Primerdesign™ Ltd

R01020

genesig™PLEX Insect-borne Real-Time PCR Multiplex Kit

Kit version: 1

Zika virus (ZIKV) - polyprotein gene

Chikungunya virus (CHIKV) - NSP

Dengue virus - 3'UTR

Tick-borne encephalitis (TBEV) - NS3

Yellow fever virus (YFV) - 5'UTR

West Nile virus (WNV) - 5'UTR into Poly gene

genesig™PLEX kit

100 tests

GENESIG

Kits by Primerdesign

Specificity of primers and probes last reviewed on:
16th April 2023

For general laboratory and research use only

Introduction

Zika virus (ZIKV)

Zika virus (ZIKV) belongs to the Flaviviridae virus family, featuring an enveloped and icosahedral structure with a non-segmented, single-stranded, positive sense RNA genome. Transmitted by infected Aedes mosquitoes, particularly Aedes aegypti, ZIKV is an emerging infectious disease. The virus has historically been limited to sub-tropical zones but it is now spreading to new regions with the vector's presence.

The ZIKV polyprotein encodes a large precursor protein that is cleaved into multiple small proteins by viral and host proteases that play vital roles in various stages of the virus's life cycle, such as viral entry, replication, transcription, translation, assembly, and release. Due to these critical functions the polyprotein is well conserved among ZIKV strains.

Incubation spans 3 to 12 days after mosquito bite, and while some remain asymptomatic, typical symptoms, lasting 2 to 7 days, encompass mild headaches, maculopapular rash, fever, malaise, conjunctivitis, and arthralgia. In 2015, ZIKV emerged in South America, causing extensive outbreaks in Brazil and Colombia, potentially linked to increased microcephaly cases due to incomplete brain development.

Chikungunya virus (CHIKV)

Chikungunya virus (CHIKV) is a single-stranded, positive sense RNA arbovirus transmitted by Aedes mosquitoes. The non-structural protein (NSP) is vital in CHIKV's lifecycle, aiding replication, immune evasion, and host cell modulation, and is conserved between strains.

CHIKV infections are most commonly found in tropical and subtropical regions, particularly in areas where the Aedes mosquitoes that transmit the virus are prevalent. CHIKV outbreaks cause dengue-like symptoms, notably prolonged joint pain in extremities. The acute febrile phase lasts 2-5 days, followed by persistent joint pain for weeks or months. Symptoms include high fever, muscle/joint pain, headache, nausea, fatigue, and occasional neurological issues.

Dengue virus

Dengue fever (DF) and dengue hemorrhagic fever (DHF) stem from four closely related yet distinct virus serotypes (DEN-1, DEN-2, DEN-3, and DEN-4) within the Flavivirus genus. These single-stranded, positive-sense RNA genomes bear a critical molecular component in their 3' untranslated region (3'UTR), influencing replication, translation, and host interactions. Despite its non-coding nature, the 3'UTR holds vital roles, featuring essential motifs and structures in the viral lifecycle.

Infection by a serotype confers lifelong immunity solely to that type, potentially leading to multiple dengue infections for those in endemic regions. DF and DHF prevail in tropical and subtropical areas, with humans and Aedes mosquitoes maintaining the virus cycle. Ranging from mild viral symptoms to severe hemorrhagic disease, infections often encompass high fever, headache, vomiting, muscle and joint pains, and rash.

DHF risk hinges on factors like virus strain, patient age, and prior infection history. Severe cases can manifest as "dengue hemorrhagic fever," involving bleeding, low platelet count, plasma leakage, or even dengue shock syndrome.

Tick-borne encephalitis virus (TBEV)

Tick-borne encephalitis virus (TBEV) is an arthropod-borne pathogen impacting the human central nervous system. It also infects ruminants, rodents, and select bird species. A member of the Flavivirus genus within Flaviviridae, TBEV is endemic in parts of Europe, Russia, and China. Three serotypes exist globally: European, Siberian, and Far Eastern. The virus is spherical (40-50 nm diameter) with a single-stranded, positive-sense RNA genome (about 11.14 kb), encoding 3 structural and 7 nonstructural proteins. The nonstructural protein NS3 is pivotal, harboring enzymatic activities vital for replication and immune evasion.

Ticks, especially Ixodes species, primarily transmit TBEV and serve as vectors and reservoirs. European TBEV uses I. ricinus ticks; Far Eastern and Siberian subtypes employ I. persulcatus. Human-to-human transmission is rare, mainly via blood transfusion or breastfeeding. Infections frequently arise during forest activities like hiking, camping, or trekking, correlating with tick abundance. Peak incidence occurs in spring and early summer but can persist year-round.

TBEV's incubation spans 4 to 28 days; many cases are asymptomatic. Early infection mimics nonspecific febrile illness with fever, malaise, muscle pain, and headache. Common diagnostic signs include leukopenia and thrombocytopenia. A second phase, affecting the central nervous system (aseptic meningitis, encephalitis, myelitis), follows remission in 20-30% of cases. About 10% of these patients require intensive care, with a 1% mortality rate post-neurological symptoms. Far Eastern subtype is most lethal (>20% mortality), while Western European subtype is milder.

Yellow fever virus (YFV)

Yellow fever virus (YFV) is a single-stranded, positive-sense RNA virus in the Flavivirus genus (Flaviviridae family). Its surface proteins form an icosahedral-like symmetry and it spreads via mosquito bites, often moving between monkeys, humans, and from person to person. These mosquitoes thrive in both jungle and urban settings.

YFV's RNA features a single open reading frame (ORF) flanked by 5' and 3' untranslated regions (UTRs), each 118 and 565 bases long. The 5'UTR plays a pivotal role, hosting structures and motifs crucial for translation, stability, and virulence. The RNA's translation yields a precursor protein, cleaved by host and viral proteases into mature viral proteins.

Symptoms of YFV infection include fever, headache, nausea, vomiting, muscle pain, backache, and jaundice from liver damage. Diagnosis involves symptom analysis and blood tests, as symptoms alone can't confirm yellow fever. YFV can result in a serious infection, causing death in about 8% of cases, but vaccination offers protection.

West Nile virus (WNV)

West Nile Virus (WNV) belongs to the Flaviviridae family. Mainly infecting birds, it can also affect humans and various species. Its 11-12kb single-stranded, positive-sense RNA genome is housed in a 45-50-nm viron, encompassed by a host-derived membrane altered by viral glycoproteins. The genome comprises 5'UTR, polyprotein, and 3'UTR, with a single open reading frame (ORF) predominantly coding a polyprotein. This is processed by viral and host proteases into structural (capsid, envelope) and non-structural proteins vital for replication.

Transmission is primarily through mosquito vectors, with rare cases involving blood transfusion, organ transplants, or breastfeeding. While most remain asymptomatic, some experience mild West Nile Fever or rarer, severe West Nile encephalitis/meningitis. Mild symptoms encompass diarrhea, fever, headache, muscle aches, nausea, rash, sore throat, swollen lymph nodes, and vomiting. Severe cases can involve disorientation, coma, muscle weakness, stiff neck, seizures, and fits. WNV can infect nerve cells, potentially breaching the central nervous system, leading to acute flaccid paralysis.

Currently, no specific treatment exists for WNV. Mild cases typically resolve in a few days, while severe cases necessitate hospitalization, involving intravenous fluids and respiratory assistance. Severe forms may cause brain damage, muscle weakness, and fatality.

Specificity

The genesig™PLEX Insect-borne kit is designed for the in vitro detection of Zika virus (ZIKV), Chikungunya virus (CHIKV), Dengue virus, Tick-borne encephalitis (TBEV), Yellow fever virus (YFV) and West Nile virus (WNV)

The assays within this kit are predicted to detect over 95% of sequences available on the NCBI database at the time of design.

This TBEV design in this kit is predicted to cross react with Greek goat encephalitis virus, Spanish sheep encephalitis virus, Spanish goat encephalitis virus, Louping ill virus and Langat virus. These would give a signal in the Cy5 channel of tube two.

The dynamics of genetic variation means that new sequence information may become available after the initial design. Primerdesign periodically reviews the detection profiles of our kits and when required releases new versions.

If you require further information or have a specific question about the detection profile of this kit, then please send an email to techsupport@primerdesign.co.uk and our team will answer your question.

Kit contents

- **Multiplex Tube 1 primer/probe mix (100 reactions BROWN)**
  FAM, VIC, ROX and Cy5 labelled (see table below)

Target	Fluorophore
Zika virus	FAM
Internal extraction control	VIC
Chikungunya virus	ROX
Dengue virus	Cy5

- **Multiplex Tube 2 primer/probe mix (100 reactions BROWN)**
  FAM, VIC, ROX and Cy5 labelled (see table below)

Target	Fluorophore
West Nile virus	FAM
Internal extraction control	VIC
Yellow Fever virus	ROX
Tick-borne Encephalitis virus	Cy5

- **Multiplex Tube 1 positive control template (RED)**
- **Multiplex Tube 2 positive control template (RED)**
- **Internal extraction control RNA (BLUE)**
- **4x Lyophilised OneStep Master Mix (GOLD)**
- **4x oasig™ resuspension buffer (BLUE)**
  for resuspension of the lyophilised master mix
- **2x Template preparation buffer (YELLOW)**
  for resuspension of the positive control templates and internal extraction control DNA
- **RNase/DNase free water (WHITE)**
  for resuspension of the primer/probe mix

Reagents and equipment to be supplied by the user

Real-time PCR Instrument

Extraction kit

This kit is recommended for use with genesig EASY DNA/RNA Extraction kit or exsig®Mag however, it is designed to work well with all processes that yield high quality RNA and DNA with minimal PCR inhibitors.

Pipettors and filter tips

Vortex and centrifuge

1.5ml microtubes

qPCR plates or reaction tubes

Kit storage and stability

This kit is stable at room temperature but should be stored at -20ºC on arrival. Once the lyophilised components have been resuspended, they should not be exposed to temperatures above -20ºC for longer than 30 minutes at a time and unnecessary repeated freeze/thawing should be avoided. The kit is stable for six months from the date of resuspension under these circumstances. Primerdesign does not recommend using the kit after the expiry date stated on the pack.

Suitable sample material

All kinds of sample material suited for PCR amplification can be used. Please ensure the samples are suitable in terms of purity, concentration, and RNA integrity. Always run at least one negative control with the samples. To prepare a negative control, replace the template RNA sample with RNase/DNase free water.

Dynamic range of test

Under optimal PCR conditions genesig kits have very high priming efficiencies of >90% with the exception of Dengue. Due to design limitations from the requirement to detect multiple subtypes, the Dengue target displays efficiencies of >80%. All targets can detect between $1X10^6$ and $1X10^2$ copies of target template.

Notices and disclaimers

This product is developed, designed and sold for research purposes only. It is not intended for human diagnostic or drug purposes or to be administered to humans unless clearly expressed for that purpose by the Food and Drug Administration in the USA or the appropriate regulatory authorities in the country of use. During the warranty period Primerdesign genesig detection kits allow precise and reproducible data recovery combined with excellent sensitivity. For data obtained by violation to the general GLP guidelines and the manufacturer's recommendations the right to claim under guarantee is expired. PCR is a proprietary technology covered by several US and foreign patents. These patents are owned by Roche Molecular Systems Inc. and have been sub-licensed by PE Corporation in certain fields. Depending on your specific application you may need a license from Roche or PE to practice PCR. Additional information on purchasing licenses to practice the PCR process may be obtained by contacting the Director of Licensing at Roche Molecular Systems, 1145 Atlantic Avenue, Alameda, CA 94501 or Applied Biosystems business group of the Applera Corporation, 850 Lincoln Centre Drive, Foster City, CA 94404. In addition, the 5' nuclease assay and other homogeneous amplification methods used in connection with the PCR process may be covered by U.S. Patents 5,210,015 and 5,487,972, owned by Roche Molecular Systems, Inc, and by U.S. Patent 5,538,848, owned by The Perkin-Elmer Corporation.

Trademarks

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Principles of the test

Real-time PCR

Individual primer and probes designed for each pathogen have been combined into a single reaction and these can be detected through the different fluorescent channels as described in the kit contents.

The primer and probe mix provided exploits the so-called TaqMan® principle. During PCR amplification, forward and reverse primers hybridize to the target DNA. Fluorogenic probes are included in the same reaction mixture which consists of a DNA probe labelled with a 5`-dye and a 3`-quencher. During PCR amplification, the probe is cleaved, and the reporter dye and quencher are separated. The resulting increase in fluorescence can be detected on a range of qPCR platforms.

Positive control

The kit contains two positive control tubes, each tube contains the templates for all three targets detected by that tube. The kit positive control will therefore give a positive signal in FAM, ROX, and Cy5 channels. Each time the kit is used, at least one positive control reaction for each primer/probe tube must be included in the run. A positive result indicates that the primers and probes for detecting each virus are working properly in that particular run. If a negative result is obtained the test results are invalid and must be repeated. Care should be taken to ensure that the positive control does not contaminate any other kit component which would lead to false positive results. This can be achieved by handling these components in a post PCR environment. Care should also be taken to avoid cross-contamination of other samples when adding the positive control to the run. This can be avoided by sealing all other samples and negative controls before pipetting the positive control into the positive control well.

Negative control

To confirm the absence of contamination, a negative control reaction should be included every time the kit is used. For this reaction, the RNase/DNase free water should be used instead of template. A negative result indicates that the reagents have not become contaminated while setting up the run

Internal RNA extraction control

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

Within the Multiplex primer/probe mix are primers and probes to detect the exogenous RNA using qPCR. The PCR primers are present at PCR limiting concentrations which allows multiplexing with the target sequence primers. Amplification of the control cDNA does not interfere with detection of the target cDNA even when present at low copy number. The Internal control is detected through the VIC channel in both tubes and gives a Cq value of 28+/-3 depending on the level of sample dilution.

Resuspension protocol

To minimise the risk of contamination with foreign RNA, we recommend that all pipetting be performed in a PCR clean environment. Ideally this would be a designated PCR lab or PCR cabinet. Filter tips are recommended for all pipetting steps.

1. **Pulse-spin each tube in a centrifuge before opening.**
   This will ensure lyophilised primer and probe mix or template is in the base of the tube and is not lost upon opening the tube.

2. **Resuspend the primer/probe mix in the RNase/DNase free water supplied, according to the table below:**
   To ensure complete resuspension, vortex the tube thoroughly.

Component – resuspend in water	Volume
Pre-PCR pack	
Multiplex Tube 1 primer/probe mix (**BROWN**)	110µl
Multiplex Tube 2 primer/probe mix (**BROWN**)	110µl

3. **Resuspend the positive control template in the template preparation buffer supplied, according to the table below:**
   To ensure complete resuspension, vortex the tube thoroughly.

Component – resuspend in template preparation buffer	Volume
Pre-PCR heat-sealed foil	
Internal extraction control RNA (**BLUE**)	500µl
Post-PCR heat-sealed foil	
Multiplex Tube 1 Positive control template (**RED**)*	500µl

Multiplex Tube 2 Positive control template (**RED**)*	500µl

*** This component contains high copy number template and is a VERY significant contamination risk. It must be opened and handled in a separate laboratory environment, away from the other components.**

4. **Resuspend the lyophilised OneStep Master Mix in oasig resuspension buffer, according to the table below:**

Component – resuspend in oasig resuspension buffer	Volume
Lyophilised OneStep Master Mix (**GOLD**)	525µl

RNA extraction

The internal extraction control RNA can be added to either lysis/extraction buffer or to the sample once it has been resuspended in lysis buffer.

DO NOT add the internal extraction control RNA directly to the unprocessed biological sample as this will lead to degradation and a loss in signal.

1. **Add 4µl of the Internal extraction control RNA (BLUE) to each sample in RNA lysis/extraction buffer per sample.**

2. **Complete RNA extraction according to the manufacturer's recommended protocols.**

OneStep RT-qPCR detection protocol

For optimum performance and sensitivity
All pipetting steps and experimental plate set up should be performed on ice. After the plate is prepared, proceed immediately to the OneStep amplification protocol. Prolonged incubation of reaction mixes at room temperature can lead to PCR artifacts that reduce the sensitivity of detection.

1. **For each RNA sample prepare a reaction mix according to the table below:**
   Include sufficient reactions for positive and negative controls.

Component	Volume
Lyophilised OneStep Master Mix (**GOLD**)	10µl
Multiplex primer/probe mix (**BROWN**)	1µl
RNase/DNase free water (**WHITE**)	4µl
Final volume	**15µl**

2. **Pipette 15µl of this mix into each well according to your qPCR experimental plate set up.**

3. **Pipette 5µl of RNA sample into each well according to your experimental plate set up.**
   For negative control wells use 5µl of RNase/DNase free water. The final volume in each well is 20µl.

4. **Pipette 5µl of positive control template into each well according to your plate set up.**
   The Tube 1 positive control contains template for Zika Virus, Chikungunya virus, and Dengue virus and Tube 2 positive control contains templates for West Nile virus, Yellow Fever virus, and Tick-borne Encephalitis virus. The final volume in each well is 20µl.

OneStep RT-qPCR amplification protocol

Amplification conditions using lyophilised OneStep Master Mix

	Step	Time	Temp
	Reverse transcription	10 mins	55°C
	Enzyme activation	2 mins	95°C
Cycling x 50	Denaturation	10 secs	95°C
	DATA COLLECTION*	60 secs	60°C

* Fluorogenic data should be collected during this step through the FAM, VIC, ROX and Cy5 channels

Interpretation of results

Positive control

Each positive control tube contains the all the templates for the targets detected by that tube and should produce positive amplification plots in the FAM, ROX, and Cy5 channels. There is no Internal control template within the positive control so the VIC channel should give no signal (flat amplification plot). The positive control signals indicate that the kit is working correctly to detect each virus.

No template control (NTC)

The NTC should give a flat line (flat amplification plots) through all channels. Signals in the NTC indicate cross contamination during plate set up.

Internal RNA extraction control

The Cq value obtained with the internal control will vary significantly depending on the extraction efficiency, the quantity of RNA added to the PCR reaction and the individual machine settings. Cq values of 28±3 are within the normal range. When amplifying a target sample with a high genome copy number, the internal extraction control may not produce an amplification plot. This does not invalidate the test and should be interpreted as a positive experimental result.

Sample data

Presence of the viruses are detected in the channels indicated in the kit contents section. Positive signals indicate positive tests for those viruses. It may be possible for samples to contain multiple viruses, therefore positive results in the FAM, ROX, and Cy5 channels may be present.

Summary of data interpretation

Multiplex Tube 1 data interpretation

Target (FAM/ROX/Cy5)	Internal extraction control (VIC)	Positive Control	Negative Control	Interpretation
FAM +	**+ / -**	**+**	**-**	**ZIKA VIRUS POSITIVE RESULT**
ROX +	**+ / -**	**+**	**-**	**CHIKUNGUNYA VIRUS POSITIVE RESULT**
Cy5 +	**+ / -**	**+**	**-**	**DENGUE VIRUS POSITIVE RESULT**
-	**+**	**+**	**-**	**NEGATIVE RESULT**

Multiplex Tube 2 data interpretation

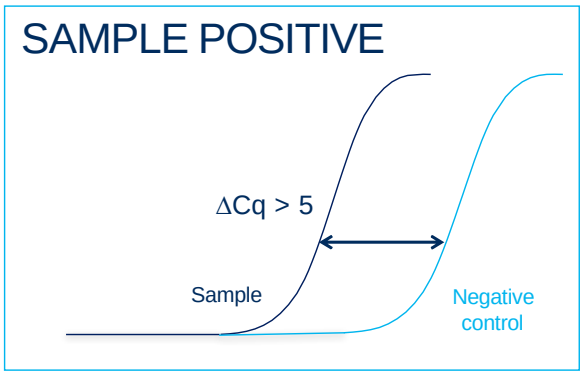
Target (FAM/ROX/Cy5)	Internal extraction control (VIC)	Positive Control	Negative Control	Interpretation
FAM +	**+ / -**	**+**	**-**	**WEST NILE VIRUS POSITIVE RESULT**
ROX +	**+ / -**	**+**	**-**	**YELLOW FEVER VIRUS POSITIVE RESULT**
Cy5 +	**+ / -**	**+**	**-**	**TICK-BORNE ENCEPHALITIS VIRUS POSITIVE RESULT**
-	**+**	**+**	**-**	**NEGATIVE RESULT**

Interpretation applicable to both tubes

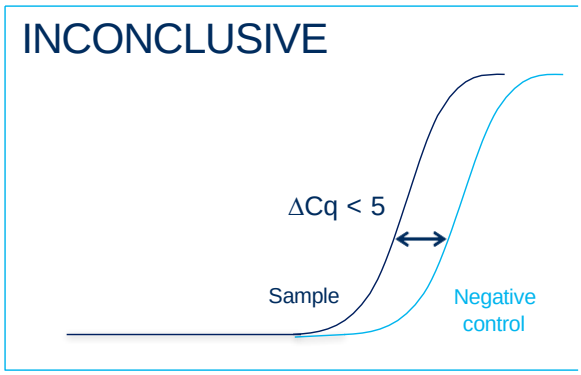
Target (FAM/ROX/Cy5)	Internal extraction control (VIC)	Positive Control	Negative Control	Interpretation
+ / -	**+ / -**	**+**	**≤35**	**EXPERIMENT FAILED Due to test contamination**
+ / -	**+ / -**	**+**	**>35**	*****
-	**-**	**+**	**-**	**SAMPLE PREPARATION FAILED**
+ / -	**+ / -**	**-**	**+ / -**	**EXPERIMENT FAILED**

Positive control template (RED) is expected to amplify between Cq 16 and 23. Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised.

* Where the test sample is positive, and the negative control is positive with a Cq >35, the sample must be reinterpreted based on the relative signal strength of the two results:



If the sample amplifies > 5 Cq earlier than the negative control then the sample should be reinterpreted (via the table above) with the negative control verified as negative.



If the sample amplifies < 5 Cq earlier than the negative control then the positive sample result is invalidated and the result should be determined inconclusive due to test contamination. The test for this sample should be repeated.