Primer Design Ltd

R00600

Dengue, Chikungunya, Zika Virus

Kit version: 2

Target Region:

Dengue Virus (3’ UTR)

Chikungunya Virus (Non-structural protein 2)

Zika Virus (Polyprotein gene)

genesig® Advanced Multiplex Kit

100 tests

GENESIG

Kits by Primerdesign

Specificity of primers and probes last reviewed on:
15 February 2022

For general laboratory and research use only

Introduction

Flavivirus is a genus of viruses in the family Flaviviridae. This genus includes the West Nile virus, dengue virus, tick-borne encephalitis virus, yellow fever virus, zika virus and several other viruses which may cause encephalitis. Chikungunya virus, however, belongs to the genus Alphavirus in the family Togaviridae.

Most of these viruses are transmitted by the bite from an infected arthropod (mosquito or tick) and hence classified as arboviruses. Human infections with these viruses are typically incidental, as humans are often dead-end hosts, unable to replicate the virus to high enough titres to reinfect the arthropods and therefore to continue the virus life cycle. The exceptions to this are the yellow fever, dengue, and zika viruses, which still require mosquito vectors, but are well-enough adapted to humans as to not necessarily depend upon animal hosts (although they continue to have important animal transmission routes, as well).

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.

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.

This kit provides a single tube discriminatory test to identify each of these three viruses individually.

Specificity

The genesig kit is designed for the in vitro detection and differentiation of Dengue virus, Zika virus (ZIKV) and Chikungunya virus (CHIKV) only. Individual tests have been designed in the conserved regions of each virus such that all isolates and subtypes will be detected simultaneously in the same test. The dengue component of the test will detect subtypes 1, 2, 3 and 4 but will not differentiate between them. A positive Dengue test result indicates that the sample contains any one of these four subtypes.

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

The dynamics of genetic variation means that new sequence information may become available after the initial design. Primer Design Ltd 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

- **1X Deng/ZIKV/CHIKV_v2.0 primer/probe mix (100 reactions, BROWN)**
  FAM, VIC, ROX and Cy5 labelled (see table below)

Target	Fluorophore
ZIKV	FAM
Dengue	VIC
CHIKV	Cy5
Internal extraction control	ROX

- **1X Endogenous control primer/probe mix (100 reactions, BROWN)**
  FAM labelled
- **1X Deng/ZIKV/CHIKV_v2.0 positive control template (RED)**
- **1X Internal extraction control RNA (BLUE)**
- **2X Lyophilised OneStep Master Mix (GOLD)**
- **2X oasig® resuspension buffer (BLUE)**
  for resuspension of the lyophilised OneStep Master Mix
- **1X Template preparation buffer (YELLOW)**
  for resuspension of the internal control template and positive control template
- **1X RNase/DNase free water (WHITE)**
  for resuspension of the primer/probe mixes

Reagents and equipment to be supplied by the user

Real-time PCR Instrument

RNA 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 tubes

qPCR plates or tubes

Kit storage and stability

This kit is stable at room temperature but should be stored at -20°C on arrival. Once the lyophilised components have been resuspended, they should not be exposed to temperatures above -20°C 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. Primer Design Ltd 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 the kit can achieve priming efficiencies between 90-110% and detect less than 100 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 Primer Design Ltd 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.

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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 cDNA. Fluorogenic probes are included in the same reaction mixture which consist 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

For a positive control, the kit contains a single positive control that contains templates for the 3 targets in the test. The kit positive control will give an ZIKV signal through the FAM channel, a Dengue signal through the VIC channel and a CHIKV signal through the Cy5 channel. Each time the kit is used, at least one positive control reaction 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 the positive control 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, or No Template Control (NTC) 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.

The primers and probe necessary to detect the internal extraction control are included in the multiplex primer and probe mix. 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 ROX channel and gives a Cq value of 28+/-3 depending on the level of sample dilution.

Endogenous control

To confirm extraction of a valid biological template, a separate primer/probe mix is included to detect an endogenous gene. Detection of the endogenous control is through the FAM channel and it is therefore NOT possible to perform a multiplex with the pathogen primers. A poor endogenous control signal may indicate that the sample did not contain sufficient biological material.

Resuspension protocol

To minimise the risk of contamination with foreign RNA, we recommend that all pipetting is 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/probe 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	
Deng/ZIKV/CHIKV_v2.0 primer/probe mix (**BROWN**)	110µl
Endogenous control primer/probe mix (**BROWN**)	165 µl

3. **Resuspend the positive control template and the internal extraction control RNA 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**)	600µl
Post-PCR heat-sealed foil	
Deng/ZIKV/CHIKV_v2.0 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 either to the RNA lysis/extraction buffer or to the RNA 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 4ul of the internal extraction control RNA (**BLUE**) to each sample in RNA lysis/extraction buffer.

2. Complete the 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
Deng/ZIKV/CHIKV_v2.0 primer/probe mix (**BROWN**)	1µl
RNase/DNase free water (WHITE)	4µl
Final volume	**15µl**

2. **For each RNA sample prepare an endogenous control reaction according to the table below (optional):**
   This control reaction provides information regarding the quality of the biological sample. The test is run in a separate well and is not a part of the multiplex.

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

3. **Pipette 15µl of these mixes into each well according to your qPCR experimental plate set up.**

4. **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.

5. **Pipette 5µl of positive control template into each well according to your plate set up.**
   The positive control contains templates for dengue, zika and chikungunya viruses. The final volume in each well is 20µl.

OneStep RT-qPCR amplification protocol

If using a machine that uses ROX as a passive reference, then the passive reference must be turned off or set to “none” for no passive reference.

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

The positive control well should give an amplification plot through the FAM channel (ZIKV), the VIC channel (Dengue) and the Cy5 channel (CHIKV). There is no internal control template within the positive control so the ROX 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.

Endogenous control

The signal obtained from the endogenous control reaction will vary according to the amount of biological material present in a given sample. An early signal indicates the presence of a good yield of biological material. A late signal suggests that little biological material is present in the sample.

Sample data

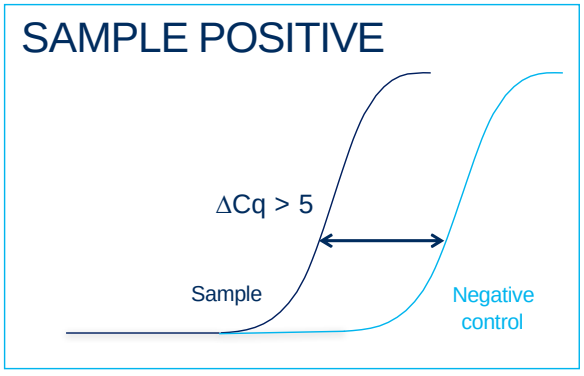
Presence of the viruses are detected in the channels indicated in the kit contents section. Positive signals indicate positive tests for those viruses. It may be possible for samples to contain multiple viruses, therefore positive results in the FAM, VIC and Cy5 channels may be present.

Summary of data interpretation

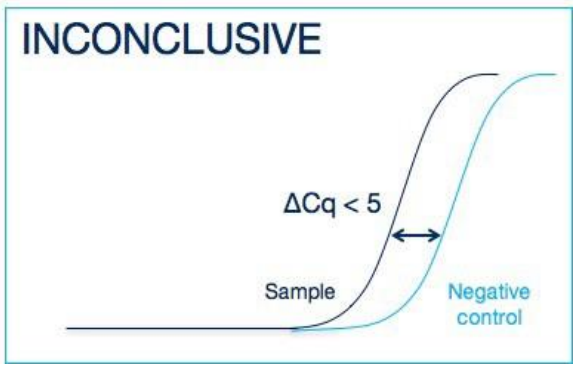
Target (FAM/VIC/Cy5)	Internal extraction control (ROX)	Positive Control	Negative Control	Interpretation
FAM +	**+ / -**	**+**	**-**	**ZIKV POSITIVE RESULT**
VIC +	**+ / -**	**+**	**-**	**Dengue POSITIVE RESULT**
Cy5 +	**+ / -**	**+**	**-**	**CHIKV POSITIVE RESULT**
-	**+**	**+**	**-**	**NEGATIVE RESULT**
+ / -	**+ / -**	**+**	**≤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.