RAS Lifesciences Pvt Ltd
Striving for better health

Amplisure™ Chikungunya RT PCR Kit

(Reverse Transcriptase Real Time PCR Kit)

EP-CHKF-25 : 25 rxns
EP-CHKF-50 : 50 rxns
EP-CHKF-100 : 100 rxns

IVD

Product Insert

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Introduction

Chikungunya disease is caused by Chikungunya virus belongs to the family Togaviridae that transmitted to humans by the bite of virus carrying *Aedes aegypti* mosquito. Chikungunya infections can be diagnosed by common laboratory tests which include virus isolation, serological tests and reverse transcriptase real time-polymerase chain reaction (RT-PCR).

Virus isolation provides the most definitive diagnosis, but takes one to two weeks for completion and moreover it must be carried out in biosafety level 3 laboratories. The technique involves exposing specific cell lines to samples from whole blood and identifying Chikungunya virus-specific responses.

Serological diagnosis requires a larger amount of blood than the other methods, and uses an ELISA assay to measure Chikungunya-specific IgM levels. Results require two to three days, and false positives can occur with infection via other related viruses.

Reverse transcriptase RT-PCR based techniques are more specific and sensitive by which Chikungunya-specific genes can be amplified from blood (K_2EDTA-Blood). Results can be determined in about three hours.

Intended Use

The Amplisure™ Chikungunya RT PCR Kit is used to detect Chikungunya Virus in human plasma. The Chikungunya infection is detected by amplification of CHK RNA in Human plasma.

Product Description

Amplisure™ Chikungunya reverse transcriptase RT PCR Kit is an *in-vitro* diagnostic kit for detection of Chikungunya Virus in human plasma. The kit contains the necessary reagents for performing Chikungunya RT PCR.

Viral detection by Real Time polymerase chain reaction (PCR) is based on the amplification of specific region (NSP gene) of the viral genome. The assay principle is based on Taqman probes which allow higher specificity and sensitivity.

In addition, the Amplisure™ Chikungunya RT PCR Kit contains a second amplification system to identify possible PCR inhibition by using an internal control (IC) without affecting the analytical sensitivity of the assay.

Recommended Work areas

Molecular Diagnostics work area includes:

a) Sample preparation area/room – for extraction of nucleic acids from clinical samples
b) Pre-PCR area/room - for setting up PCR reaction
c) PCR area/room – for performing PCR using the thermo cyclers

As part of Good Laboratory Practices (GLP), it is recommended to have dedicated areas to avoid cross contamination.

General Precautions

Precautions while extracting Nucleic acid

Always wear proper attire (nitrile gloves, facemask and Head cap) before starting the nucleic acid extraction procedure. During preparation of samples, compliance with good laboratory practices are essential to minimize the risk of cross-contamination between samples, and the inadvertent introduction of ribonucleases (RNases) into samples during and after the extraction procedure. Proper aseptic technique should always be used when working with RNA.

The Sample Preparation Area is dedicated to processing samples. All reagents used in the Sample Preparation Area should remain in this dedicated area at all times.

Laboratory coats, pipettes, pipette tips and vortex mixer used in the Sample Preparation Area must remain in this area and not be moved to the Pre-PCR/PCR area. Discard the gloves before leaving this area. Do not bring amplified product into the Sample Preparation Area. Usage of filter tips is recommended while sample preparation and should be performed in a Biosafety cabinet.

Precautions while setting up a PCR reaction

PCR assay is sensitive and any accidental introduction of product from previous amplification reactions leads to incorrect results. Hence, measures should be taken to reduce the risk of contamination in the laboratory which includes physically separating the activities involved in performing PCR and complying with good laboratory practices.
It is recommended to have proper cleaning procedures to minimize the risk of cross contamination and carry over contamination (e.g. DNA OUT™, RNase OUT™, 0.1% Sodium Hypochlorite, Fumigation etc.).

It is recommended that areas should be defined in Pre-PCR room for preparation of mastermix and addition of templates. Laboratory coats and equipment used in the Pre-PCR Area must remain in this area and should not be moved to the Sample Preparation Area.

Precautions for post PCR or equipment area/room

The Real time PCR instrument/s should be kept in a separate segregated area away from Sample preparation area and Pre-PCR area.

Precautions after completion of Real time PCR assay

The reaction tubes or strips should be properly discarded without opening the caps, after the completion of run to avoid carry over contamination.

Usage Limitations

1. The kit and all its components are for *in-vitro* diagnostics only.
2. The product is to be used by personnel specially trained in the *in-vitro* diagnostics procedures only.
3. Follow the product insert strictly for optimal PCR results.
4. Do not use the kit beyond the expiry date mentioned on the kit box.
5. Follow the guidelines provided in product insert for sample collection, storage and transport.
6. For ideal performance, store the kit under recommended conditions only.

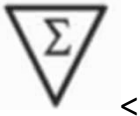
Safety Precautions

1. All patient specimens should be considered as potentially infectious and handled in a BSL2 biosafety hood with BSL3 practices.
2. Wear personal protective equipment, including gloves, head cap, face mask and lab coats when handling kit reagents/sample extraction. Wash hands thoroughly using detergents before and after performing the test.
3. Do not smoke, drink or eat in areas where kit reagents and/or human specimens are being used.
4. Dispose the unused kit reagents and human specimens as per regulatory guidelines.

Storage Conditions and Product Stability

1. All the kit reagents should be stored at -20 °C. Replace all the kit components immediately at -20 °C after usage.
2. Repeated thawing and freezing (more than 6 x) of all kit reagents should be avoided, as it reduces assay sensitivity. If needed, make aliquots of the kit reagents according to the volume used in the protocol prior to freezing.
3. Allow reagents to be thawed completely on Ice/4 °C prior to use.
4. Kit reagents are stable through the end of the expiration date indicated on the box when stored at -20 °C.

Symbols

Description of Symbol	Denotation
	*in-vitro* Diagnostic medical device
	Consult Instruction manual (Product Insert) for use
	Lot Number of the kit or Kit contents
	Catalogue number of Kit
 <N>	Contains sufficient for <N> reactions (Pack Size)
	Manufacturer
	Temperature limitation (Storage Condition)
	Use by MMM-YYYY (Expiry Date)
	Biological risk (handle carefully)
	Important Note

Kit Components

Color Coding (Caps)	Contents	Description	25 rxns (EP-CHKR-25)	50 rxns (EP-CHKR-50)	100 rxns (EP-CHKR-100) 2 x 50 rxns
Yellow	RAS qRNA PCR Mix	DNA Amplification Reagent	1 x 375µL	1 x 750µL	2 x 750µL
Red	RAS RT Mix	cDNA Amplification Reagent	1 x 175µL	1 x 350µL	2 x 350µL
Brown	RAS CHK PPM	Primer-Probe Mix	1 x 50 µL	1 x 100 µL	2 x 100 µL
Lilac	RAS CHK PC	Positive control	1 x 30 µL	1 x 60 µL	2 x 250 µL
Natural	RAS IC-B PCR Mix	Internal Controls	1 x 25 µL	1 x 50 µL	2 x 50 µL
Natural	RAS R-IC-B EX Mix		1 x 250 µL	1 x 500 µL	2 x 500 µL
White	MBGW	Molecular Biology Grade water	1 x 0.5 mL	1 x 0.5 mL	2 x 0.5 mL

Materials required but not supplied

The materials which are required but not supplied are listed below:

1. Viral RNA Extraction kit
2. Biosafety Cabinet
3. PCR Hood
4. Calibrated variable micropipettes
5. Sterile pipette filter tips (aerosol free)
6. Vortex mixer
7. Dry Bath

8. Benchtop centrifuge with rotor for 1.5 mL reaction tubes
9. Real Time PCR machine
10. Strip Tubes and Caps (0.2 mL) or PCR Tubes (0.2 mL) or 96 well plate
11. Cooling block (96 x 0.2 mL tubes)
12. 1.5 mL centrifuge tubes
13. 1.5 mL centrifuge tube stand
14. Cooling block (2 mL tubes)
15. Sterile nitrile gloves
16. Facemask
17. Head cap
18. Lab coats

Quality Systems

In accordance with ISO-certified Quality Management System (9001:2008 and 13485: 2003) of RAS Lifesciences, each lot of Amplisure™ Chikungunya RT PCR Kit is tested against predetermined specifications to ensure consistent product quality.

Sample Type/Collection/Storage/Transport

Sample Type

Plasma (K_2EDTA-Blood)
Heparinized Blood must not be used as they inhibit the PCR reaction

Sample Collection, Storage and Transport

3-5 mL of blood has to be drawn into a K_2EDTA vaccutainer. Cap and swirl the tubes for uniform mixing of blood and K_2EDTA. When K_2EDTA is used, whole blood can be collected in tubes with or without a gel separator. The K_2EDTA blood samples are centrifuged (20 minutes at 1000–1500 x *g)* to separate plasma from cellular material and in the case of a non gel separator tube, the plasma should be removed to a secondary sterile tube within 4 hours of phlebotomy.

Collection and storage of unstabilized whole blood is not recommended for PCR analysis, because RNA degradation occurs in blood stored *ex vivo*. The sensitivity of the assay can be reduced if whole blood samples are frozen or stored for a longer period of time.

Plasma separated in a gel separator tube may be transported to the laboratory *in situ.* Plasma should be shipped at 2 to 8 °C and stored at -20°C as it is stable for up to five days at 2 to 8 °C and longer if frozen at -20°C or -70°C or lower. Do not store plasma samples in a "frost -free" freezer as the temperature is cycled several times per day on this type of freezer, causing degradation of nucleic acid targets.

Sample material should be transported in a leak proof, unbreakable transport container to avoid leakage of sample. The samples should be transported following the local and national instructions for the transport of pathogen material.

Assay Procedure

RNA Extraction

Amplisure™ Chikungunya RT PCR Kit has been validated using the following Viral RNA extraction kits:

1- Roche High Pure Viral RNA kit (Cat. No. 11858882001)
2- QIAamp Viral RNA Mini Kit (Cat. No. 52904)

Follow the manufacturer's instructions mentioned in the manual for Viral RNA extraction. Different pack sizes of the above mentioned kits can be used. However, the customer can also validate their own extraction process using other Viral RNA extraction Kits.

Recommended sample volume for extraction and elution are as follows:

S. No.	Name of the RNA Isolation Kit	Recommended Sample volume *(to be taken for RNA Extraction)*	Recommended Final Elution volume
1.	Roche High Pure Viral RNA kit (Cat. No. 11858882001)	200 µL	50 µL
2.	QIAamp Viral RNA Mini Kit (Cat. No. 52904)	140 µL	50 µL

The analytical sensitivity of the assay in consideration of the purification was determined using the above defined volumes.

Use of Internal Control (IC-B)

Internal controls are supplied (RAS R-IC-B Ex Mix and RAS IC-B PCR Mix) along with Amplisure™ Chikungunya RT PCR Kit. This allows the user to control the Viral RNA isolation procedure as well as to check for possible PCR inhibition.

ⓘ Internal control should only be used once, either at the Extraction step or at the PCR step

Usage of Internal Control at the RNA extraction step

If internal control (IC) is required to be added at the time of RNA extraction, add 10 µL of RAS *R-IC-B Ex mix* per isolation to the lysis buffer along with other components of kit used for lysis (as per kit instructions) and vortex for 5 seconds prior to usage.

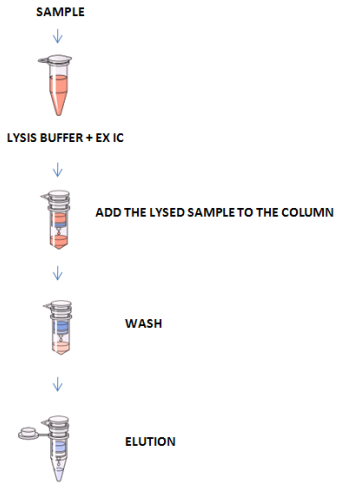


Fig.1 Viral RNA Extraction Overview

Usage of Internal Control at the Real time PCR step

The internal control can optionally be used exclusively to check for possible PCR inhibition. For this application, add the internal control directly to the PCR master mix as described below.

qPCR Protocol

Preparation of Reaction Mastermix

Detection procedure with Amplisure™ Chikungunya RT PCR Kit involves *single step RT qPCR*. It is recommended that a minimum of three standards and a negative control (MBGW should be used as negative control) are required to be included in a single run for acquiring proper results.

Set up a real time single step RTPCR reaction as below

1- qPCR reaction mix composition without Internal Control (When RAS R-IC-B Ex Mix is added during RNA extraction)

Components	Volume per reaction (µL) (for final vol. of 30 µL)
RAS qRNA PCR Mix	15.0
RAS RT Mix	7.0
RAS CHK PPM	2.0
RNA/CHK PC/ MBGW	5.0
MBGW	1.0

2- qPCR reaction mix composition with Internal Control (When RAS R-IC-B Ex Mix is not added during RNA extraction)

Components	Volume per reaction (µL) (for final vol. of 30 µL)
RAS qRNA PCR Mix	15.0
RAS RT Mix	7.0
RAS CHK PPM	2.0
RNA/CHK PC/ MBGW	5.0
RAS IC-B PCR Mix	1.0

1. **Addition of IC-B PCR mix (if required) should be done at the time of preparation of master mix.**
2. **The results may be inconsistent, if the IC-B PCR mix is added individually.**

Place the PCR plate/tubes/strips in real time thermocycler.

PCR Programming

The Amplisure™ Chikungunya RT PCR Kit is validated on the following instruments:

- Rotor-Gene™ 6000
- Rotor-Gene™ Q 5plex
- ABI 7500 DX Real-Time PCR System
- ABI 7300 Real-Time PCR System
- Eppendorf Realplex 4
- Bio-Rad™ CFX 96

Plate Setup

1. Program the plate setup by labeling the slots as per tube/strip/plate labels. The sequence of labeling of slots should be the same way as the tube/strip/plate is kept in the machine.
2. Select the type of sample (Unknown/PC/NTC) for each slot.

3. Select the channel for acquisition (FAM/Yakima Yellow).

Sl. No.	Name of channel	Source wavelength (nm)	Detection wavelength (nm)
1.	FAM (Pathogen target)	470	510
2.	Yakima Yellow (Internal Control)	530	555

4. For background calibration in different instruments, follow the procedure described below:

   Rotor-Gene™ 6000 - Perform 'Gain optimization'
   Rotor-Gene™ Q 5plex - Perform 'Gain optimization'
   ABI 7500 DX Real-Time PCR System - Select Passive Reference dye 'ROX'
   ABI 7300 Real-Time PCR System - Select Passive Reference dye 'ROX'

Preparation of reaction mastermix and cycling conditions are same for all the instruments listed in the product insert. For instrument specific protocols, please contact our technical support team at amplisure@raslifesciences.com

Cycling conditions

1. Configure the following program in the machine.

Steps	No. of cycles	Temperature (°C)	Time
1 (cDNA synthesis)	1	42	15 min.
2(Initial Denaturation)	1	95	10 min
3 (PCR cycling)	45	95	30 sec.
		60*	1 min
* **Plate Read/Data Acquisition in FAM / Yakima Yellow channel**			

2. Set the reaction volume as 30 µL.
3. Plate read/Data Acquisition for FAM and VIC channel should be incorporated in the second stage of step 3 (60 °C/ 1 min).
4. The ideal run time for the assay is 120 minutes. Note: *In case of Eppendorf Realplex 4, select RAMP rate as 35%.*

Preparation of reaction mastermix and cycling conditions are same for all the instruments listed in the product insert. For instrument specific protocols, please contact our technical support team at amplisure@raslifesciences.com

Data Analysis

Analyze the data after completion of the run. Check the R_n/*Cycle* amplification plot and ΔR_n/*Cycle* amplification plot to observe the amplification signal generated by different samples in the run. Compare both the plots for data analysis. Also look for noisy signals, if observed as it might not give you a proper result.

Setting the threshold for the qPCR Data analysis

The threshold should be set either automatically (by the machine itself)/ or manually just above the background signal of the negative controls and negative samples by referring to R_n/*Cycle* amplification plot. The mean threshold value calculated from these experiments will most likely work for the majority of future runs, but the user should nevertheless review the generated threshold value at regular intervals.

Result

The values for unknown samples would appear in the result column in *Ct* in FAM Channel. Samples showing no amplification in FAM channel should show amplification in FAM channel, and then only results should be considered. The negative control should not show any value in the result column.

Interpretation

Interpret the values for unknown samples based on the observations as described in the following table and there should be no amplification in negative control.

Observation	Interpretation	Conclusion
Amplification signal detected in CHK channel (FAM) and in Internal control channel (Yakima yellow)	Chikungunya RNA detected	Proceed for further Analysis
Amplification signal detected in CHK channel (FAM) but no signal in Internal Control channel (Yakima yellow)	Chikungunya RNA detected	
Amplification signal not detected in CHK channel (FAM) but detected in Internal control channel (Yakima yellow)	Chikungunya RNA not detected	

No Amplification signal detected in CHK channel (FAM) as well as Internal control channel (Yakima yellow) in unknown samples	Possible inhibition of PCR	Dilute the RNA sample (1:100) and repeat the assay

Troubleshoot

Observation	Possible cause	Solution
No amplification signal for Samples in FAM channel	1. One of the components may not have been added. 2. Incorrect channel selection 3. Incorrect programming of the real time machine.	1. Repeat the assay by following the correct protocol and addition of reagents 2&3. Please recheck the PCR program 4. Contact manufacturer of thermocycler for technical
Weak amplification signal for samples (Signal below threshold) in FAM Channel	1. Improper PCR programming. 2. Inaccurate dispensing of reagents 3. Possible deterioration of kit components due to improper storage	1. Repeat the assay by following the correct protocol 2. Minimize Pipetting errors/Check for calibration status of pipettes
Identical/Similar Ct values observed in FAM channel	1. Possible contamination of Kit reagents / Positive Control/Work area.	1. Use fresh aliquots of Positive Control/Kit Reagents (if available) 2. Clean the PCR rack/Pipettes thoroughly as per GLP 3. Clean and Fumigate the work area overnight prior to use

For any other technical query; please contact amplisure@raslifesciences.com

Assay Characteristics

Analytical Sensitivity of PCR assay

Total 5 samples ranging from 50 copies/ PCR to 3 copies /PCR were prepared by diluting Chikungunya RNA and analytical sensitivity is determined by testing these concentrations in replicates of 8.

The Lower Detection Limit (LOD) of the Amplisure™ Chikungunya RT-PCR assay is defined as 0.6 copies/µL which is equivalent to 3 copies /PCR as we are using 5µL of RNA as template.

Specificity

The specificity of the Amplisure™ Chikungunya RT PCR Kit is ensured by the selection of the primers and probes, as well as the selection of stringent reaction conditions. The primers and probes were checked for possible homologies to all published sequences (Genbank) by BLAST analysis to avoid any homology with other organisms.

Cross Reactivity Data

A potential cross-reactivity of the Amplisure™ Chikungunya RT PCR Kit was tested using the control group listed below. None of the tested pathogens has been reactive. No cross-reactivity appeared with mixed infections.

Moreover, the specificity was validated with 50 different healthy plasma specimens/other various sample types. These did not generate any signals with the CHK specific primers and probes, which are included in the Amplisure™ Chikungunya RT PCR Kit.

Pathogen Tested	Cross reactivity with the Chikungunya Primers/Probes
Chikungunya negative samples	-
Herpes Simplex Virus-1	-
Herpes Simplex Virus-2	-
Epstein Barr virus	-
Human Immunodeficiency Virus	-
Hepatitis B virus	-

Hepatitis C virus	-
Parvovirus	-
Adenovirus	-
Dengue Virus	-
Cytomegalovirus	-
BK Virus	-
E.coli	-

Precision

For studying the precision stock RNA was serially diluted to 100 copies/PCR, 50 copies/PCR and 25 copies/PCR. Samples were tested with Amplisure™ Chikungunya RT PCR Kit continuously in duplicates for 9 days.

No. of Days of testing	Sample 1		Sample 2		Sample 3	
	SD	CV%	SD	CV%	SD	CV%
9	0.262	0.008%	0.339	0.010%	0.770	0.022%

The Amplisure™ Chikungunya RT PCR Kit amplified the samples with good consistency.

Performances study on clinical samples

Biological performances of the Amplisure™ Chikungunya RT PCR Kit kit on plasma samples have been evaluated in an Indian Microbiology Laboratory, using samples collected during the laboratory's routine activity.

The laboratory's routine technique is a published gel based qualitative PCR which was used to amplify 354 BP NSp1 region of chikungunya genome. The results were expressed as detected and not detected.

The samples of plasma were extracted with the Roche High Pure Viral RNA kit (Cat. No.11858882001) using the protocol recommanded by the manufacturer on 200 µL with an elution volume of 50 µL. The extracted samples were amplified on ABI Veriti end point PCR machine for performing published method. Amplisure chikungunya Real time PCR was performed on rotagene Q following manufacturers instructions.

A total of 57 samples were tested.

Comparison of qualitative results- RAS assay compared to gel based qualitative PCR :

		Routine technique		**Total**
		Detected	**Not detected**	
Amplisure™ Chikungunya RT PCR Kit	**Detected**	19	21 **	**40**
	Not Detected	0	17	**17**
	Total	**19**	**38**	**57**

*** Among the 21 samples not detected by the routine technique, most of them had a Ct around 36-37 with Amplisure Chikungunya Real time. Beside the routine technique (PCR and gel electrophoresis), all of these 21 samples when subjected to a second test by nested PCR and gel electrophoresis. All were confirmed as positive for Chikungunya by the size of the band by electrophoresis. These bands were eluted and sequenced on 5 samples confirming Chikungunya, in accordance with Amplisure Chikungunya Real time PCR results.*

Positive percent agreement= 19/19 = 100.0% [82.4 - 100] % (95% confidence interval)
Negative percent agreement = 17/38 =44.7 % [28.6 – 61.7] % (95% confidence interval)
Overall percent agreement = 36/57 = 63.1% [49.3 – 75.6] % (95% confidence interval)

Abbreviations

Abbreviation	***Expansion***
CHK	Chikungunya Virus
RNA	Ribonucleic Acid
IC	Internal Control

DNases	Deoxyribonucleases
PCR	Polymerase Chain Reaction
BSL2	Bio Safety Level 2
BSL3	Bio Safety Level 3
mL	Milli Liters
µL	Micro Liters
K_2EDTA	Potassium Ethylene Diamine Tetra Acetate
G	Relative Centrifugal Force
qPCR Protocol	Quantitative PCR protocol
MBGW	Molecular Biology Grade Water
RT PCR	Real Time PCR
NTC	No Template Control
FAM	Carboxyfluorescein
FAM	Cyanine
ROX	Carboxy-X-rhodamine
BLAST	Basic Local Alignment Search Tool
Sps	Species
Rxn	Reaction

References

1. Panning M, Charrel RN, Mantke OD, *et al*. Coordinated Implementation of Chikungunya Virus Reverse Transcription–PCR. Emerging Infect Dis 2009; 15 (6): 469-71.
2. Lakshmi V, Neeraja M, Subbalaxmi MV, *et al*. Clinical features and molecular diagnosis of Chikungunya fever from South India. Clin Infect Dis. 2008; 46:1436–42.
3. F. Hasebe, Z. Saat, M.C. Parquet, *et al.*Combined Detection and Genotyping of Chikungunya Virus by a Specific Reverse Transcription-Polymerase Chain Reaction. Journal of Medical Virology 67:370–374 (2002).

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