Japanese encephalitis virus [JEV] Real-time PCR Kit

For use with: Agilent, Bio-Rad, Roche Lightcycler-96, Roche-Z480/Cobas-480, Applied Bio systems [ABI], Thermo-Piko-Real, Rotor gene 5/6plex, Alta-96, Cepheid Real time PCR machines.

INSTRUCTION MANUAL

FOR ELISA KIT No: LT025600RE (JEV)

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INTENDED USE

This kit is a nucleic acid amplification kit for the detection and quantification of JEV specific RNA. For *in vitro* use only.

MANUAL VERSION 1.02

ASSAY SUMMARY

Japanese encephalitis virus [JEV] Real-time PCR Kit constitutes a ready-to-use system for the detection of JEV specific RNA using polymerase chain reaction (PCR). It contains reagents and enzymes for the specific amplification of the conserved region of the JEV viral genome, and for the direct detection of the specific amplicon in FAM channel. In addition, it contains an internal control amplification system to identify possible PCR inhibition and RNA purification efficiency. External positive control is supplied, which can be used as both qualitative and quantitative to determination the amount of viral load.

CHARACTERISTICS

 Specificity: JEV primer and probe have been designed for the specific and exclusive in vitro quantification of JEV. The target sequence is highly conserved and sequences in this kit have 100% homology with a broad range of clinically relevant reference sequences based on a comprehensive bioinformatics analysis.

 Analytical Sensitivity: The analytical sensitivity is defined as the concentration of RNA molecules (copies / µl) that can be detected with a positivity rate of 95%. The analytical sensitivity was determined by analysis of dilution series of quantified JEV specific pDNA from 0.001copies to 10copies/ µl in triplicates. Under optimal PCR conditions, the analytical sensitivity is 0.95 copies per µl.

Dynamic linear range: The linear range was evaluated by analyzing a logarithmic dilution series of RNA concentrations ranging from 1.00E+09 to 1.00E+00 copies/ µl. At least six replicates per dilution were analyzed. The linear range is 1.00E+09 to 1.00E+00 copies/ µl.

PRODUCT INFORMATION

MATERIALS SUPPLIED AND STORAGE CONDITIONS

Components	Volume Per reaction	Number of vials	Volume Per vials
One step RT-PCR Master Mix	8µl	1	200µl
RT-Taq enzyme mix	2µl	1	50µl
JEV Primer Probe Mix [JEV PP mix]	2.5µl	1	65µl
Internal control Primer Probe Mix [IC PP Mix]	2.5µl	1	65µl
Internal control template [IC template]	5µl	1	125µl
JEV Positive control [QS1]	10µl	1	250µl
Water, PCR grade		1	4ml

The kit is shipped on gel ice. Upon arrival, all components should be stored in -20 ° C. They are stable until the expiration date stated on the label. Repeated thawing and freezing should be avoided, as this might affect the performance of the assay. If the reagents are to be used only intermittently, they should be frozen in aliquots. Storage at 2 to 8 ° C should not exceed a period of 5 hours.

MATERIALS REQUIRED BUT NOT SUPPLIED

- Real-time PCR instrument having FAM & HEX channels
- Automatic Nucleic acid extraction system or spin column based purification kit for the purification of nucleic acids
- Desktop centrifuge having 13000rpm or above with a rotor for .5/2 ml reaction tubes
- Centrifuge with a rotor for PCR strips/tubes and 96 well plates
- Optical cap qPCR tubes or strips or 96 wells
- Micro Pipettes (variables)
- Micro Pipette tips with filters (disposable)
- Powder-free gloves (disposable)

RNA PURIFICATION

Purified RNA is the starting material for the Real-time PCR assay. The quality of the purified RNA has a profound impact on the performance of the entire test system. It has to be ensured that the purification system used for RNA purification is compatible with real-time PCR technology. If you are using a spin column based sample preparation procedure having washing buffers containing ethanol, it is highly recommended to perform an additional centrifugation step for 10 min at approximately 17000 x g (~ 13000 rpm), using a new collection tube, prior to the elution of the RNA.

INTERNAL CONTROL TEMPLATE

When performing RNA extraction, it is often advantageous to have an exogenous source of nucleic acid template that is spiked into the lysis buffer. This internal control nucleic acid template is then co-purified with the sample RNA and can be detected as a positive control for the extraction process. Successful co-purification and real-time PCR for the control template also indicates that PCR inhibitors are not present at a high concentration.

The primer and probe present at PCR limiting concentrations which allows multiplexing with the target sequence primers. Amplification of the internal control template does not interfere with detection of the pathogen target gene even when present at low copy number. The Internal control is detected through the HEX channel and gives a CT value of 23 +/-6.

Add 5µl of the internal control template to each test sample. Do not add directly to test sample. Add after adding lysis buffer to the test sample [sample/lysis buffer mix]. Complete purification according to the manufacturer's protocols

ASSAY PROCEDURE

Preparation of standard curve dilution series:

1. Pipette 90µl of PCR grade water into three 1.5ml microcentrifuge tubes and label as QS2 to QS4.
2. Pipette 10µl of Positive control-QS1 into tube QS2.
3. Vortex thoroughly and spin down briefly.
4. Change pipette tip and pipette 10µl from tube QS2 into tube QS3.
5. Vortex thoroughly and spin down briefly.
6. Repeat steps 4 and 5 to complete the dilution series.
7. Use 10µl per reaction.
8. Prepare every time fresh and use.

Detection Protocol

Things to do before starting:

・Before use, all kit components need to be thawed completely, mixed by gently inverting and centrifuged briefly.

・Make sure that Positive and Negative control is included in every run.

・Make sure that internal control template is added during RNA purification. If not, pipette 2.5µl of the internal control template directly into the purified RNA.

・Include 0.5 reaction volume for pipetting error while calculating the volume for total number of reactions.

Components	Volume per reaction
One step RT-PCR Master Mix	8µl
RT-Taq enzyme mix	2µl
JEV PP Mix	2.5µl
IC PP Mix	2.5µl
	15µl
Purified RNA	**10µl**
Final reaction volume	25µl

Negative Control setup [NTC]

Add 10µl of PCR grade water.

Qualitative Positive Control setup

Add 10µl of any one of the Positive controls [From QS1 to QS4]

Quantitative Positive controls setup

10µl of all Positive controls prepared from QS1 to QS4.

Centrifuge PCR vials briefly before placing into thermal cycler.

[Note: There should not be any bubbles in the reaction mix. Bubbles interfere with fluorescence detection.]

Programming the Thermal Cycler

Sample volume	25µl
Fluorescence Dyes	FAM & HEX
Passive reference	None
Ramping rate	Default

Thermal Profile

	Step	Time	Temp
	Reverse transcriptase	20min	50°C
	Taq enzyme activation / Hold	15min	95°C
40 cycles	Denaturation	20sec	95°C
	Annealing/Data collection*	20sec	60°C
	Extension	20sec	72°C

Data collection/Acquisition	Targets
FAM	JEV
HEX	Internal control

READING THE GRAPH

Step-1 – Internal control Validation

Select the test samples alone for the internal control analysis. Select HEX dye and view the graph of internal control amplification. A successful amplification Ct value must be within Ct 23 +/- 6. This range indicates NO PCR inhibition in the reaction. Any sample value goes beyond Ct 31 indicates that either sample has

some issues in the purification or inhibiting PCR reaction. Internal control will not get amplified in the negative and positive controls. Ignore a late noise HEX amplification graph in the NTC and Positive control wells.

Step-2 – FAM - Negative and Positive control validation

Select the NTC and Positive control [Qualitative] or Standards wells [Quantitative], select FAM channel, and view the graph of amplification. The NTC must be flat with no Ct value. If required adjust the threshold value just above the NTC. The PC or Standards must be amplified as per their copy numbers. NTC justifies NO contamination in the reagent as well as fine pipetting and its environment. PC justifies the reagents storage conditions and reaction parameters are as prescribed.

Step-3 –FAM - Test Sample status

In FAM channel, select test sample well one by one, analyze the graph/amplification

Qualitative interpretation of results:

Test Sample	Negative control	Positive control	Internal Control	Interpretation
Positive	Negative	Positive	Positive	JEV specific RNA detected
Negative	Negative	Positive	Positive	No JEV specific RNA Detected. Sample does not contain detectable amounts of JEV specific RNA.
Negative	Negative	Negative	Negative	Experiment fail
Positive	Positive	Positive	Positive	Experiment fail

Calculating copies per ml

Input the machine indicated copy number into the following formula

$$\text{Result (copies/ml)} = \frac{\text{Result (copies/µl)} \times \text{Elution Volume (µl)}}{\text{Sample Volume (ml)}}$$

Note:

Elution volume: must be typed in micro liter format, example 30µl, 60µl or 100µl.

Sample volume: must be typed in milliliter format, example 0.2ml or 0.5ml

Limitations

Good laboratory practice is essential for proper performance of this assay. Strict compliance with the instructions for use is required for optimal results. Analysts should be trained and familiar with testing procedures and interpretation of results prior to performing the assay. A false negative result may occur if inadequate numbers of organisms are present in the sample due to improper collection, transport or handling. Appropriate specimen collection, transport, storage and processing procedures are required for the optimal performance of this test. Extreme care should be taken to preserve the purity of the components of the kit and reaction setups. All reagents should be closely monitored for impurity and contamination. Any suspicious reagents should be discarded. The presence of PCR inhibitors may cause under quantification, false negative or invalid results. Potential mutations within the target regions of the pathogen's genome covered by the primers and/or probes used in the kit may result in under quantification and/or failure to detect. As with any kit, this JEV Real-time PCR results need to be

interpreted in consideration of all other laboratory or clinical findings.

Product Use Limitations

· The product is to be used by personnel specially instructed and trained in Molecular Biology.

· Strict compliance with the user manual is required for optimal PCR results.

· Attention should be paid to expiration dates printed on the box and labels of all components. Do not use expired components.

· Wear protective disposable powder-free gloves, a laboratory coat and eye protection when handling specimens and kit components.

· Avoid microbial and nuclease (RNAse/RNase) contamination of the specimens and the components of the kit.

· Always use RNAse/RNase-free disposable pipette tips with aerosol barriers.

· Use separated and segregated working areas for sample preparation, reaction setup and amplification/detection activities.

· The workflow in the laboratory should proceed in unidirectional manner. Always wear disposable gloves in each area.

· Store positive and/or potentially positive material separated from all other components of the kit.

· Do not open the reaction tubes/plates post amplification, to avoid contamination with amplicons.

· Additional controls may be tested according to guidelines or requirements of local, state and/or federal regulations or accrediting organizations.

· 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.