Primerdesign™ Ltd

Dengue, Chikungunya, Zika Virus

(Multiplex kit)

genesig® kit

100 tests

For general laboratory and research use only

genesig®

DNA testing

Everything...
Everyone...
Everywhere...

Introduction to Flaviviruses

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 usually unable to replicate the virus to high enough titers to reinfect the arthropods needed to continue the virus lifecycle – man is then a dead end host. 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, Chikungunya, Zika Virus

The clinical presentation of these three arboviruses in humans can be very similar and they are endemic within the same geographical regions. This is because all three are spread by *Aedes* mosquito species having seasonality that reflects the mosquito breeding season. There are however subtle differences in the eventual clinical outcome and this means that differential diagnostic is an important diagnostic capability especially at a time when there is wide spread interest in tracking the spread of Zika virus in new populations.

The current kit provides a single tube differential test for these three viruses and is able to identify each individually.

Specificity

The genesig® is designed for the 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 results indicates that the sample has either one of these four subtypes.

The primers and probe sequences in this kit have 100% homology with a broad range of clinically relevant reference sequences based on a comprehensive bioinformatics analysis. They therefore have a very broad quantification profile.

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

Kit Contents

- Multiplex primer/probe mix (100 reactions BROWN)
  FAM, VIC, CY5 and ROX labelled (see table below)

Target	Fluorophore
ZIKV	FAM
Dengue	VIC
CHIKV	CY5
Internal extraction control	ROX

- Endogenous control primer/probe mix (150 reactions BROWN)
  FAM labelled
- Multiplex positive control template (Dengue, Chikungunya virus and Zika virus) (RED)
- Internal extraction control RNA (BLUE)
- Lyophilised oasig OneStep Mastermix
- Oasig resuspension buffer (BLUE)
- RNAse/DNAse free water (WHITE)

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. However, it is designed to work well with all processes that yield high quality RNA with minimal PCR inhibitors.

Pipettors and Tips

Vortex and Centrifuge

Thin walled 1.5ml PCR reaction 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 re-suspended 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.

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/DNA 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% and can detect between $1X10^8$ 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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The PCR process is covered by US Patents 4,683,195, and 4,683,202 and foreign equivalents owned by Hoffmann-La Roche AG. BI, ABI PRISM® GeneAmp® and MicroAmp® are registered trademarks of the Applera Genomics (Applied Biosystems Corporation). BIOMEK® is a registered trademark of Beckman Instruments, Inc.; iCycler™ is a registered trademark of Bio-Rad Laboratories, Rotor-Gene is a trademark of Corbett Research. LightCycler™ is a registered trademark of the Idaho Technology Inc. GeneAmp®, TaqMan® and AmpliTaqGold® are registered trademarks of Roche Molecular Systems, Inc., The purchase of the Primerdesign ™ reagents cannot be construed as an authorization or implicit license to practice PCR under any patents held by Hoffmann-LaRoche Inc.

Principles of the test

Real-time PCR

Individual primer and probes design for each virus 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 consists of a DNA probe labeled 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 real-time PCR platforms.

Positive control

For a positive control, the kit contains a single positive control that contains templates for each for the three viruses under test. 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 controls do 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 real-time PCR 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. The amplification of the internal control does not affect the sensitivity of the test and is detected separately through the ROX channel. The Internal control will give a Cq value of 28+/-3 but this can vary greatly depending on the efficiency of sample extraction and level of sample dilution.

Endogenous control

To confirm extraction of a valid biological template, a primer and probe mix is included to detect an endogenous gene. Detection of the endogenous control is through the FAM channel and it is NOT therefore 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.

Reconstitution Protocol

To minimize the risk of contamination with foreign DNA, 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 is in the base of the tube and is not spilt upon opening the tube.

2. Reconstitute the kit components in the RNAse/DNAse free water supplied, according to the table below:

To ensure complete resuspension, vortex each tube thoroughly.

Component – resuspend in water	Volume
Pre-PCR pack	
Multiplex primer/probe mix (BROWN)	110 µl
Endogenous control primer/probe mix (BROWN)	165 µl
Pre-PCR heat-sealed foil	
Internal extraction control RNA (BLUE)	600 µl
Post-PCR heat-sealed foil	
Positive Control Template (RED)*	500 µl

* 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.

3. Reconstitute the Lyophilised oasig OneStep Mastermix in oasig resuspension buffer, according to the table below:

Component – resuspend in oasig resuspension buffer	Volume
Lyophilised oasig OneStep Mastermix (RED)	525 µl

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 4µl 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-PCR detection protocol

For optimum performance and sensitivity

All pipetting steps and experimental plate set up should be performed on ice. After the plate is poured 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
oasig™ OneStep Mastermix	10 µl
Multiplex primer/probe mix (BROWN)	1 µl
RNAse/DNAse free water (WHITE)	4 µl
Final volume	**15 µl**

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 separate well and is not a part of the multiplex.

Component	Volume
oasig™ OneStep Mastermix	10 µl
Endogenous control primer/probe mix (BROWN)	1 µl
RNAse/DNAse free water (WHITE)	4 µl
Final volume	**15 µl**

3. **Pipette 15µl of these mixes into each well according to your real-time PCR experimental plate set up.**

4. **Pipette 5µl of RNA template 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.

Onestep 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 oasig™ OneStep Mastermix

	Step	Time	Temp
	Reverse Transcription	10 mins	55°C
	Enzyme Activation	2 mins	95°C
Cycling x50	Denaturation	10 secs	95°C
	DATA COLLECTION*	60 secs	60°C

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

Interpretation of Results

Positive control

The positive control well should give an amplification plot through the FAM channel (ZIKV), 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 plots). 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.

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

Each sample should give a signal through the ROX channel which indicates successful extraction of the sample. Presence of the viruses are detected in the channels indicated in the kit contents section. Positive signals indicate positive tests for those viruses. It is anticipated that each sample is likely to have only one virus within it and hence multiple positive results in multiple channels should be treated with caution.

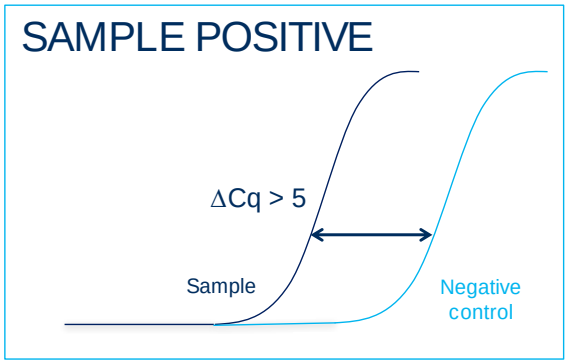
Summary of data interpretation

Target (FAM/VIC/CY5)	Internal control (ROX)	Positive Control	Negative Control	Interpretation
FAM +	+ / -	+	-	ZIKV POSITIVE RESULT
VIC +	+ / -	+	-	Dengue POSITIVE RESULT
CY5 +	+ / -	+	-	CHIKV POSITIVE RESULT
-	+	+	-	NEGATIVE RESULT

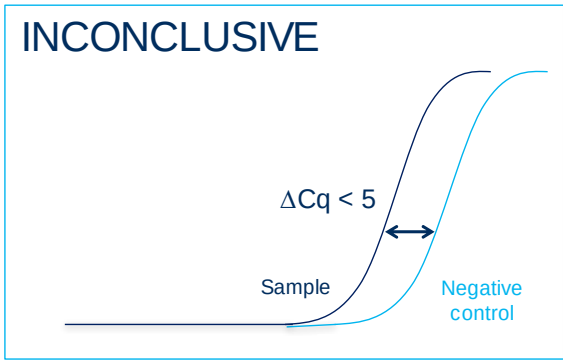
+ / -	+ / -	+	≤35	EXPERIMENT FAILED Due to test contamination
+ / -	+ / -	+	>35	*
-	-	+	-	SAMPLE PREPARATION FAILED
+ / -	+ / -	-	+ / -	EXPERIMENT FAILED

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.