanghai ZJ Bio-Tech Co. submitted documentation in support of safety and performance for Liferiver Zika Virus (ZIKV) Real Time RT-PCR Kit as per the "*Invitation to manufacturers of in vitro diagnostics for Zika virus to submit an application for emergency use assessment and listing by WHO*". The information submitted in the product application was reviewed by WHO staff and external experts (reviewers) appointed by WHO. The findings of the reviews were reported in accordance with "Instructions for Submission Requirements: In vitro diagnostics (IVDs) Detecting Zika Virus Nucleic Acid or Antigen" (WHO document PQDx_0240).

Safety and performance documentation for Emergency Use Assessment and Listing conclusion: **Acceptable**

Laboratory evaluation

Analytical testing

Testing was conducted with the international standard (IS, code 11468/16) developed by the Paul-Ehrlich-Institut (PEI), Langen, Germany on behalf of WHO. The standard has been assigned a potency of 7.70 $\log_{10}$ IU/ml based on the results of an international collaborative study and was approved by the Expert Committee on Biological Standardization (ECBS) in October 2016.

The study was performed under the BSL-2 conditions. Reference material was reconstituted in 0.5 ml of nuclease-free water.

[1] Invitation to manufacturers of in vitro diagnostics for Zika virus to submit an application for emergency use assessment and listing by WHO.

One hundred and forty µl sample volume was used for the extraction. The final elution volume was 2 x 40 µl of which 5 µl were used for the amplification/detection reaction (corresponding to a sample volume equivalent of 8.75 µl). Testing was performed on the Bio-Rad CFX 96 (Bio-Rad Laboratories GmbH, Munich, Germany) instrument in accordance with manufacturer's recommendations and as per the instrument's user manual.

Testing in plasma

Three independent dilution series of 11468/16 were prepared in Zika virus-negative pooled human plasma for the evaluation. In the initial testing, 11468/16 was diluted in 10-fold dilution steps down to 10^8 IU. Sufficient volume was prepared so that duplicate extractions could be performed. Dilutions between log 10^{-3} and 10^{-8} ($5x10^4$ to $5x10^1$ IU/ml) were tested in order to determine the end-point. The duplicate RNA extracts were each tested in duplicate PCR reactions.

Subsequently, 11468/16 was tested in half $\log_{10}$ dilution steps between -4.5 $\log_{10}$ and -7.0 $\log_{10}$ ($1.58x10^3$ to $1.58x10^0$ IU/ml). Five replicates of each dilution were extracted and PCR was performed singly on each sample, per dilution.

A third round of testing was performed using 11468/16 tested in half $\log_{10}$ dilution steps – between -5.0 $\log_{10}$ and -7.5 $\log_{10}$ ($5x10^2$ to $1.58x10^0$ IU/ml). Five replicates of each dilution were extracted and PCR was performed singly on each sample, per dilution.

The analytical sensitivity (95% limit of detection [LoD]) of Liferiver Zika Virus (ZIKV) Real Time RT-PCR Kit in plasma specimens was determined by Probit analysis. The LoD (at 95% hit rate) was 118.7 IU/ml (combined value from all three runs), with the 95% confidence interval (CI) ranging from (95% CI: 64.0 – 220.1) IU/ml.

Testing in urine

Three independent dilution series of 11468/16 were prepared in Zika virus-negative human urine for the evaluation. In the initial testing, 11468/16 was diluted in half $\log_{10}$ dilution steps between -4.5 $\log_{10}$ and -7.0 $\log_{10}$ ($1.58x10^3$ to $1.58E10^0$ IU/ml). Five replicates of each dilution were extracted and PCR was performed singly on each sample, per dilution. In the case of one -6.5 $\log_{10}$ dilution, the sample had an internal control failure and was excluded from analysis. In addition, four samples at the -7.0 $\log_{10}$ dilution also had internal control failures and were also excluded from the analysis.

Subsequently, 11468/16 was tested in half $\log_{10}$ dilution steps between -4.5 $\log_{10}$ and -7.0 $\log_{10}$ ($1.58x10^3$ to $1.58x10^0$ IU/ml). Five replicates of each dilution were extracted and PCR was performed singly on each sample, per dilution. Internal control failures were reported for four samples at the -6.0 and -6.5 $\log_{10}$ dilutions as well as two internal control failures for the -7.0 $\log_{10}$ dilution and these results were excluded from the analysis.

For the third dilution series, 11468/16 was tested in half $\log_{10}$ dilution steps between -4.5 $\log_{10}$ and -7.0 $\log_{10}$ (($5x10^3$ to $1.58x10^0$ IU/ml). Five replicates of each dilution were extracted and PCR was performed singly on each sample per dilution. There was a single internal control failure for one sample at the -7.0 $\log_{10}$ dilution and this was excluded from the analysis.

The analytical sensitivity (95% limit of detection [LoD]) of Liferiver Zika Virus (ZIKV) Real Time RT-PCR Kit in urine specimens was determined by Probit analysis. The LoD (at 95% hit rate) was 223.8 IU/ml (combined value from all three runs), with the 95% confidence interval (CI) ranging from (95% CI: 108.2 - 463.1) IU/ml.

Table 1 Dose for centile 95 (95% LoD) and confidence interval (CI)

	Plasma	Urine
95% LoD (CI) IU/ml	118.7 IU/ml (95% CI: 64.0 -220.1)	223.8 IU/ml (95% CI: 108.2 - 463.1)

Clinical Testing:
Clinical testing was performed at the Institut Pasteur in Cayenne, French Guiana.

Specimens were collected from patients exhibiting symptoms of Zika virus infection between the 1st and the 8th day following the onset of disease. A total of 206 clinical specimens were obtained from 152 patients (including 52 patients with paired (sera and urine) and/or sequential samples). The specimen types collected were: 101 serum, 1 plasma and 104 urine specimens.

All specimens were tested with the Liferiver Zika Virus (ZIKV) Real Time RT-PCR Kit as well as the RealStar® Zika Virus RT-PCR Kit 1.0, used as a benchmark assay.

Of the 206 specimens included in the comparison study 90 (43.6%) were considered as positive for Zika virus RNA with the Liferiver Zika Virus (ZIKV) Real Time RT-PCR Kit. Eleven specimens (5.3%), with no amplification for Zika and for internal control, were classified as undetermined. All eleven undetermined specimens tested positive with the RealStar® Zika Virus RT-PCR Kit 1.0.

The results are summarized in the following table:
Table 2 Overall performance with serum and urine specimens

Results from Liferiver Zika Virus real time RT-PCR kit	**Results from RealStar® Zika Virus RT-PCR Kit**		
	Positive	**Negative**	**Total**
Positive	90	0	90
Negative	19	86	105
Undetermined*	2	9	11
Total	111	95	206

*Undetermined: with no positive amplification result at FAM (=Zika) and VIC (=IC) channel (Liferiver interpretation: “PCR inhibition, no diagnosis can be concluded”)

Overall performance of the Liferiver Zika Virus (ZIKV) Real Time RT-PCR Kit:
Including undetermined as negative:
- Positive Percent Agreement: 81.1% (90/111) - 95% C.I. 72.5-87.9%
- Negative Percent Agreement: 100.0% (95/95) - 95% C.I. 96.2-100%

Excluding undetermined results:
- Positive Percent Agreement: 82.6% (90/109) - 95% C.I. 74.1-89.8%
- Negative Percent Agreement: 100.0% (86/86) - 95% C.I. 95.8-100%

Performance in serum/plasma specimens:

Table 3 Performance with serum/plasma specimens

Results from Liferiver Zika Virus real time RT-PCR kit	**Results from RealStar® Zika Virus RT-PCR Kit U.S.**		
	Positive	**Negative**	**Total**
Positive	46	0	46
Negative	16	40	56
Total	62	40	102

Positive Percent Agreement: 74.2% (46/62, 95% C.I. 61.5-84.5%)
Negative Percent Agreement: 100% (40/40, 95% C.I. 91.2-100%)

Performance in urine specimens:
Table 4 Performance with urine specimens

Results from Liferiver Zika Virus real time RT-PCR kit	**Results from RealStar® Zika Virus RT-PCR Kit U.S.**		
	Positive	**Negative**	**Total**
Positive	44	0	44
Negative	5	55	60
Total	49	55	104

Positive Percent Agreement: 89.8% (44/49, 95% C.I. 77.8-96.6%)
Negative Percent Agreement: 100% (55/55, 95% C.I. 93.5-100%)

Cross reactivity:
Forty four Dengue positive serum specimens were used:
Dengue 1: 9 specimens

Dengue 2: 10 specimens
Dengue 3: 1 specimen
Dengue 4: 10 specimens

All 30 specimens tested negative with the Liferiver Zika Virus real time RT-PCR kit.

Ten Chikungunya positive serum specimens were also tested. All 10 specimens tested negative with the Liferiver Zika Virus real time RT-PCR kit.

Laboratory evaluation for Emergency Use Assessment and Listing conclusion:
Acceptable

WHO Emergency Use Assessment and Listing Decision

Based on the review of the manufacturer's submitted data, as well as data generated from the limited laboratory evaluation Liferiver Zika Virus real time RT-PCR kit is eligible for WHO procurement.

Post market surveillance to monitor the performance of Liferiver Zika Virus real time RT-PCR kit in comparison is highly recommended.

Scope and duration of procurement eligibility

Liferiver Zika Virus real time RT-PCR kit with product code **ER-0360-02**, manufactured by **Shanghai ZJ Bio-Tech Co.** is considered to be eligible for WHO procurement. This listing does not infer that the product meets WHO prequalification requirements and does not mean that the product is listed as WHO prequalified.

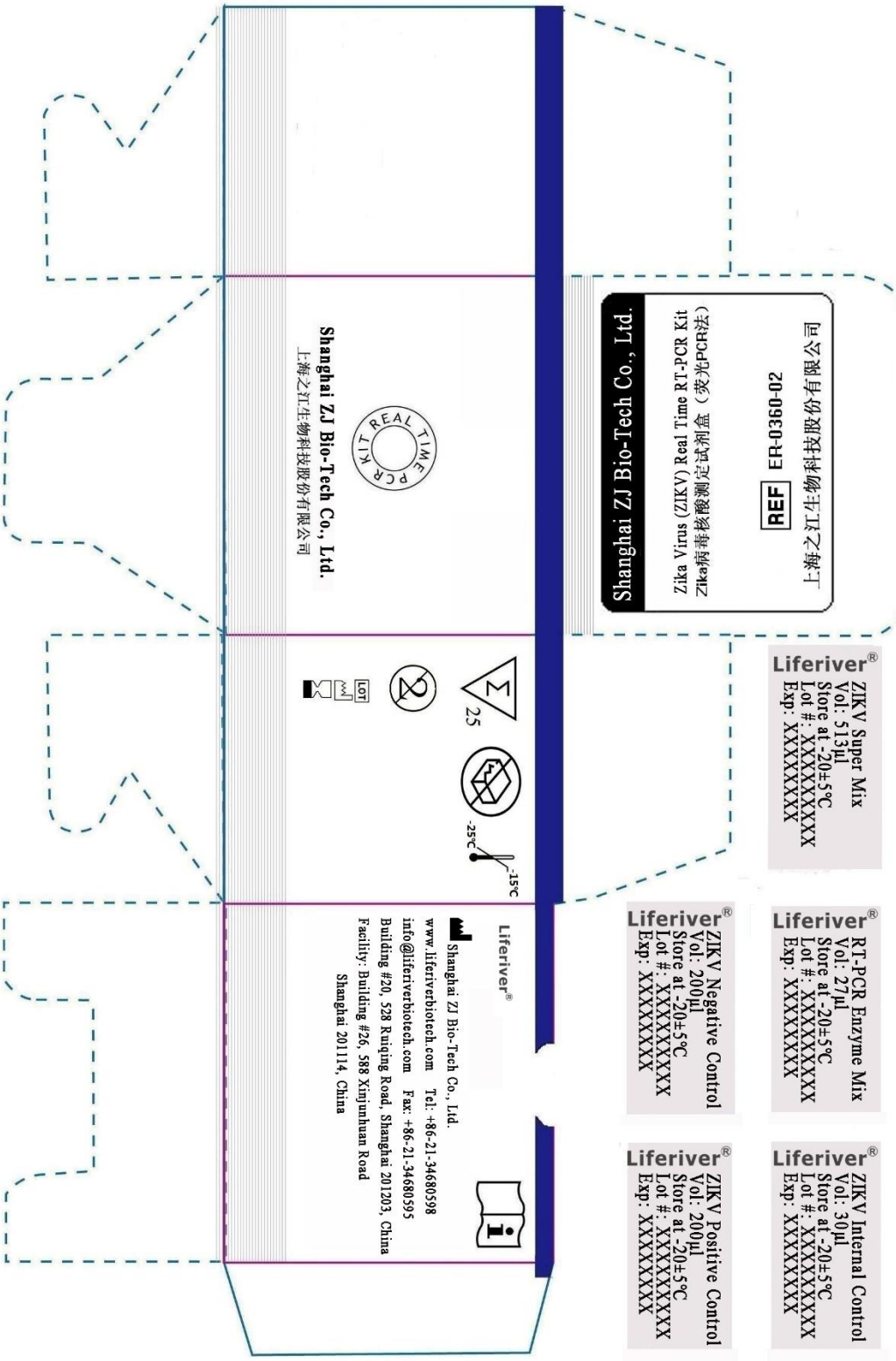
As part of the on-going requirements for listing as eligible for WHO procurement **Shanghai ZJ Bio-Tech Co.** must engage in post-market surveillance activities to ensure that the product continues to meet safety, quality and performance requirements. **Shanghai ZJ Bio-Tech Co.** is required to notify WHO of any complaints, including adverse events related to the use of the product within 7 days of receipt. Furthermore, WHO will continue to monitor the performance of the assay in the field.

WHO reserves the right to rescind eligibility for WHO procurement, if additional information on the safety, quality and performance comes to WHO's attention during post-market surveillance activities.

Labelling

1. **Labels**
2. **Instructions for use**

1. Labels


Shanghai ZJ Bio-Tech Co., Ltd.
Zika Virus (ZIKV) Real Time RT-PCR Kit
Zika病毒核酸测定试剂盒（荧光PCR法）
REF ER-0360-02
上海之江生物科技股份有限公司
Liferiver®
ZIKV Super Mix
Vol: 513μl
Store at -20±5℃
Lot #: XXXXXXXXX
Exp: XXXXXXXX
Liferiver®
RT-PCR Enzyme Mix
Vol: 27μl
Store at -20±5℃
Lot #: XXXXXXXXX
Exp: XXXXXXXX
Liferiver®
ZIKV Negative Control
Vol: 200μl
Store at -20±5℃
Lot #: XXXXXXXXX
Exp: XXXXXXXX
Liferiver®
ZIKV Internal Control
Vol: 30μl
Store at -20±5℃
Lot #: XXXXXXXXX
Exp: XXXXXXXX
Liferiver®
ZIKV Positive Control
Vol: 200μl
Store at -20±5℃
Lot #: XXXXXXXXX
Exp: XXXXXXXX
Shanghai ZJ Bio-Tech Co., Ltd.
上海之江生物科技股份有限公司
REAL TIME PCR KIT
Σ 25
-25℃ -15℃
LOT
Liferiver®
Shanghai ZJ Bio-Tech Co., Ltd.
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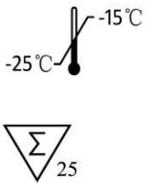
2. Instructions for use

Liferiver®

Revision No.: ZJ0003
Issue Date: February 28, 2018

Zika Virus (ZIKV) Real Time RT-PCR Kit

User Manual



For In Vitro Diagnostic Use Only

REF ER-0360-02

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

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Table of Contents

General Information

1 Intended Use

The Liferiver Zika Virus (ZIKV) Real Time RT-PCR Kit is an in vitro diagnostic medical device, based on real time RT-PCR technology utilizing reverse-transcriptase (RT) reaction to convert RNA into complementary DNA (cDNA). It is intended for the qualitative detection of Zika virus RNA. Serum, saliva and urine specimens are validated for use. The function of the assay is for the diagnosis or aid for diagnosis. Reactive results should be confirmed by details of confirmatory step which have been introduced in the Interpretation of Results. The assay is used to detect, define or differentiate a condition or risk factor of interest. The assay is manually operated and to be used with the RNA extraction kits and PCR instruments listed below. The assay is for use by a laboratory professional trained to use real time PCR in a laboratory setting.

The use of RT-PCR method shortens the testing window, which is conducive to earlier identification and treatment of infection.

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 a 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 a PCR with the advantage of detecting the amount of DNA formed after each cycle with a fluorescently-tagged oligonucleotide probe. The probe is 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 is quenched by the quencher dye and so no fluorescence can be detected in the absence of target sequence. 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 real time PCR instrument. Testing data is generated, collected and analyzed by specialized software.

3 Product Description

Zika virus (ZIKV) is an emerging mosquito-borne virus that was first identified in Uganda in 1947 in rhesus monkeys. It was subsequently identified in humans in 1952 in Uganda and the United Republic of Tanzania. Zika virus disease outbreaks were reported for the first time from the Pacific in 2007 and 2013 (Yap and French Polynesia, respectively), and in 2015 from the Americas (Brazil and Colombia) and Africa (Cape Verde). In addition, more than 13 countries in the Americas have reported sporadic Zika virus infections indicating rapid geographic expansion of Zika virus.

Zika virus is a member of the virus family Flaviviridae, enveloped and icosahedral with a non-segmented, single-stranded, positive sense RNA genome. It is most closely related to the Spondweni virus and is one of the two viruses in the Spondweni virus clade. Zika virus is transmitted to people primarily through the bite of an infected *Aedes* species mosquito. The recent reported cases indicate that the virus could possibly spread through blood transfusion and sexual contact.

People with Zika virus disease usually have symptoms that can include mild fever, skin rashes, conjunctivitis, muscle and joint pain, malaise or headache. These symptoms normally last for 2-7 days. Data suggests that infection with Zika virus during pregnancy may result in microcephaly.

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

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.

4 Warnings and Precautions

- Read this user manual carefully before using the kit.
- Before first use please check the product and its components for integrity, completeness, correct labeling and that the kit components are frozen upon arrival.
- 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.
- Dedicate supplies and equipment to the separate working areas and do not move them from one area to another.
- Specimens should always be treated as infectious and/or biohazardous in

accordance with safe laboratory procedures.

- Avoid microbial and nuclease (DNase/RNase) contamination of the specimens and the components of the kit.
- Store positive and/or potentially positive material separated from all other components of the kit.
- Use of this product is limited to personnel specially instructed and trained in the techniques of real time PCR and *in vitro* diagnostic procedures.
- Wear protective disposable powder-free gloves, a laboratory coat and eye protection when handling specimens.
- Always use DNase/RNase-free disposable pipette tips with aerosol barriers.
- Always wear protective disposable powder-free gloves when handling kit components.
- Additional controls may be tested according to guidelines or requirements of local, state and/or federal regulations or accrediting organizations.
- Do not open the reaction tubes/plates post amplification, to avoid contamination with amplicons.
- Do not autoclave reaction tubes after the PCR, since this will not degrade the amplified nucleic acid and will bear the risk to contaminate the laboratory area.
- Discard sample and assay waste according to your local safety regulations.
- Do not use components of the kit that have passed the expiration date.

5 Kit Contents

Reagents	Quantity Sufficient for 25 Tests	Color Coding
ZIKV Super Mix	1 vial, 513µl	transparent
RT-PCR Enzyme Mix	1 vial, 27µl	green
ZIKV Internal Control	1 vial, 30µl	orange
ZIKV Negative Control	1 vial, 200µl	green
ZIKV Positive Control	1 vial, 200µl	white
User Manual	1	-

Control materials

- ZIKV Negative Control is saline that will serve as an external negative specimen during RNA extraction procedure. It should be used whenever RNA extraction procedure takes place.
- ZIKV Positive Control is a pseudovirus containing partial ZIKV RNA fragment that will serve as an external positive control during RNA extraction procedure. The ZIKV 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. It should be used whenever RNA extraction procedure takes place.
- 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 pseudovirus will be amplified by the primers used to amplify target sequence, but it will be detected by another probe. IC should be used whenever RNA extraction procedure takes place.

6 Storage

- All reagents should be stored at -20±5°C.
- All reagents should be used before expiration date indicated on kit.
- Repeated thaw-&-freeze for more than 3 times should be avoided as this may reduce the sensitivity of the assay.

7 Specimen Requirements

7.1 Specimen Types

- Serum

Take 2ml of venous blood with a sterile syringe. The blood is collected in a disposable sterile tube. After coagulation of blood, centrifuge the blood at 3,000rpm for 10 minutes.

- Saliva

Collect saliva with 1 or 2 swabs from anterior floor of mouth and near Stenson's ducts. Place swabs into viral transport media (FLOQSwabs™, COPAN DIAGNOSTICS, INC).

- Urine

Collect 10-20ml of midstream clean-voided urine in a sterile container.

7.2 Specimen Storage

- Keep refrigerated (2~8°C) if it is to be processed within 24 hours.
- Keep frozen (-20±5°C) if it is to be processed after the first 24 hours or within 7 days.
- Keep frozen (-70±5°C) if it is to be processed after a week. The sample can be preserved for extended periods.

7.3 Shipment

- Ship within 48 hours. Ship (insofar as possible) with dry ice; at the very least, maintain the cold chain with cooling gels. Always use triple packaging.
- If shipment is delayed and facilities are available, the specimens should be frozen at -70°C and shipped on dry ice.

8 RNA Extraction Procedure for Specimens and Controls

The nucleic acid extraction reagents are not supplied in this kit and three commercial kits are recommended for ZIKV RNA extraction:

1) QIAamp Viral RNA Mini Kit (Qiagen, Cat. # 52904 or 52906);
2) RNA Isolation Kit (Paramagnetic Beads Column) (Liferiver, Cat. # ME-0010);
3) Ribonucleic Acid (RNA) Isolation Kit (Preloaded for Auto-Extraction) (Liferiver, Cat. # ME-0014).

It is noted that the ZIKV positive control and ZIKV 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 1μl/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 below:

- Freezer (-20±5°C, -70±5°C) and refrigerator (2-8°C),
- Vortex
- Microcentrifuge† (with rotor for 1.5ml and 2ml 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.
- Pipets† and pipette tips (To prevent cross-contamination, we strongly recommend the use of pipette tips with aerosol barriers)
- Ethanol (96–100%)*
- QIAamp Viral RNA Mini Kit (Qiagen, Cat. # 52904 or 52906) or RNA Isolation Kit (Paramagnetic Beads Column) (Liferiver, Cat. # ME-0010) or Ribonucleic Acid (RNA) Isolation Kit (Preloaded for Auto-Extraction) (Liferiver, Cat. # ME-0014) associated with EX2400 Automated Nucleic Acid Extraction System (Liferiver, Cat. # 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 serum, saliva or urine specimens. The specimens can be stored at 2–8°C for up to 24 hours. For long-term storage, freezing at –20±5°C or –70±5°C in aliquots is recommended (refer to Section 7.2). Frozen specimens must not be thawed more than once. Repeated freeze–thawing leads to denaturation and precipitation of proteins, results in reduced viral titers and therefore reduces yields of viral nucleic acids. In addition, cryoprecipitates might be formed during freeze–thawing. 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 QIAGEN--QIAamp Virus RNA Mini Kit

Note: QIAamp Mini spin columns should be stored dry at room temperature (15–25°C); storage at higher temperatures should be avoided. All solutions should be stored at room temperature unless otherwise stated. QIAamp Mini spin columns and all buffers and reagents can be stored under these conditions until the expiration date on the kit box without showing any reduction in performance.

Lyophilized carrier RNA can be stored at room temperature until the expiration date on the kit box. Carrier RNA should be dissolved in Buffer AVE; dissolved carrier RNA should be immediately added to Buffer AVL as described on page 14. This solution should be prepared fresh, and is stable at 2–8°C for up to 48 hours. Buffer AVL–carrier RNA develops a precipitate when stored at 2–8°C that must be redissolved by warming at 80°C before use. Unused portions of carrier RNA dissolved in Buffer AVE should be frozen in aliquots at –20°C. Do not freeze–thaw the aliquots of carrier RNA more than 3 times.

DO NOT warm Buffer AVL–carrier RNA solution more than 6 times. DO NOT incubate at 80°C for more than 5 min. Frequent warming and extended incubation will cause degradation of the carrier RNA, leading to reduced recovery of viral RNA and eventually false negative RT-PCR results, particularly when low-titer samples are used.

1) Pipette 560µl of prepared Buffer AVL containing carrier RNA into a 1.5ml microcentrifuge tube.
2) Add 140µl of specimen, or positive control or negative control and 1µl internal control to the Buffer AVL-carrier RNA in the microcentrifuge tube. Mix 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 560µl 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 2ml

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 clean 2ml collection tube (from the QIAamp Mini Kit) and add 500µl 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 2ml collection tube (from the QIAamp Mini Kit). Carefully open the QIAamp spin column and add 500µl 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 1.5ml RNase-free microcentrifuge tube (not provided). Discard the old collection tube containing the filtrate.
15) Carefully open the QIAamp Mini column and add 60µl 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 (do not exceed 3) repeated freeze-thaw cycles.

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

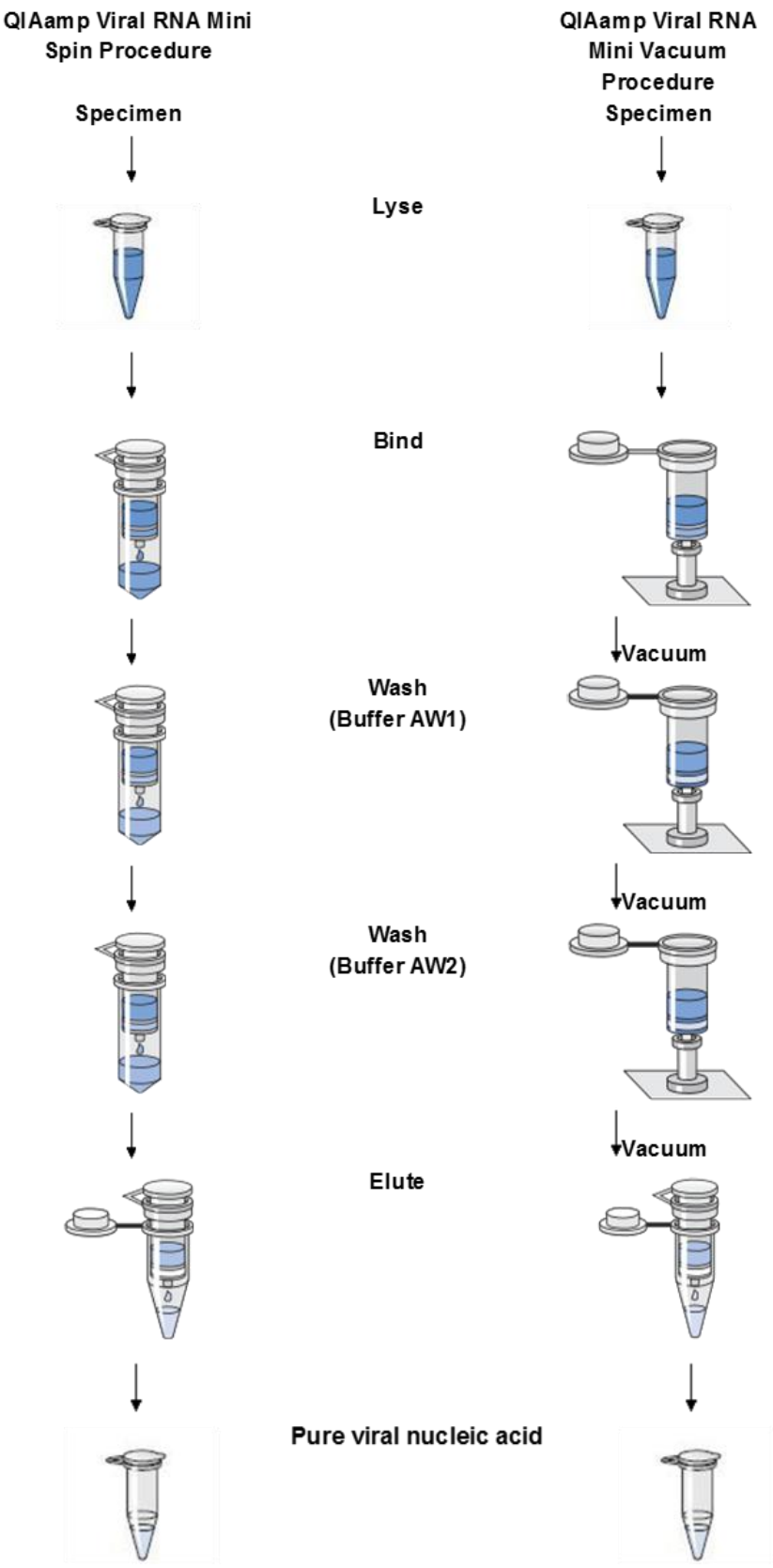


Figure 1. Viral RNA Isolation Procedure using QIAamp Viral RNA Mini Kit. The illustration shows the simple steps (lyse, bind, wash, and elute) of the spin protocol and the vacuum protocol.

8.2.2 Liferiver® RNA Isolation Kit (Paramagnetic Beads Column)

1) Pipette 526µl of prepared Binding Solution containing 20µl of magnetic beads suspension and 6µl of carrier RNA into each 1.5ml RNase-free tube.
2) Transfer 140µl of specimen, or ZIKV positive control or ZIKV negative control and 1µl 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 liquid of 667µl in Step 3 to a Binding Column
5) Centrifuge the tube at 16,000 × 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 500µl of Washing Buffer A to each specimen and centrifuge the tube at 16,000 × g for 40 sec. Discard the filtrate in the collection tube.
7) Repeat Step 6.
8) Add 500µl of Washing Buffer W to each specimen and centrifuge the tube at 16,000 × g for 15 sec. Discard the liquid in the collection tube.
9) Repeat Step 8.
10) Centrifuge the binding columns at 16,000 × g for 2 min to thoroughly dry the membrane.
11) Add 50µl of Elution Buffer (preheated to 65°C) to each column, and incubate for 2 min at room temperature.
12) Centrifuge the tube at 16,000 × g for 2 min, and store the purified RNA in the 1.5ml RNase-free tube at –20 °C.

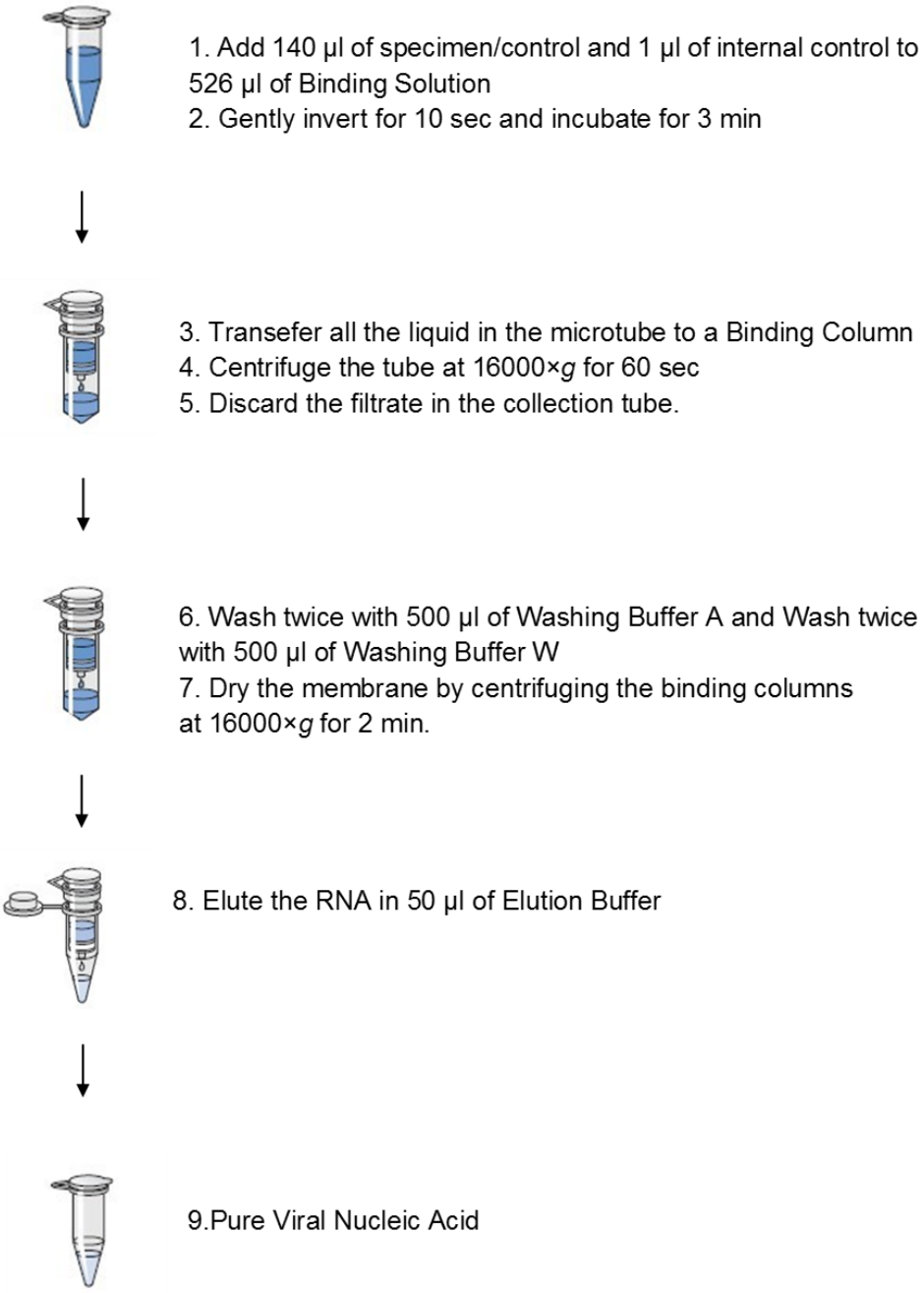


Figure 2. Viral RNA Isolation Procedure using Liferiver® RNA Isolation Kit (Paramagnetic Beads Column). The illustration shows the simple steps (lyse, bind, wash, and elute) of the protocol.

8.2.3 Liferiver® RNA Isolation Kit (Preloaded for Auto-Extraction)

ZIKV nucleic acid extraction can be automated using Liferiver RNA Isolation Kit (Preloaded for Auto-Extraction) on the EX2400 Automated Nucleic Acid Extraction System (Liferiver, Cat. # IE-0001).

8.2.3.1 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 (Add 350μl of Elution Buffer to each tube before use)	room temp	6μl

Note:

a) Do not freeze Preparing Plate.

b) Wells in Row A of Preparing Plate tend to be crystallized at low temperature（eg.＜10℃*), so dissolve it at 37*℃ *before use.*

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

8.2.3.2 RNA Isolation Procedure using Liferiver® RNA Isolation Kit (Preloaded for Auto-Extraction)

1) Pipette 1μl of ZIKV 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 of Carrier RNA and 200μl of mix (specimen, ZIKV positive control or ZIKV negative control and ZIKV internal control) into the wells A1-A12 of preparing plate.
4) Put preparing plate on the transport platform carefully and insert the magnetic cap.
5) Choose “RNA Isolation” program, and press “START” to run the test.
6) After the program is finished, discard the magnetic cap, take out preparing plate and transfer the liquid in wells E1-E12 into RNase-free EP tubes. It can be used for immediate experiment or stored at -20°C.

9 PCR Setup

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

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

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

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

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

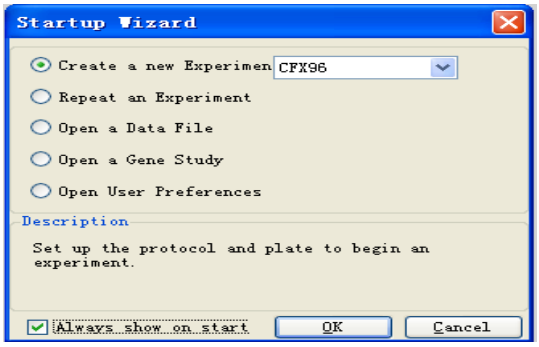
Instrument Specific Instructions

10 Operation Procedure on Bio-Rad CFX 96

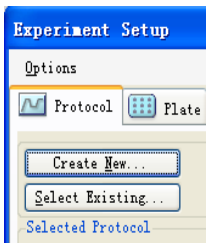
10.1 Double-click the icon 

to open the Bio-Rad CFX software.

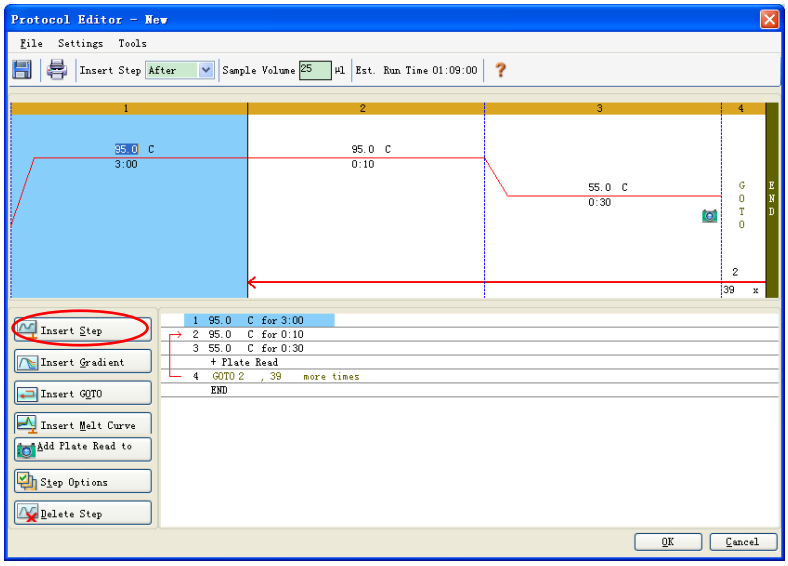
10.2 Create a new Experiment and choose the type of the instrument, click “OK”.



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.



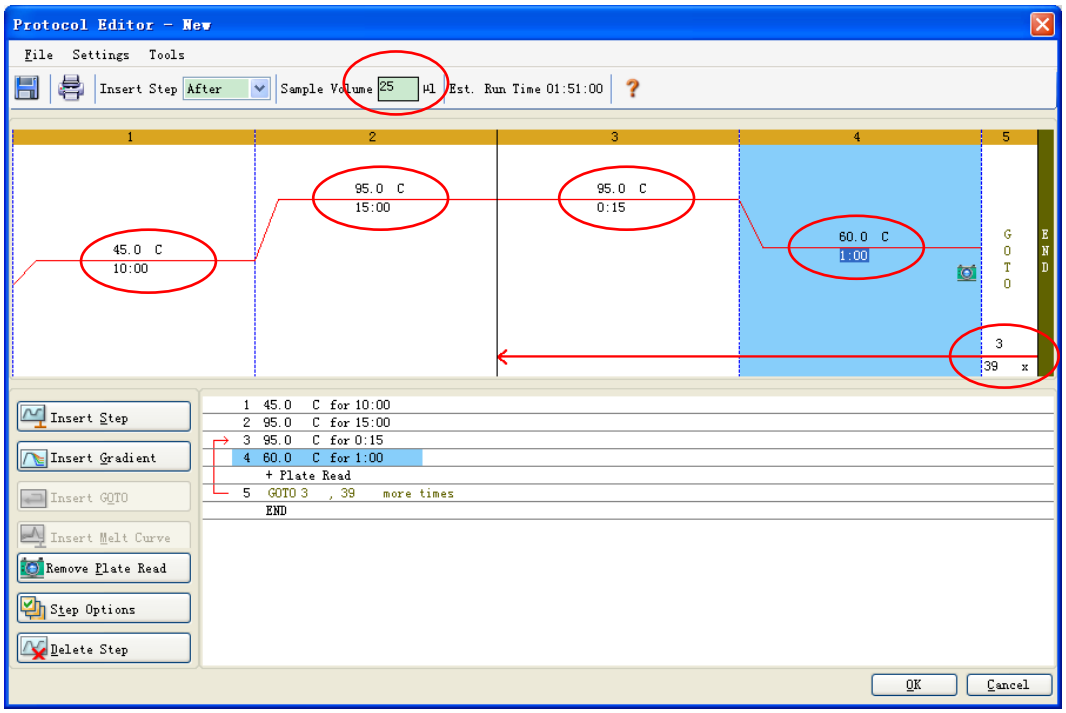
10.4 After clicking “Create New” button, you can see the interface below. Insert a step first.



10.5 Parameter setting:

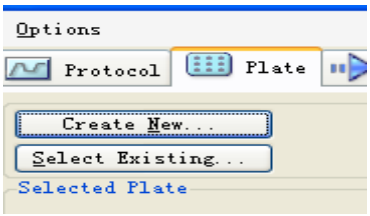
Step 1	45°C for 10 min	1 cycle
Step 2	95°C for 15 min	
Step 3	95°C for 15 sec	40 cycles
Step 4	60°C for 1 min *Fluorescence measured at 60°C	

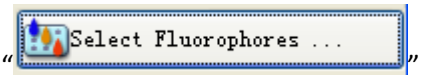
Sample volume: 25µl



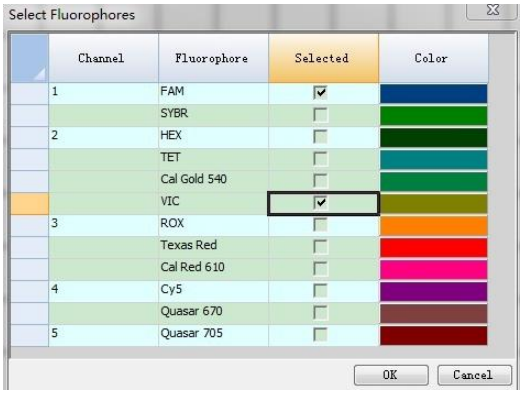
10.6 Click “Ok”. Save the protocol.

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

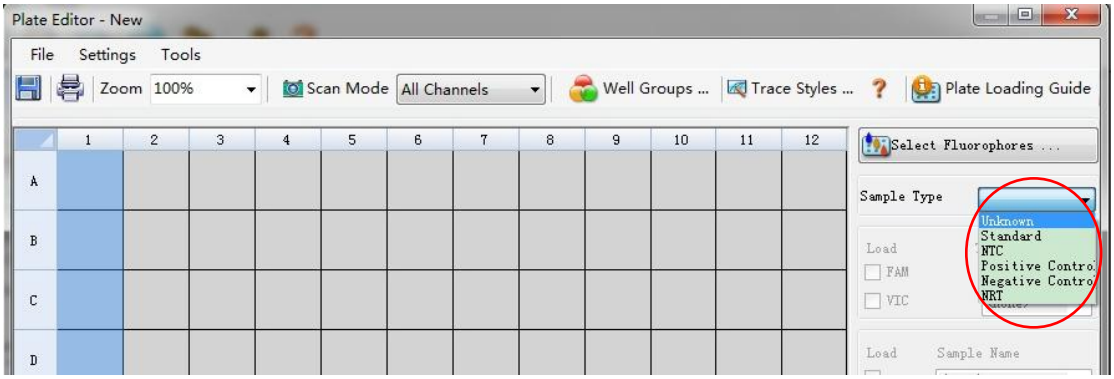


10.8 Select the fluorescence channel: Click “ 

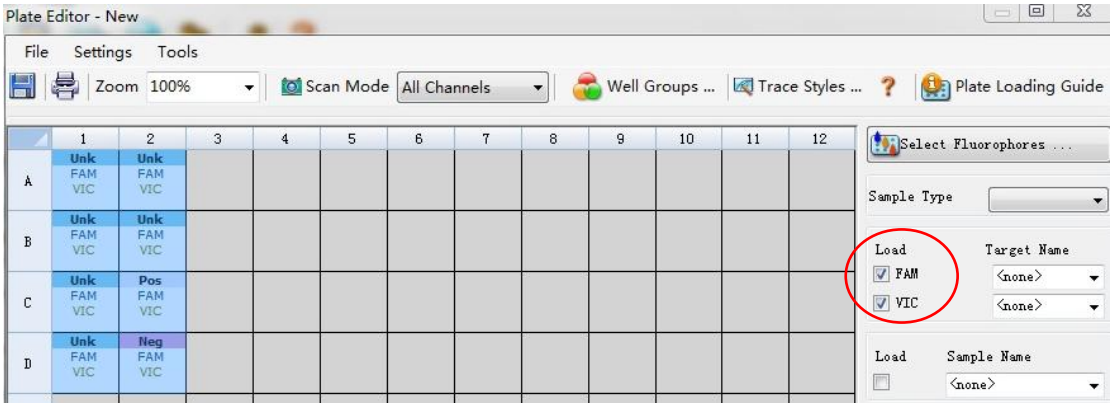
” to choose “FAM” and “VIC” channel, click “OK”.



10.9 Choose the well and select sample type in the drop-down list box. Choose “Unknown” for sample, “Positive Control” for positive control and “Negative Control” for negative control.

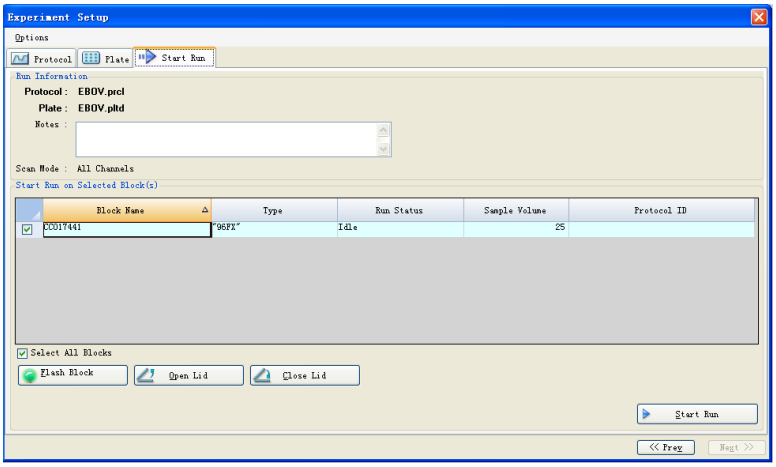


10.10 Choose all the wells in the experiment. Click “FAM” and “VIC”.

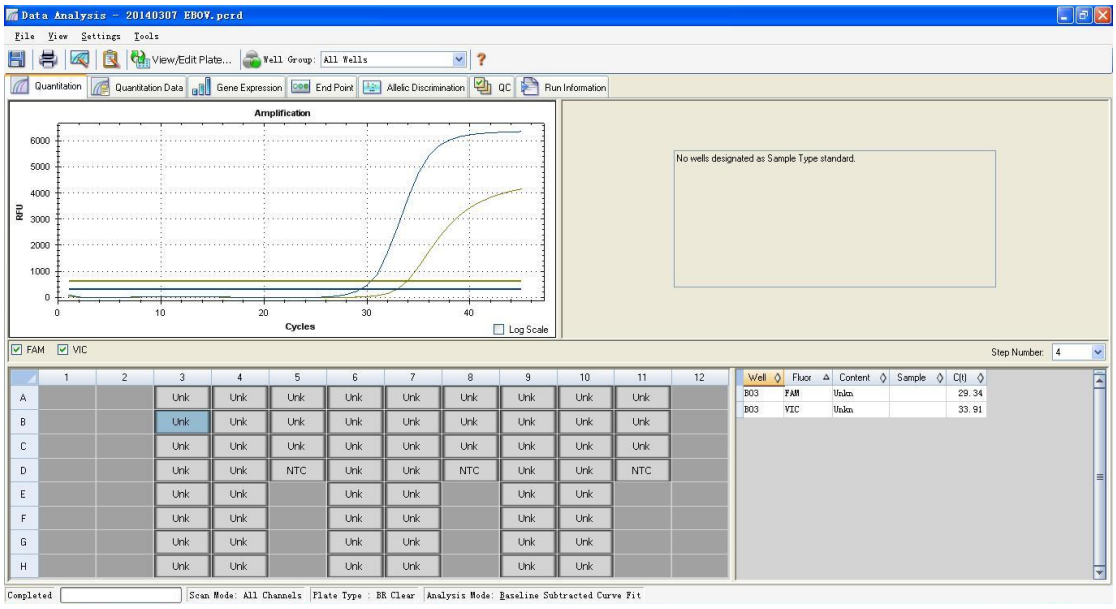


10.11 Click “OK”. Save the plate.

10.12 Under “Start Run” interface, click “Open Lid”. Add the plate into the instrument and then click “Close Lid”. Click “Start Run”, and then save the run file. The experiment begins.



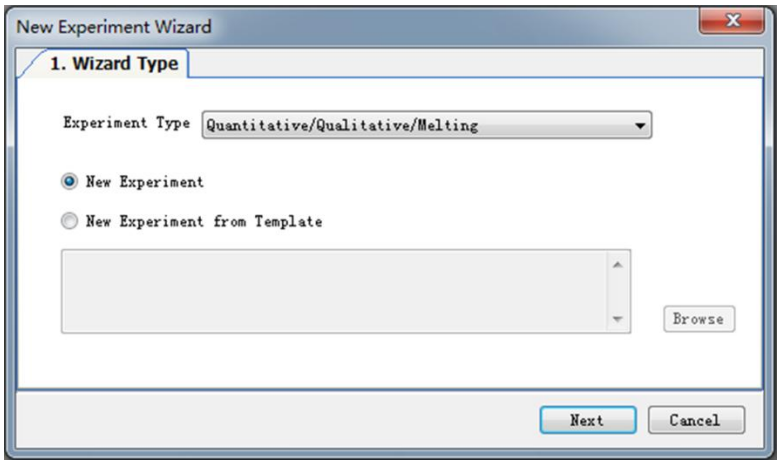
10.13 PCR Analysis: After the run is completed, click the well to read the Ct value on the lower right and amplification curve on the upper left.



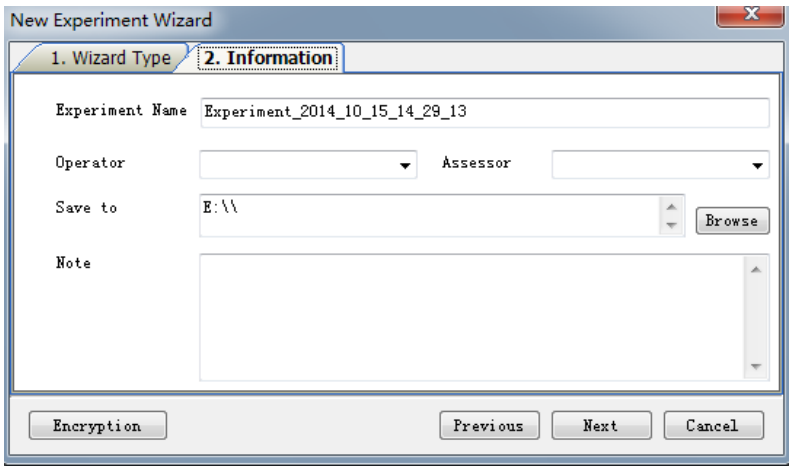
11 Operation Procedure on SLAN®-96

11.1 Enter the SLAN-96 PCR program by double clicking on the SLAN-96 system icon on the desktop.

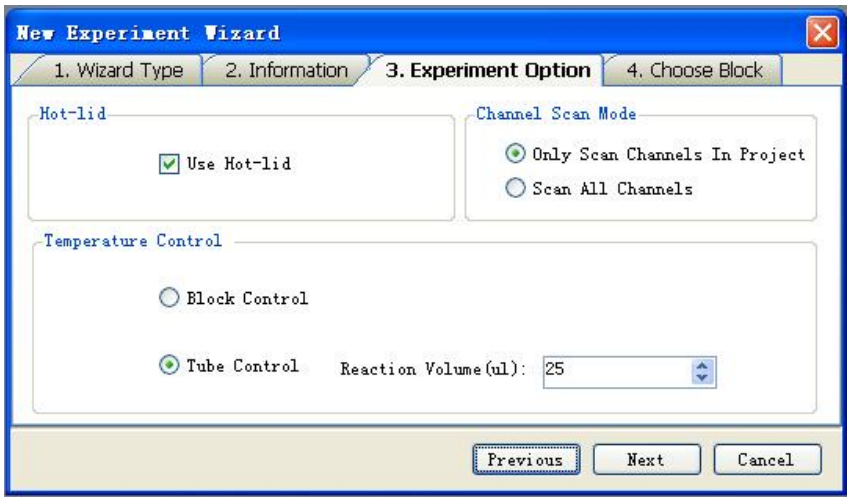
11.2 Select “Create New Document” from the Quick start up menu. The “New Experiment Wizard” screen will appear.



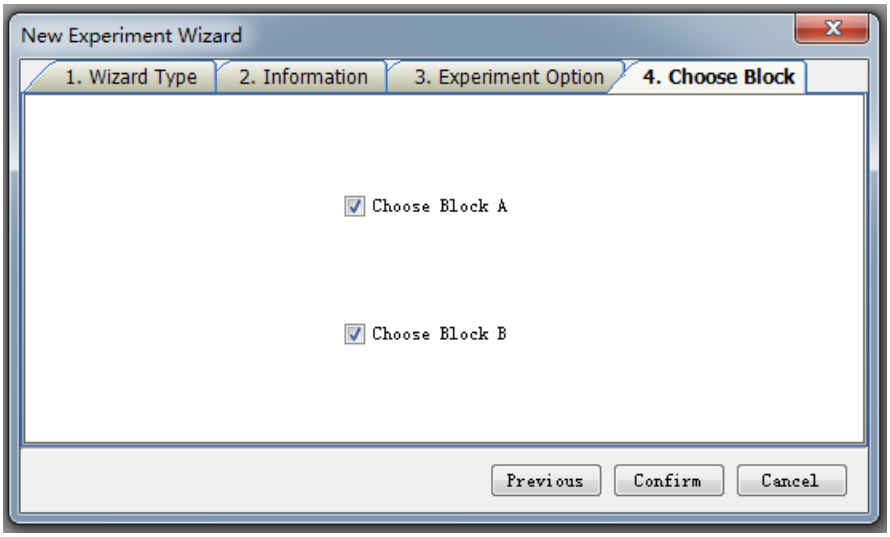
11.3 Click “Next”, enter the experiment name and save path in the new interface.



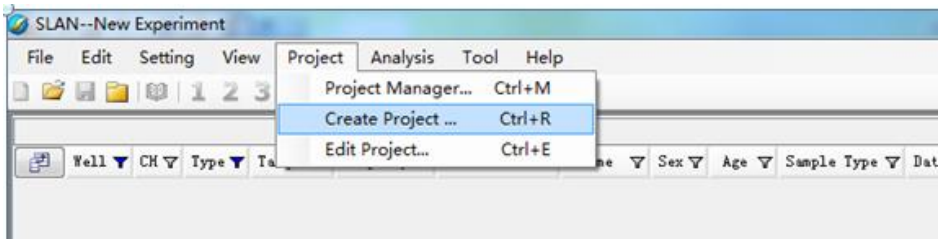
11.4 Click “Next”. Under temperature control, select “Tube Control”, and enter PCR reaction volume: 25μl.



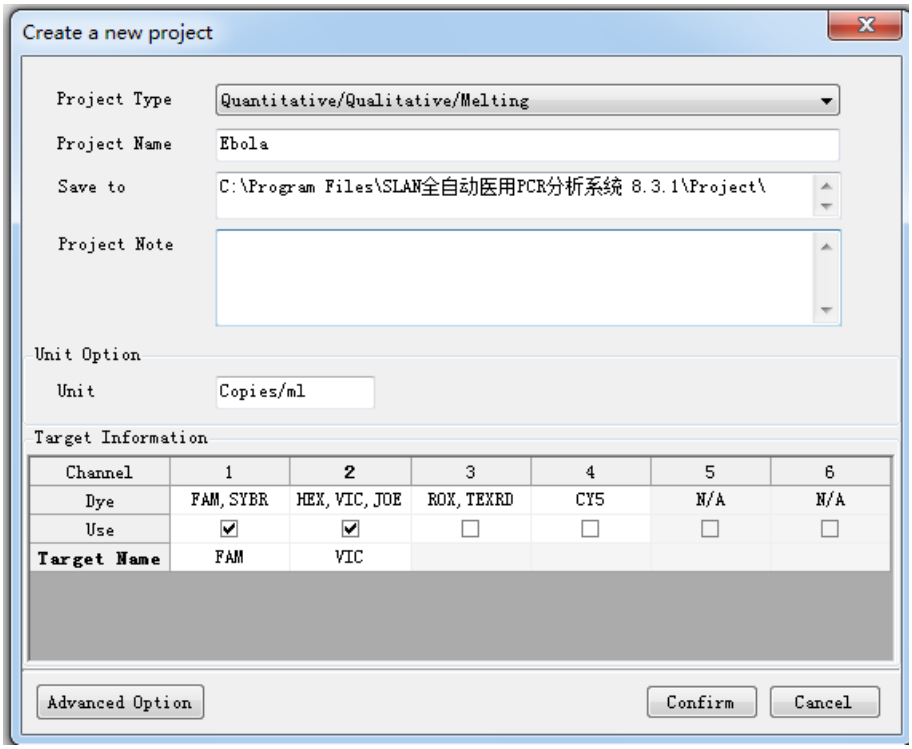
11.5 Click “Next”. Choose block A and/or block B where the PCR tubes are.



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



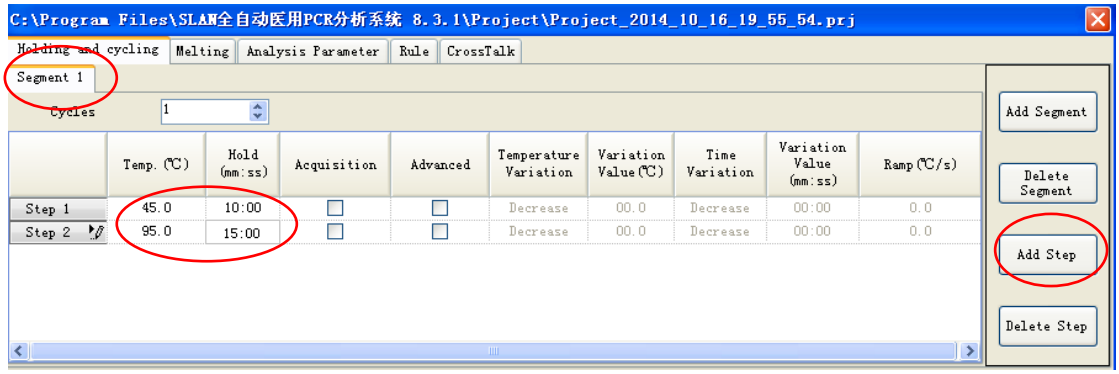
11.7 The following window will appear. Enter project name and save path, select the channel (FAM & VIC).



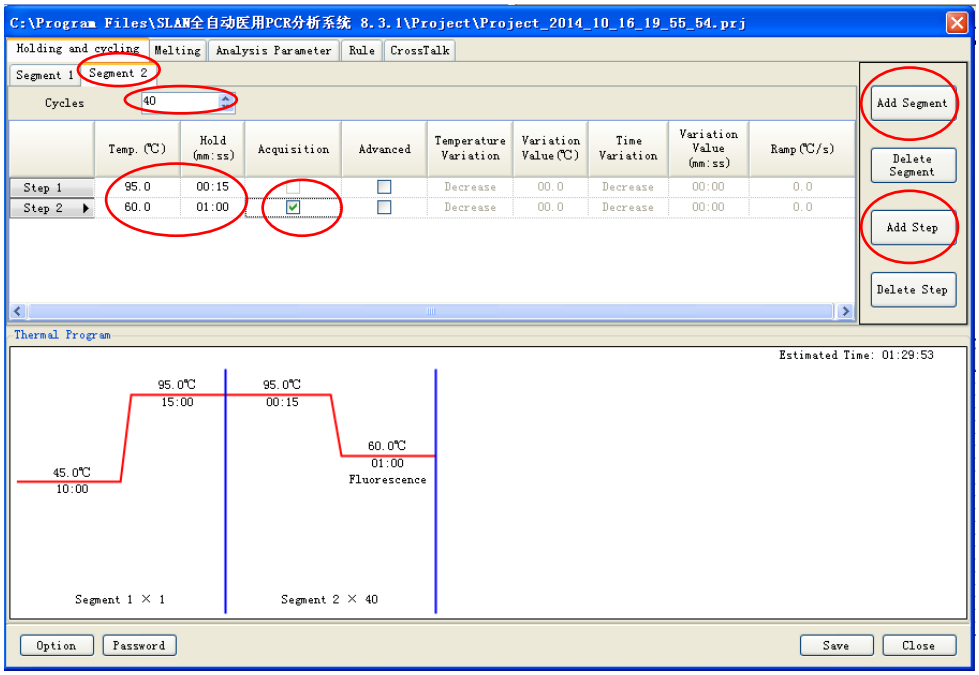
11.8 Click “Confirm”. Set parameters in the new window.

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

11.8.1 Segment 1: Click “Add Step” and set parameters.

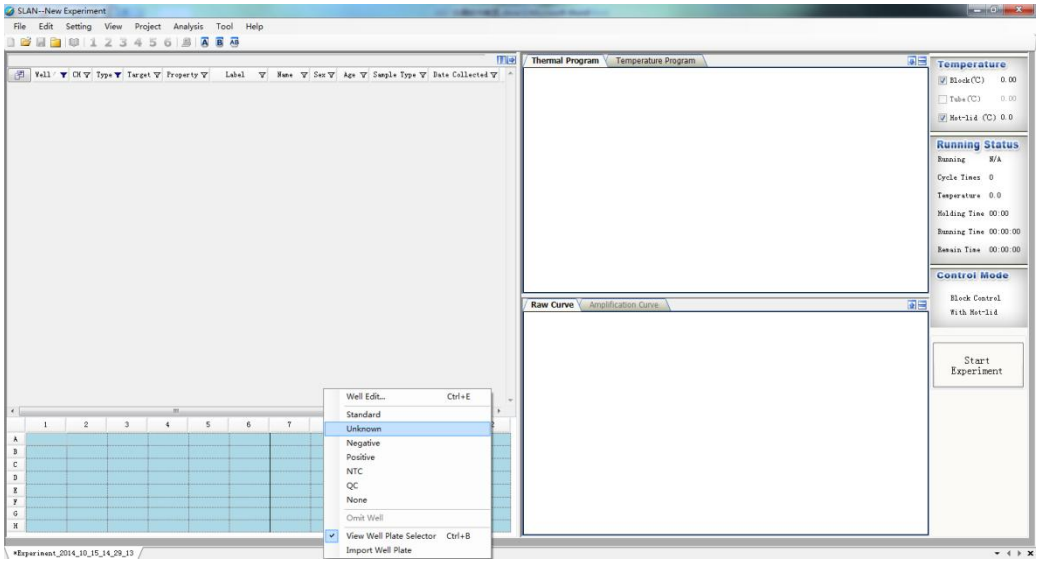


11.8.2 Click “Add Segment”. Under Segment 2: Click “Add Step” and set parameters.

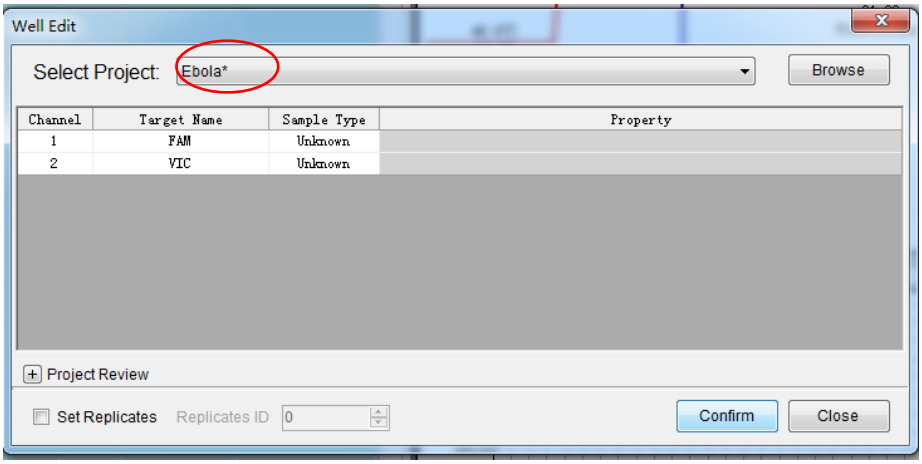


11.9 Click “Save” and then “Close” to exit.

11.10 Well Edit: Select the well where the tubes are and right-click. For samples, choose “Unknown”; for negative control or positive control, choose “Negative” or “Positive”.



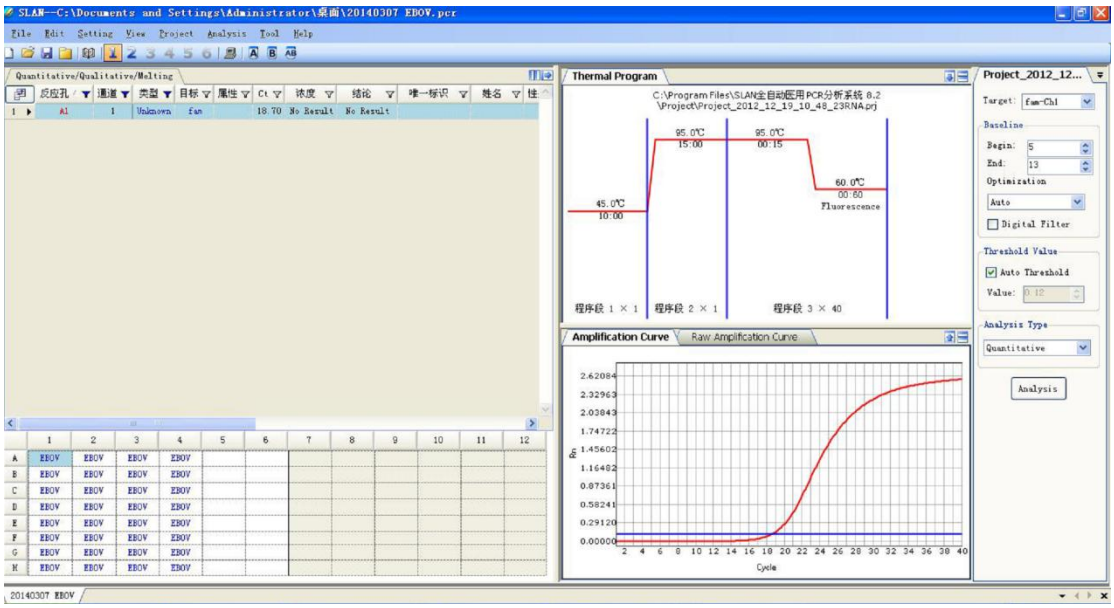
11.11 Take a sample well as an example. Click “Unknown” and the following screen will appear. Select the project setup in 11.7, and then click “Confirm”.



11.12 Click block A or block B or block A&B to unlock the hot lid needed. Then open the machine lid to load the samples.

11.13 Start run by clicking “Start Experiment” button.

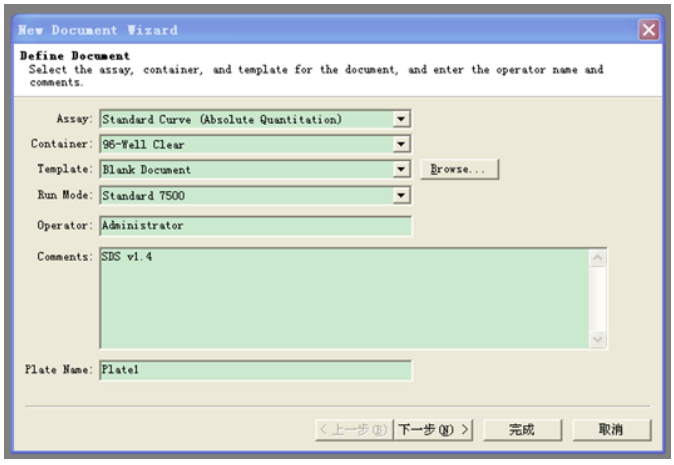
11.14 PCR Analysis: After the run is completed, click the well to read the result. View and adjust the raw data on the right. Selecting “Baseline” and “Threshold Values”, then click ‘Analysis’. The Ct value is on the left and the amplification curve on the lower right.



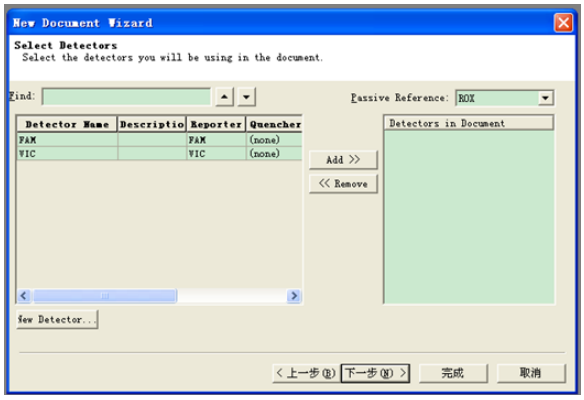
12 Operation Procedure on ABI Prism®7500

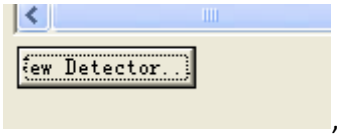
12.1 Double click on the ABI system icon on the desktop to start the program.

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

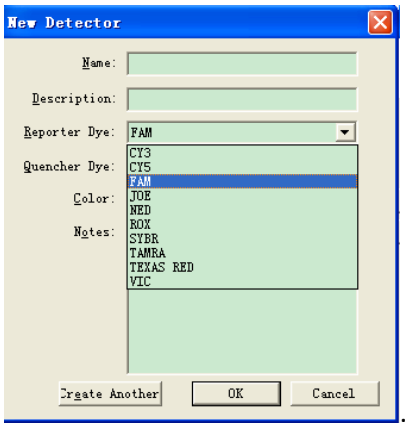


12.3 Click “Next” and a new screen will appear as below.



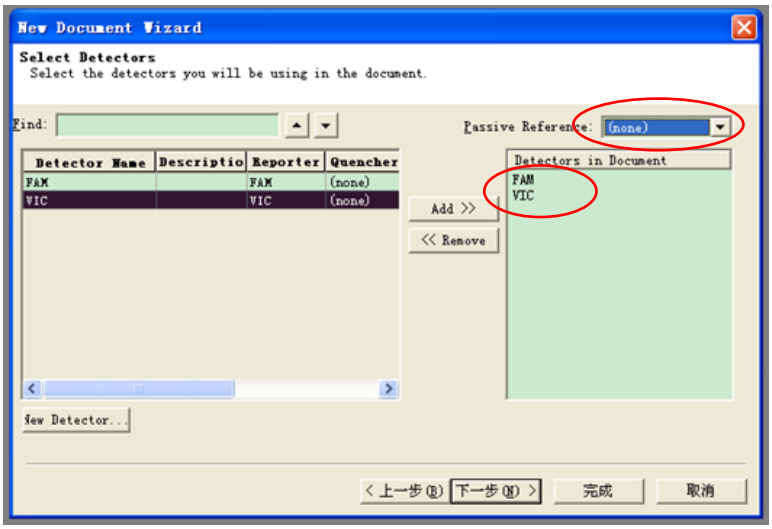
12.4 If there is no “FAM” and “VIC”, click “New Detector” 

,

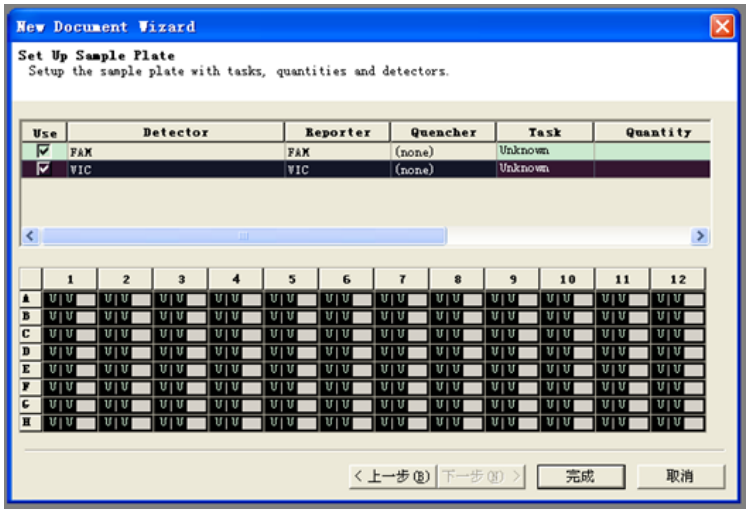


. Enter the name.

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



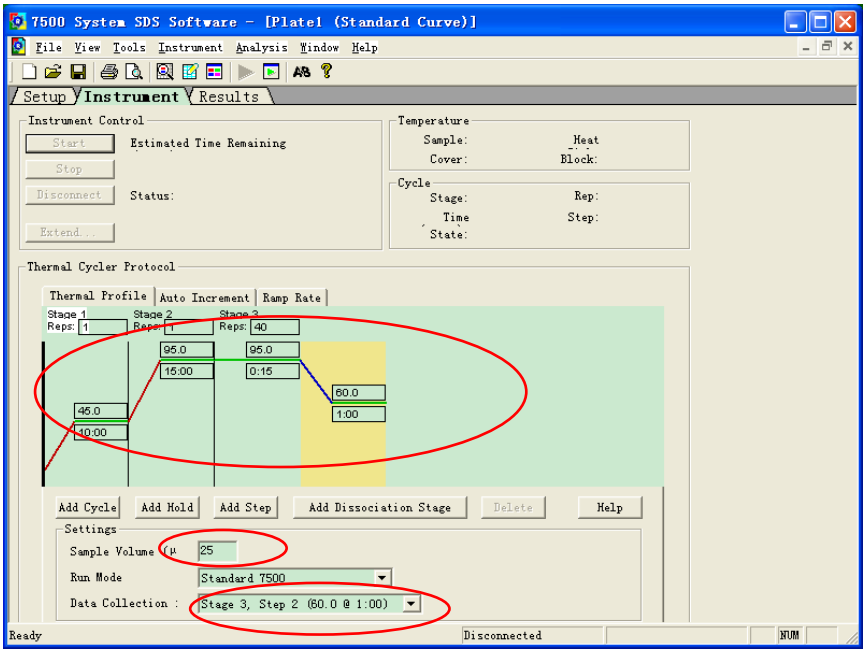
12.6 Click “Next”, select the well containing the samples and controls, and then click the Detector.



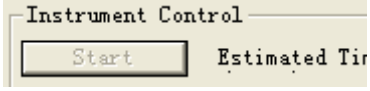
12.7 Click “Finish”.

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

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

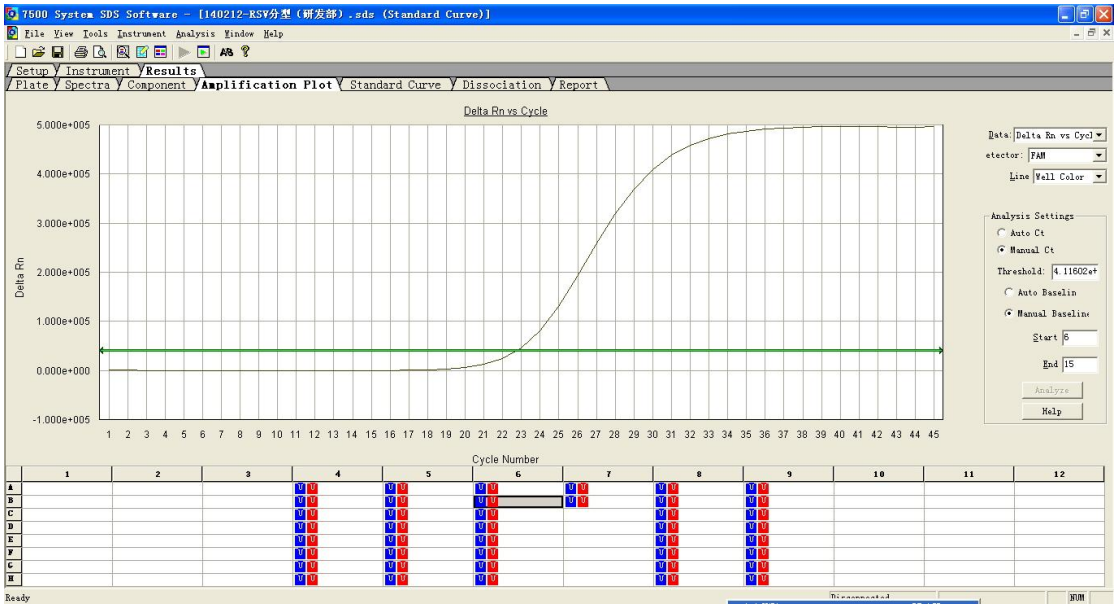


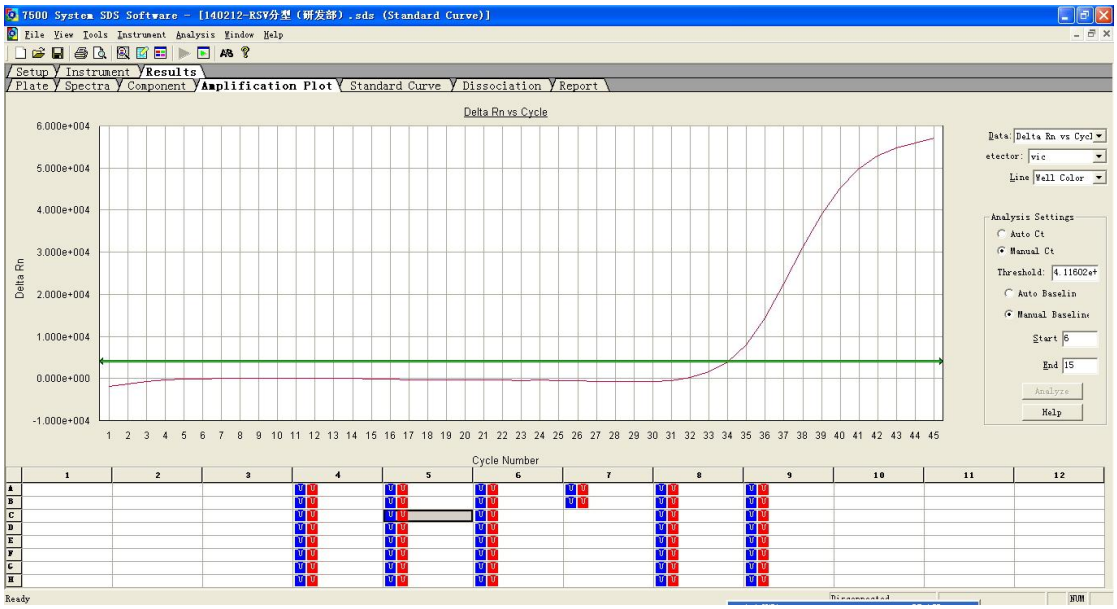
12.9  Save the document and then click “Start” to run the evaluation.



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

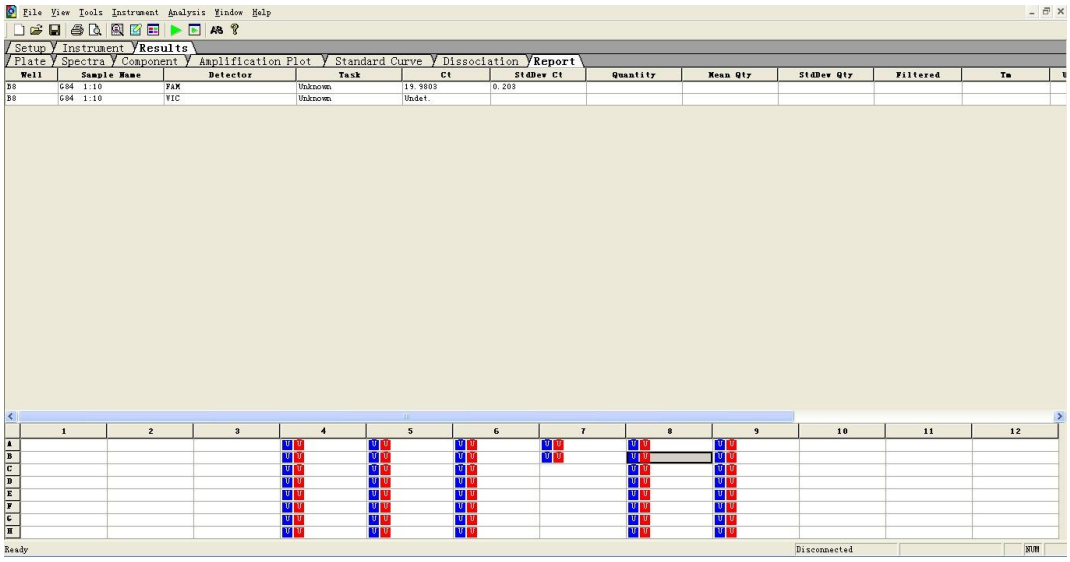
- In the “Data” window, “Delta Rn vs Cycle” should be selected.
- In the “Detector” window, “FAM” and “VIC” should be selected.
- The “Start (cycle)” window should read “6.” The “End (cycle)” should be 15.
- Lastly, be sure to click “Analyze” icon to update the analysis.





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

Liferiver


File View Tools Instrument Analysis Window Help
Setup Instrument Results
Plate Spectra Component Amplification Plot Standard Curve Dissociation Report
Well Sample Name Detector Task Ct StdDev Ct Quantity Mean Qty StdDev Qty Filtered Tm
D8 G04 1:10 FAM Unknown 19.9803 0.203
D8 G04 1:10 VIC Unknown Undet.
1 2 3 4 5 6 7 8 9 10 11 12
A B C D E F G H
Ready
Disconnected
NUM

Interpretation of Results

13 Interpretation of Controls and Clinical Specimens

13.1 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, with Ct value ≤35. If failed, it is recommended to re-test the run using new reagents (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	--

13.2 Specimen

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

13.2.2 A specimen is considered positive for Zika virus if the specimen has a positive amplification result at FAM channel, with Ct value ≤38 (see Table 2).

13.2.3 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 of the same result, the specimen is reported as below the detection limit or negative for Zika virus (see Table 2).

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

13.2.5 If it has no positive amplification result at FAM and VIC channel, no diagnosis can be concluded 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#	≤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

14 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 Zika 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

Limit of Detection

ZIKV pseudovirus was examined in the sensitivity tests. The pseudovirus was diluted linearly 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 ZIKV concentration were further repeated for 20 times. The results suggested that the minimum detection for Zika pseudovirus is 1×10^3 copies/ml. The upper measurement limit can reach $1x10^{11}$ copies/ml. Ct value is not available for cases of higher concentration due to possible instrument limitation.

Analytical Specificity

Eighteen microorganisms and human genomic DNA samples were examined with Zika Virus (ZIKV) Real Time RT-PCR Kit in the specificity tests. The tests were performed with three lots of kits (Lot# P20140401, P20140402, P20140403). All tests were performed according to the User Manual of Zika Virus (ZIKV) Real Time RT-PCR Kit. The tests were conducted from April 2014 to June 2014. Our results showed that the kit only detected Zika virus; whereas negative ZIKV detection was observed using other 18 microorganisms and human genomic DNA samples, suggesting high specificity of the kit for Zika virus detection. The test results with 18 microorganisms and human genomic DNA samples are summarized in the following table (see Table 3):

Table 3: Specificity Test Results

Sample	Lot #					
	P20140401		P20140402		P20140403	
	ZIKV	Internal Control	ZIKV	Internal Control	ZIKV	Internal Control
Hepatitis C virus	-	+	-	+	-	+
Dengue virus type 1	-	+	-	+	-	+
Dengue virus type 2	-	+	-	+	-	+
Dengue virus type 3	-	+	-	+	-	+
Dengue virus type 4	-	+	-	+	-	+
Coxsackie virus A 6	-	+	-	+	-	+
Coxsackie virus A 10	-	+	-	+	-	+
Coxsackie virus A 16	-	+	-	+	-	+
Enterovirus 71	-	+	-	+	-	+
Hepatitis B virus	-	+	-	+	-	+
Chikungunya virus	-	+	-	+	-	+
Japanese encephalitis virus	-	+	-	+	-	+

Rubella Virus	-	+	-	+	-	+
Human parvovirus	-	+	-	+	-	+
Aedes aegypti genome	-	+	-	+	-	+
Cytomegalovirus	-	+	-	+	-	+
Measles virus	-	+	-	+	-	+
Rickettsia	-	+	-	+	-	+
Human genomic DNA	-	+	-	+	-	+

Precision

Precision covers repeatability and reproducibility (i.e. inter-lot, inter-day, inter-run, inter-site, inter-operator and inter-instrument), for which 6 lots of assays and 3 different PCR instruments in total were employed for the study.

Repeatability

Repeatability study was performed with 3 lots of assays on 3 different PCR instruments (Bio-Rad CFX96, SLAN-96 and ABI Prism®7500). We tested 2 different concentrations (1E+04 copies/ml, 1E+07 copies/ml), with 10 replicates in a run for each concentration. The CV of within-run of each instrument was computed with Ct values. The results are shown in Tables 4~6.

It shows that the maximum CV among 3 lots and 3 instruments is 2.35% at concentration of 1E+04 copies/ml while 1.71% at concentration of 1E+07 copies/ml.

Table 4: Bio-Rad CFX96

Sample		Within-Run Imprecision		
		P20140401	P20140402	P20140403
1E+04 copies/ml	Zika	1.80%	1.63%	1.49%
1E+07 copies/ml	Zika	0.96%	1.02%	1.35%

Table 5: SLAN-96

Sample		Within-Run Imprecision		
		P20140401	P20140402	P20140403
1E+04 copies/ml	Zika	1.60%	2.35%	1.89%
1E+07 copies/ml	Zika	1.38%	1.71%	1.45%

Table 6: ABI Prism®7500

Sample		Within-Run Imprecision		
		P20140401	P20140402	P20140403
1E+04	Zika	1.19%	2.27%	1.68%

copies/ml				
1E+07 copies/ml	Zika	1.38%	1.26%	1.21%

Reproducibility

We studied the performance difference under the following scenarios: inter-lot, inter-day, inter-run, inter-site, inter-operator and inter-instrument, for which 3 different PCR instruments and 6 lots of assays were used.

(1) Inter-lot variability

Inter-lot study was performed with 3 different PCR instruments (Bio-Rad CFX96, SLAN-96 and ABI Prism®7500) and repeated once. The 1st test studied the inter-lot variability among three lots of P20140401, P20140402 & P20140403; the 2nd test studied the inter-lot variability among three lots of P20150701, P20150702 & P20150703. Each test was carried out as follows--

We tested 2 different concentrations (1E+04 copies/ml, 1E+07 copies/ml), with 10 replicates for each lot of each concentration with each instrument. The CV among 3 lots was computed with the Ct values of 3 lots (It is computed separately for different concentration and different instrument). See Tables 7~9 for the results.

It shows that the maximum CV among 3 lots and 3 instruments at concentration of 1E+04 copies/ml is 1.96% while 1.55% at concentration of 1E+07 copies/ml.

Table 7: Bio-Rad CFX96

Sample		Inter-Lot Imprecision (P20140401, P20140402, P20140403)
1E+04 copies/ml	Zika	1.63%
1E+07 copies/ml	Zika	1.20%

Sample		Inter-Lot Imprecision (P20150701, P20150702, P20150703)
1E+04 copies/ml	Zika	0.98%
1E+07 copies/ml	Zika	0.51%

Table 8: SLAN-96

Sample		Inter-Lot Imprecision (P20140401, P20140402, P20140403)
1E+04 copies/ml	Zika	1.96%
1E+07	Zika	1.55%

copies/ml		

Sample		Inter-Lot Imprecision (P20150701, P20150702, P20150703)
1E+04 copies/ml	Zika	0.77%
1E+07 copies/ml	Zika	0.59%

Table 9: ABI Prism®7500

Sample		Inter-Lot Imprecision (P20140401, P20140402, P20140403)
1E+04 copies/ml	Zika	1.72%
1E+07 copies/ml	Zika	1.49%

Sample		Inter-Lot Imprecision (P20150701, P20150702, P20150703)
1E+04 copies/ml	Zika	1.84%
1E+07 copies/ml	Zika	1.43%

(2) Inter-day variability

Inter-day study was performed with 1 lot of assay and 2 different PCR instruments (Bio-Rad CFX96 & SLAN-96) in 5 days.

We tested 2 different concentrations (1E+04 copies/ml, 1E+07 copies/ml), with 2 replicates for each concentration with each instrument per day and repeated for 5 days. The CV of inter-day was computed with the Ct values of 5 days (It is computed separately for different instrument and different concentration). See Tables 10~11 for the results.

It shows that the maximum inter-day CV with both instruments at concentration of 1E+04 copies/ml is 0.92% while 0.57% at concentration of 1E+07 copies/ml.

Table 10: Bio-Rad CFX96

Sample		Inter-Day Imprecision (P20150701)
1E+04 copies/ml	Zika	0.69%
1E+07 copies/ml	Zika	0.57%

Table 11: SLAN-96

Sample		Inter-Day Imprecision (P20150701)
1E+04 copies/ml	Zika	0.92%
1E+07 copies/ml	Zika	0.15%

(3) Inter-run variability

Inter-run study was performed with 2 lots of assays and 2 different PCR instruments (Bio-Rad CFX96 & SLAN-96).

We tested 2 different concentrations (1E+04 copies/ml, 1E+07 copies/ml), with 3-4 replicates for each instrument and each concentration in each run; in total, we had 3 runs. The CV of inter-run was computed with the Ct values of 3 runs (It is computed separately for different instrument and different concentration). See Tables 12~13 for the results.

It shows that the maximum inter-run CV with both instruments is 0.93% at concentration of 1E+04 copies/ml while 0.61% at concentration of 1E+07 copies/ml.

Table 12: Bio-Rad CFX96

Sample		Inter-Run Imprecision (P20150702)
1E+04 copies/ml	Zika	0.93%
1E+07 copies/ml	Zika	0.61%

Table 13: SLAN-96

Sample		Inter-Run Imprecision (P20150703)
1E+04 copies/ml	Zika	0.68%
1E+07 copies/ml	Zika	0.31%

(4) Inter-site variability

Inter-site study was performed with 2 lots of assays and 2 different PCR instruments (Bio-Rad CFX96 & SLAN-96) at 3 different testing sites.

We tested 2 different concentrations (1E+04 copies/ml, 1E+07 copies/ml), with 3-4 replicates for each instrument and each concentration at each site. The CV of inter-site was computed with the Ct values from the tests at 3 sites (It is computed separately for different instrument and different concentration). See Tables 14~15 for the results.

It shows that the maximum inter-site CV with both instruments is 1.61% at concentration of 1E+04 copies/ml while 1.16% at concentration of 1E+07 copies/ml.

Table 14: Bio-Rad CFX96

Sample		Inter-Site Imprecision (P20150703)
1E+04 copies/ml	Zika	1.24%
1E+07 copies/ml	Zika	0.34%

Table 15: ABI Prism®7500

Sample		Inter-Site Imprecision (P20150701)
1E+04 copies/ml	Zika	1.61%
1E+07 copies/ml	Zika	1.16%

(5) Inter-operator variability

Inter-operator study was performed with 1 lot of assay and 2 different PCR instruments (SLAN-96 & ABI Prism®7500) by 3 different lab technicians.

We tested 2 different concentrations (1E+04 copies/ml, 1E+07 copies/ml), with 3-4 replicates for each instrument and each concentration for every technician. The CV of inter-operator was computed with the Ct values from these 3 technicians (It is computed separately for different instrument and different concentration). See Tables 16~17 for the results.

It shows that the maximum inter-operator CV with both instruments is 1.85% at concentration of 1E+04 copies/ml while 1.88% at concentration of 1E+07 copies/ml.

Table 16: SLAN-96

Sample		Inter-Operator Imprecision (P20150702)
1E+04 copies/ml	Zika	0.75%
1E+07 copies/ml	Zika	0.94%

Table 17: ABI Prism®7500

Sample		Inter-Operator Imprecision (P20150702)
1E+04 copies/ml	Zika	1.85%
1E+07 copies/ml	Zika	1.88%

(6) Inter-instrument variability

Inter-instrument study was performed with 1 lot of assay and 3 units of ABI Prism®7500.

We tested 2 different concentrations (1E+04 copies/ml, 1E+07 copies/ml), with 3-4 replicates for each concentration with every unit of ABI Prism®7500. The CV of inter-instrument was computed with the Ct values from these 3 units of ABI Prism®7500 (It is computed separately for different concentration). See Table 18 for the results

It shows that the maximum inter-instrument CV is 1.82% at concentration of 1E+04 copies/ml while 1.06% at concentration of 1E+07 copies/ml.

Table 18: ABI Prism®7500

Sample		Inter-Instrument Imprecision (P20150703)
1E+04 copies/ml	Zika	1.82%
1E+07 copies/ml	Zika	1.06%

Clinical Testing

Clinical testing was performed at the Institut Pasteur in Cayenne, French Guiana.

Specimens were collected from patients exhibiting symptoms of Zika virus infection between the 1st and the 8th day following the onset of disease. A total of 206 clinical specimens were obtained from 152 patients (including 52 patients with paired (sera and urine) and/or sequential samples). The specimens of each type collected were: 101 serum, 1 plasma and 104 urine specimens.

All specimens were tested with Liferiver Zika Virus (ZIKV) Real Time RT-PCR Kit as well as the RealStar® Zika Virus RT-PCR Kit 1.0, used as the benchmark assay.

Of the 206 specimens included in the comparison study, 90 (43.6%) were tested positive for Zika virus RNA with Liferiver Zika Virus (ZIKV) Real Time RT-PCR Kit. Eleven specimens (5.3%), with no amplification for Zika and for internal control, were classified as undetermined. All 11 undetermined specimens tested positive with the RealStar® Zika Virus RT-PCR Kit 1.0.

The results are summarized in the following table:

Overall performance with serum and urine specimens

Results of Liferiver Zika Virus (ZIKV) Real Time RT-PCR kit	**Results of RealStar® Zika Virus RT-PCR Kit**		
	Positive	**Negative**	**Total**
Positive	90	0	90

Negative	19	86	105
Undetermined*	2	9	11
Total	111	95	206

*Undetermined: With no positive amplification result at FAM (=Zika) and VIC (=IC) channel (Liferiver interpretation: "PCR inhibition, no diagnosis can be concluded")

Overall performance of the Liferiver Zika Virus (ZIKV) Real Time RT-PCR Kit:

Including undetermined as negative:

- Positive Percent Agreement: 81.1% (90/111) - 95% C.I. 72.5-87.9%
- Negative Percent Agreement: 100.0% (95/95) - 95% C.I. 96.2-100%

Excluding undetermined results:

- Positive Percent Agreement: 82.6% (90/109) - 95% C.I. 74.1-89.8%
- Negative Percent Agreement: 100.0% (86/86) - 95% C.I. 95.8-100%

Performance with serum/plasma specimens:

Performance with serum/plasma specimens

Results of Liferiver Zika Virus (ZIKV) Real Time RT-PCR Kit	**Results of RealStar® Zika Virus RT-PCR Kit**		
	Positive	**Negative**	**Total**
Positive	46	0	46
Negative	16	40	56
Total	62	40	102

Positive Percent Agreement: 74.2% (46/62, 95% C.I. 61.5-84.5%)

Negative Percent Agreement: 100% (40/40, 95% C.I. 91.2-100%)

Performance with urine specimens:

Performance with urine specimens

Results of Liferiver Zika Virus (ZIKV) Real Time RT-PCR Kit	**Results of RealStar® Zika Virus RT-PCR Kit**		
	Positive	**Negative**	**Total**
Positive	44	0	44
Negative	5	55	60
Total	49	55	104

Positive Percent Agreement: 89.8% (44/49, 95% C.I. 77.8-96.6%)

Negative Percent Agreement: 100% (55/55, 95% C.I. 93.5-100%)

Cross reactivity:

Dengue positive serum specimens were used:

Dengue 1: 9 specimens
Dengue 2: 10 specimens
Dengue 3: 1 specimen
Dengue 4: 10 specimens

All 30 specimens tested negative with Liferiver Zika Virus (ZIKV) Real Time RT-PCR Kit.

Ten Chikungunya positive serum specimens were also tested. All 10 specimens tested negative with Liferiver Zika Virus (ZIKV) Real Time RT-PCR Kit.

Traceability of Calibrators and Control Material Values

We have generated Zika pseudovirus 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 value of ZIKV pseudovirus is 15.26.

We also generated five ZIKV negative references from clinical samples. Additionally, we have generated ZIKV 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	15.88	14.30
Ct Value after Dilution	23.45	29.83

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