A Multiplex Real-Time PCR (qPCR) Assay for Detection of Dengue (DENV), Chikungunya (CHIKV) and Zika (ZIKV) Viruses

Product Insert
*ab*TES™ DEN/CHIKU/ZIKA 3 qPCR I Kit
Kit Version: 2.0

CE IVD

REF 300169 (50 Reactions)
300170 (100 Reactions)

Store at -25°C to -15°C

AITbiotech Pte Ltd
25 Pandan Crescent #05-15
TIC TECH Centre
Singapore 128477

EC REP Medevice Group"
Jurmalas gatve 32, Riga,
LV-1083, Latvia.
www.medevice-group.com

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For use on Bio-Rad CFX96, ABI 7500 Fast and *ab*CyclerQ only.

1. Pathogen Information

Dengue virus (DENV) is the cause of dengue fever. It is a mosquito-borne single positive-stranded RNA virus of the family *Flaviviridae*; genus *Flavivirus*. Dengue fever and Dengue hemorrhagic fever (DHF) are viral diseases transmitted by *Aedes* mosquitoes, usually *Aedes aegypti*. They are found in most tropical and subtropical areas of the world, and have become the most common arboviral disease of humans. Human infections are acquired by the bite of infected *Aedes aegypti* mosquitoes, and epidemics are sustained by human-mosquito-human transmission. There are four virus serotypes, Dengue 1 (DEN1), Dengue 2 (DEN2), Dengue 3 (DEN3) and Dengue 4 (DEN4), each serotype is sufficiently different so that there is no cross-protection and epidemics caused by multiple serotypes can occur.

Chikungunya virus (CHIKV) is an RNA virus with a positive-sense single-stranded genome of about 11.6kb. Because it is transmitted by arthropods, namely mosquitoes, it can also be referred to as an arbovirus. Chikungunya fever has recently re-emerged after an interval of several decades to affect millions of people, particularly in India and Southeast Asia. CHIKV epidemic cycle is similar to that of dengue, characterized by sudden onset of chills, fever, headache, nausea, vomiting and rash.

Zika virus (ZIKV) is an enveloped and icosahedral RNA virus and has a non-segmented, single-stranded, 10 kb positive-sense RNA genome which belongs to the *Flaviviridae* family and the *Flavivirus* genus. It is spread mostly by the bite of an infected *Aedes* species mosquito (*Ae. aegypti* and *Ae. albopictus*) during the day. With the recent outbreak of Zika viruses in Brazil since April 2015, the transmission of the ZIKV to other countries in South America, Central America, North America, and the Caribbean were recorded. In August 2016, localized community spread of ZIKV infection was reported in Singapore. Fever, rash, joint pain and conjunctivitis are common symptoms and signs of Zika infections.

2. Test Description

The *ab*TES™ DEN/CHIKU/ZIKA 3 qPCR I Kit is a multiplex real-time polymerase chain reaction (qPCR) kit for the simultaneous detection of DENV, CHIKV and ZIKV in one reaction tube. The kit contains all the necessary PCR reagents for rapid, sensitive and reproducible real-time detection of DENV, CHIKV and ZIKV RNA using highly specific primer pairs and double-dye hydrolysis probes. An **Internal control (IC)** is also supplied to check for possible PCR inhibition. The kit adopts multiplexed PCR strategy for detection and differentiation of RNA from Dengue, Chikungunya and Zika viruses in serum and for the detection of Zika virus RNA in urine.

3. Storage Conditions

The components of *ab*TES™ DEN/CHIKU/ZIKA 3 qPCR I Kit should be stored in the dark, at -20 °C in a **NON**-frost-free freezer. Frost-free freezers go through freeze-thaw cycles to remain frost-free and may cause accelerated degradation of enzymes and nucleic acids. Avoid repeated thawing and freezing (max 3 times) as this may lower the sensitivity. If reagents will be used intermittently, it is suggested to keep the reagents frozen in aliquots.

4. Kit Components

Tubes	Items	300169 (50 rxns)	300170 (100 rxns)
1	2x RT-PCR Reaction Mix	1x 500 µL	2 x 500 µL
2	RT Enzyme Mix	1x 20 µL	2 x 20 µL
3	Primer/Probe Mix	1x 100 µL	2 x 100 µL
4	Internal Control Template	1x 200 µL	2 x 200 µL
5	Nuclease-free Water	1x 600 µL	2 x 600 µL
6	DEN/CHIKU/ZIKA PC ALL	1x 100 µL	1x 100 µL

5. Additional Materials Required but not Provided

- Disposable powder-free gloves
- Nucleic acid extraction kit
- Vortex mixer
- Pipettes and pipette tips with filter
- Desktop centrifuge with rotor
- Real-time thermal cycler
- 0.2 mL PCR tubes/ 96-well PCR plates
- Ice box/cooling block

6. Limitations and General Precautions

- The use of this product and its data interpretation are intended for personnel trained in real-time PCR techniques and *in vitro* diagnostics procedures only.
- It is advisable to analyze the real time PCR graph at the end of the run to determine the validity of the Ct data.
- Appropriate specimen collection, transport, storage and nucleic acid extraction procedures are required for reliable results.
- Wear disposable gloves, laboratory coats and eye protection when handling samples and reagents. Wash hands thoroughly thereafter.
- Use sterile pipette tips with filters and replace the tip for every procedure.
- Store and extract positive materials (specimens, controls and amplicons) separately from all other reagents and add to the reaction mix in a separate facility, if possible.
- Thaw all the components thoroughly at room temperature before starting an assay.
- When the components are thawed, mix the components and centrifuge briefly.
- Do not use the kit after its expiration date or mix components from different lots.

7. Procedures

7.1 Nucleic Acids (NA) Extraction

Standard NA extraction kits are compatible with this assay. Please carry out NA extraction as per instructed in the manufacturer's extraction kit manual.

7.2 PCR Reaction Setup

Thoroughly thaw all components, mix and spin briefly. Keep all components and samples on ice. Prepare your PCR reaction based on the following pipetting scheme:

Reagent	Volume per reaction			
	Test sample reaction	Positive control reaction	Negative control reaction	Non template reaction
2x RT-PCR Mix	10.0 µL	10.0 µL	10.0 µL	10.0 µL
RT/ *Taq* Enzyme Mix	0.4 µL	0.4 µL	0.4 µL	0.4 µL
Primer/Probe Mix	2.0 µL	2.0 µL	2.0 µL	2.0 µL
Nuclease-Free Water	1.6 µL	1.6 µL	1.6 µL	7.6 µL
Internal control	1.0 µL	1.0 µL	1.0 µL	-
Positive Control	-	5.0 µL	-	-
Extracted Test Sample	5.0 µL	-	-	-
Total Volume	**20 µL**	**20 µL**	**20 µL**	**20 µL**

7.3 PCR Cycling Conditions

The following cycling conditions were established and validated on Bio-Rad CFX96, ABI 7500 Fast and *ab*CyclerQ. You may need to adjust these conditions for other real-time platforms. **Cy5** (ZIKV), **FAM** (Internal control), **Texas Red** (DENV), and **HEX/VIC** (CHIKV) channels should be chosen and the fluorescence is measured at the annealing phase of each cycle.

Phase	Description	No. of Cycles	Temperature	Duration
1	cDNA synthesis	1	52 °C	15 min
2	*Taq* activation	1	95 °C	2 min 30 sec
3	Amplification	45	95 °C	15 sec
			*61.8 °C	30 sec

**Data acquisition at annealing & extension phase*

For ABI 7500 Fast, ensure the following settings are established before the run.

ABI 7500 Fast Settings	
Ramp speed	Standard
Passive reference	None

8. Performances Characteristics

8.1. Analytical sensitivity

Analytical sensitivity (limit of detection) is defined as the lowest concentration at which the assay can detect with a positivity rate of at least 95%.

The limit of detection of the *ab*TES™ DEN/CHIK/ZIKA 3 qPCR I Kit was determined by analyzing a dilution series of Dengue, Chikungunya and Zika in-vitro transcribed (*IVT*) RNA Controls from 0.08 to 5.0 x 10^3copies/µl, 0.08 to 5.0 x 10^3copies/µl and 0.08 to 1.0 x 10^4 copies/µl respectively. The testings were carried out in either 10-replicates (for concentrations ≥5.0 x 10^2copies/ul) or 14-replicates (for concentrations <5.0 x 10^2copies/ul) on the Bio-Rad CFX96 Real-Time PCR System and ABI 7500 Fast.

The analytical sensitivity was estimated by probit analysis using SPSS release 16.0.0 followed by a confirmation of 20 replicates of each target at its estimated limit-of-detection concentration using Bio-Rad CFX96 and ABI 7500 Fast real-time PCR system with a mean Ct value of 37.25, 37.22 and 37.02 for DENV, ZIKV and CHIKV respectively.

The analytical sensitivity (not in consideration of extraction) was determined to be the following:

Target	Detecting Channel	Analytical Sensitivity (95% confidence)
DENV	Texas Red	4.44 copies/ µl
CHIKV	HEX	4.02 copies/ µl
ZIKV	Cy5	4.68 copies/ µl

8.2. Analytical specificity

The assay was tested for potential cross-reactivity against the following closely related organisms. All organisms were tested in 1000 copies/µl concentration except otherwise stated. No cross-reactivity was observed.

Organisms Tested for Analytical Specificity			
Organism	**DENV (Texas Red)**	**CHIKV (HEX)**	**ZIKV (CY5)**
Yellow Fever Virus	Not detected	Not detected	Not detected
St Louis Encephalitis Virus	Not detected	Not detected	Not detected
Parvovirus b19	Not detected	Not detected	Not detected
West nile virus (New York 99)	Not detected	Not detected	Not detected
Dengue 1 virus (500pg)	Detected	Not detected	Not detected
Dengue 2 virus (500pg)	Detected	Not detected	Not detected
Dengue 3 virus (500pg)	Detected	Not detected	Not detected
Dengue 4 virus (500pg)	Detected	Not detected	Not detected
Chikungunya virus	Not detected	Detected	Not detected
Zika Virus	Not detected	Not detected	Detected

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(65) 6778 6822 • sales@aitbiotech.com • www.aitbiotech.com

8.3. Precision/Reproducibility

The inter-assay (variability between different runs) and intra-assay (variability within one run) precision of the abTES™ DEN/CHIKU/ZIKA 3 qPCR I was determined by performing the assay once per day in 5-replicates over a period of 2 days for 4 samples of different concentrations (total of 40 reactions per target). To also account for variability between technicians, the testing over the 2 days were tested by 2 different technicians.

For all the 3 targets, the qualitative results of all 40 reactions were 100% reproducible for both inter-assay and inter-technician.

The coefficient of variation (%CV) of the cycle threshold (Ct) for the intra- and inter-assay precision are as follows:

DENV	**10000 copies/ul**	**1000 copies/ul**	**100 copies/ul**	**20 copies/ul**
Inter-assay	1.2%	1.0%	0.6%	1.7%
Intra-assay	1.0%	0.5%	0.6%	1.4%
CHIKV	**10000 copies/ul**	**1000 copies/ul**	**100 copies/ul**	**10 copies/ul**
Inter-assay	0.9%	0.5%	0.7%	2.6%
Intra-assay	0.9%	0.4%	0.7%	2.6%
ZIKV	**10000 copies/ul**	**1000 copies/ul**	**100 copies/ul**	**10 copies/ul**
Inter-assay	0.4%	0.7%	0.5%	1.7%
Intra-assay	0.2%	0.6%	0.4%	1.7%

8.4. Diagnostic Evaluation

A total of 44 clinically extracted Dengue and Chikungunya positive samples were used for diagnostic evaluation. These samples were previously analyzed by other molecular method (nested PCR/Sanger Sequencing/ real-time PCR assay).

The diagnostic sensitivity and specificity of DENV and CHIKV is tabulated as below.

Diagnostic sensitivity and specificity for *DENV*

Reference Method	***ab*TES (*n*=44)**		**Sensitivity/ Specificity**
	***DENV* Positive**	***DENV* Negative**	
DENV Positive	41	0	100% sensitivity
DENV Negative	0	3	100% specificity
Total	41	3	

Diagnostic sensitivity and specificity for *CHIKV*

Reference Method	***ab*TES (*n*=44)**		**Sensitivity/ Specificity**
	***CHIKV* Positive**	***CHIKV* Negative**	
CHIKV Positive	3	0	100% sensitivity
CHIKV Negative	0	41	100% specificity
Total	3	41	

As there are no Zika clinical samples available, we participated in the Quality Control for Molecular Diagnostics (QCMD) 2016 Zika Virus EQA Pilot Study which includes eight Zika samples (African and French Polynesian strains) in transport medium, one of each dengue and chikungunya sample in transport medium.

The samples were purified using Geneall *Ribospin vRD II Kit.* The initial sample input volume was 100ul and finally eluted in 40ul. Real-time detection was performed with abTES DEN/CHIKU/ZIKA 3 qPCR I Kit as well as another commercial assay targeting only Zika virus.

The result was 100% concordant to the QCMD Zika Virus Pilot study as summarized in the table below.

Sample ID	**Sample Content**	***ab*TES™ DEN/CHIKU/ZIKA 3 qPCR I Kit**		
		Ct DENV	**Ct CHIKV**	**Ct ZIKV**
ZIKA16-01	Dengue Virus Type 2, West Nile Virus (NY99), Yellow Fever Virus	31.9	-	-
ZIKA16-02	Zika Virus (African)	-	-	35.7
ZIKA16-03	Zika Virus (French Polynesian)	-	-	32.5
ZIKA16-04	Zika Virus (French Polynesian)	-	-	30.8
ZIKA16-05	Zika Virus Negative	-	-	-

Sample ID	**Sample Content**	***ab*TES™ DEN/CHIKU/ZIKA 3 qPCR I Kit**		
		Ct DENV	**Ct CHIKV**	**Ct ZIKV**
ZIKA16-06	Zika Virus (French Polynesian)	-	-	30.1
ZIKA16-07	Chikungunya Virus	-	30.2	-
ZIKA16-08	Zika Virus (African)	-	-	33.7
ZIKA16-09	Zika Virus (African)	-	-	29.8
ZIKA16-10	Zika Virus (African)	-	-	32.7

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9. Interpretation of Data

A sample will be considered as having a positive result if an amplification signal is detected in the respective fluorescence channel.
The result is regarded as true negative for DENV, CHIKV, and ZIKV when the Texas Red, HEX and Cy5 are negative and the FAM IC channel is positive.

Target	DENV (Texas Red)	CHIKV (HEX)	ZIKV (Cy5)	Internal Control (FAM)
DENV positive	+	-	-	+/-
CHIKV positive	-	+	-	+/-
ZIKV positive	-	-	+	+/-
DEN/CHIK/ZIKA PC ALL	+	+	+	+/-
Non-template Control	-	-	-	+

10. Troubleshooting

10.1. No signal of internal control and detection in analytical channel observed with positive control

- Check programmed temperature settings against the protocol given.
- Affirm if proper storage was done and check the expiry date on the kit; use a newer kit to repeat experiments if needed.
- PCR inhibition has possibly occurred
- Repeat PCR if needed.

10.2. Signal detected for non-template control

- A contamination in the reagents or samples is highly possible.
- Repeat experiment protocol and take steps to locate source of contamination.

10.3. No signal of the internal control and detection in analytical channel observed with unknown samples

- A possible PCR inhibition has occurred. Re-extract the sample to remove inhibitors and repeat PCR, if needed.
- Affirm if proper storage was done and check the expiry date on the kit. Use a new kit to repeat the test if needed.

11. Explanation of Symbols

IVD	*In vitro* diagnostic medical device
REF	Catalogue number
	Store at -25°C to -15°C
	Manufacturer
EC REP	Authorized representative in the European community
LOT	Lot number
	Use by
	Contains sufficient for <n> tests

Electronic copy of the product insert can be downloaded at http://aitbiotech.com/denv-chikv-zikv/

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