Sacace BIOTECHNOLOGIES

IVD For *in Vitro* Diagnostic Use

CE

Dengue Real-TM Genotype

Handbook

Real Time PCR kit for detection and differentiation of Dengue virus genotypes 1, 2, 3 and 4 in clinical material

REF V63-50FRT

Σ **50**

NAME

Dengue Real-TM Genotype

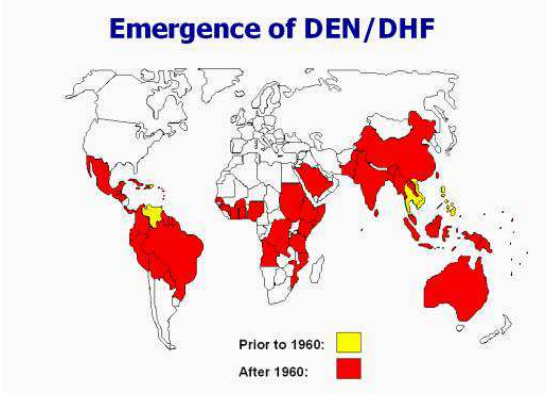
INTRODUCTION

Dengue is one of the most important arthropod-borne viral diseases with large global burden. The disease is caused by dengue virus (DENV), a member of Flaviviridae family, with four distinct serotypes (DENV-1, -2, -3, and -4) circulating in tropical and subtropical regions in the world. DENV is transmitted to human by *Aedes* mosquitoes as vector. Dengue clinical manifestations vary from asymptomatic or mild flu-like syndrome known as classic Dengue Fever (DF) to more severe form known as Dengue Hemorrhagic Fever (DHF) and the potentially fatal Dengue Shock Syndrome (DSS). Similar to other RNA viruses, DENV possess diverse genetic characteristics as shown by the presence of various genotypes within serotypes.

There are four distinct dengue virus (DENV) serotypes that share antigenic relationships (DENV-1, DENV-2, DENV-3, and DENV-4), and although infection with one serotype confers lifelong protection against that serotype, it does not necessarily protect against a secondary infection with a heterologous serotype. Indeed, non protective but cross-reactive antibodies may enhance disease severity.

There are evidence that secondary infection with a heterologous DENV serotype, or primary infection in infants born to dengue-immune mothers, is an important individual risk factor for DHF/DSS. During secondary infection with a different serotype, the presence of low levels of heterotypic neutralizing antibodies may reduce disease severity. Alternatively, in the absence of such neutralizing antibodies, heterotypic cross-reactive antibodies may form complexes with the virus and the Fc-receptors on these complexed antibodies may attach to mononuclear phagocytes, thus enhancing the efficiency of infection and thereby increasing the number of infected mononuclear phagocytes. This phenomenon is known as antibody dependent enhancement (ADE).

The geographical areas in which dengue transmission occurs have expanded in recent years and all four dengue virus serotypes (DENV-1–4) are now circulating in Asia, Africa and the Americas, a dramatically different scenario from that which prevailed 30 or 40 years ago.



Estimates of the global incidence of dengue infections per year have ranged between 50 million and 200 million; however, recent estimates using cartographic approaches suggest this number is closer to almost 400 million.

The expansion of dengue is expected to increase due to factors such as the modern dynamics of climate change, globalization, travel, trade, socioeconomics, settlement and also viral evolution. No vaccine or specific antiviral therapy currently exists to address the growing threat of dengue.

Acute infection with dengue virus is confirmed when the virus is isolated from serum or autopsy tissue specimens, or the specific dengue virus genome is identified by reverse transcription-polymerase chain reaction (RT–PCR) from serum or plasma, cerebrospinal fluid, or autopsy tissue specimens during an acute febrile illness. Methods such as one-step, real time RT–PCR are now widely used to detect dengue viral genes in acute-phase serum samples, approximately the first 5 days of symptoms. This detection coincides with the viremia and the febrile phase of illness onset.

INTENDED USE

DENGUE Real-TM Genotype PCR kit is an *in vitro* nucleic acid amplification test for detection and differentiation of RNA of Dengue virus type 1-4 in the clinical materials (blood plasma, blood serum) and in the human's autopsy materials (brain, liver, spleen tissues), in animal's materials (brain, spleen tissues), in mosquitoes by using real-time hybridization-fluorescence detection of amplified products.

The results of PCR analysis are taken into account in complex diagnostics of disease.

PRINCIPLE OF ASSAY

Dengue virus detection includes RNA isolation from biological materials and reverse transcription of RNA into cDNA combined with real-time PCR amplification of cDNA. *Dengue* detection by the polymerase chain reaction (PCR) is based on the amplification of pathogen genome specific region using specific *Dengue* genotype primers. In real-time PCR, the amplified product is detected using fluorescent dyes. These dyes are linked to oligonucleotide probes which bind specifically to the amplified product. The real-time monitoring of fluorescence intensities during the real-time PCR allows detection of the amplified product without re-opening the reaction tubes after the PCR run. **Dengue Real-TM Genotype** PCR kit uses "hot-start", which greatly reduces the frequency of nonspecifically primed reactions.

The *DENGUE* genotype 1 cDNA is detected in the FAM/Green channel.

The *DENGUE* genotype 2 cDNA is detected in the JOE/HEX/Yellow channel

The *DENGUE* genotype 3 cDNA is detected in the Rox/Texas Red/Orange channel.

The *DENGUE* genotype 4 cDNA is detected in the Cy5/Red channel

The signal of the IC is detected in the Cy5.5/Crimson/ Quasar705 channel*

**only for 5-channels Real Time PCR instruments. On 4-channels RT PCR instrument kit can be used without IC detection.*

The Positive Control of Amplification, Positive Control DV 1-4 types/IC is detected in FAM/Green (genotype 1), JOE/HEX/Yellow (genotype 2), ROX/Texas Red/Orange (genotype 3), Cy5/Red (genotypes 4) and Cy5.5/Crimson/ Quasar705 (Internal Control) channels.

MATERIALS PROVIDED

- **RT-G-mix-2,** 0,015 ml;
- **PCR-mix-1-FRT DV,** 0,6 ml;
- **RT-PCR-mix-2-TM,** 0,3 ml;
- **Hot Start TaqF Polymerase,** 0,03 ml;
- **M-MLV Revertase,** 0,015 ml;
- **cDNA *DV* 1-4 types/IC (C+),** 0,2 ml;
- **RNA-buffer,** 0,2 ml;
- **Negative Control (C–)*,** 2 x 1,2 ml;
- **Internal Control (IC)**,** 5 x 0,12 ml;

Dengue Real-TM Genotype PCR kit is intended for 60 reverse transcription and amplification reactions including controls.

* *Must be used in the isolation procedure as Negative Control of Extraction: add 100 µl of C– (Negative Control) to labeled Cneg;*

** *add 10 µl of Internal Control during the RNA isolation directly to the sample/lysis mixture (see Ribo-Virus REF K-2/C/A or DNA/RNA-Prep REF K-2-9 protocol).*

MATERIALS REQUIRED BUT NOT PROVIDED

- Real Time Thermalcycler
- RNA isolation kit
- Desktop microcentrifuge for “eppendorf” type tubes
- Vortex mixer
- Disposable gloves, powderless
- Biohazard waste container
- Refrigerator, Freezer
- Workstation
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Disposable polypropylene PCR tubes or strips
- Tube racks

STORAGE INSTRUCTIONS

All components of the **DENGUE Real-TM Genotype** PCR kit are to be stored at or below minus 20ºC. They are stable until the expiration date indicated on the label. The kit can be shipped at 2-8°C but should be stored at -20°C immediately on receipt.

STABILITY

DENGUE Real-TM Genotype PCR kit is stable up to the expiration date indicated on the kit label. The product will maintain performance through the control date printed on the label. Exposure to light, heat or humidity may affect the shelf life of some of the kit components and should be avoided. Repeated thawing and freezing of these reagents should be avoided, as this may reduce the sensitivity.

QUALITY CONTROL

In accordance with Sacace's ISO 13485-Certified Quality Management System, each lot is tested against predetermined specifications to ensure consistent product quality.

WARNINGS AND PRECAUTIONS

IVD

***In Vitro* Diagnostic Medical Device**

For *In Vitro* Diagnostic Use Only

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol barriers and use new tip for every procedure.
- Store extracted positive material (samples, controls and amplicons) away from all other reagents and add it to the reaction mix in a separate area.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use a kit after its expiration date.
- Dispose of all specimens and unused reagents in accordance with local authorities' regulations.
- Specimens should be considered potentially infectious and handled in a biological cabinet in accordance with appropriate biosafety practices.
- Clean and disinfect all sample or reagent spills using a disinfectant such as 0.5% sodium hypochlorite, or other suitable disinfectant.
- Avoid sample or reagent contact with the skin, eyes, and mucous membranes. If skin, eyes, or mucous membranes come into contact, rinse immediately with water and seek medical advice immediately.
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in the techniques of DNA amplification.
- The laboratory process must be one-directional, it should begin in the Extraction Area and then move to the Amplification and Detection Areas. Do not return samples, equipment and reagents to the area in which the previous step was performed.

Some components of this kit contain sodium azide as a preservative. Do not use metal tubing for reagent transfer.

PRODUCT USE LIMITATIONS

All reagents may exclusively be used in in vitro diagnostics. Use of this product should be limited to personnel trained in the techniques of DNA amplification (UNI EN ISO 18113-2:2012). Strict compliance with the user manual is required for optimal PCR results. Attention should be paid to expiration dates printed on the box and labels of all components. Do not use a kit after its expiration date.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

Note: Handle all specimens as if they are potentially infectious agents.

Dengue Real-TM Genotype PCR kit is intended for analysis of RNA/DNA extracted with RNA/DNA extraction kits from the clinical/biological material blood plasma, blood serum, human's autopsy materials (brain, liver, spleen tissues), animal's materials (brain, spleen tissues) and mosquitoes.

- *Blood plasma:* EDTA tubes may be used with the **Dengue Real-TM Genotype**. Follow sample tube manufacturer's instructions. Whole blood collected in EDTA should be separated into plasma and cellular components by centrifugation at 800-1600 x g for 20 min within six hours. The isolated plasma has to be transferred into a sterile polypropylene tube. Plasma may be stored at 2-8°C for an additional 3 days. Alternatively, plasma may be stored at -18°C for up to one month or 1 year when stored at -70°C.Do not freeze whole blood. Specimens anti-coagulated with heparin are unsuitable for this test. Thaw frozen specimens at room temperature before using. Whole blood must be transported at 2-25°C and processed within 6 hours of collection. Plasma may be transported at 2-8°C or frozen.
- Autopsy materials (brain, liver, spleen tissues). This material is homogenized by using sterile porcelain mortars and pestles, after that 10 % suspension is made on the sterile saline solution or phosphate buffer. For RNA extraction 30 µl of suspension should be taken.
- Mosquitoes. For making mosquito suspension sterile porcelain cap and sterile pestle are used. At first pools of mosquitoes should be formed (not more than 25 species of mosquitoes of Aedes class). Mosquitoes are homogenized in the saline solution or in the phosphate buffer in proportion 1 mosquito – 40 µl solution. The samples are centrifuged at 10 000 g for 1 min. After that 100 µl of supernatant is taken away for RNA extraction.

The above-mentioned biological material can be stored for 24 hour period before the test at 2-8 °C or for a week at temperature from minus 24 to minus 16 °C. For autopsy materials and mosquitoes the following storage conditions are provided: internal tissues and mosquitoes are stored for a week at temperature from minus 24 to minus 16 °C, for longtime storage period at temperature minus 68 °C. Transportation of clinical specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.

RNA ISOLATION

Any commercial RNA/DNA isolation kit, if IVD-CE validated for the specimen types indicated herein at the "SAMPLE COLLECTION, STORAGE AND TRANSPORT" paragraph, could be used.

Sacace Biotechnologies recommends to use the following kits:

- ⇨ **Ribo-Virus** – plasma (Sacace, REF K-2/C)
- ⇨ **DNA/RNA-Prep –** plasma, tissue, mosquitoes (Sacace, REF K-2-9)
- ⇨ **SaMag Viral Nucleic Acids Extraction kit** – plasma (Sacace, REF SM003)

Please carry out the RNA extraction according to the manufacturer's instructions.

The purified RNA can be stored at 2–8 °C for at most 4 h, at temperatures not higher than minus 16 °C for 1 month, and at temperatures not higher than minus 68 °C for one year.

PROTOCOL: PREPARING TUBES FOR PCR

1. Before starting work, thaw and vortex all reagents of the kit. Make sure that there are no drops on the caps of the tubes.
2. Take the required number of PCR tubes for amplification of clinical and control samples (including one negative control of extraction and two controls of amplification).
3. To prepare the reaction mixture, mix in a new sterile tube the reagents per one reaction:
   - **10 µl** of **PCR-mix-FRT DV,**
   - **5 µl** of **RT-PCR-mix-2-TM,**
   - **0.25 µl** of **RT-G-mix-2,**
   - **0.5 µl** of **Hot Start TaqF Polymerase,**
   - **0.25 µl** of **MMLV Revertase**).

Thoroughly vortex the mixture, make sure that there are no drops on the caps of the tubes.

4. Add **15 µl** of the prepared reaction mixture to each PCR tube.
5. Add **10 µl** of **RNA samples** isolated from the clinical samples to each PCR tube.
6. Run the **control reactions**:

C– - Add **10 µl** of the **RNA sample** extracted from the Negative Control to the tube labeled C– (Negative Control of Extraction)

C+ - Add **10 µl** of **Positive Control cDNA *DV* 1-4 types/IC (C+)** to the tube labeled C+ (Positive Control of Amplification).

NCA - Add **10 µl** of **RNA-buffer** to the tube labeled NCA (Negative Control of Amplification).

Make sure that there are no drops on the tube walls, otherwise vortex the tubes briefly.

Table. REACTION MIXTURE PREPARATION

		Reaction volume (with one extra sample)				
Reagent volume for one reaction, µl		**10.00**	**5.00**	**0.25**	**0.50**	**0.25**
N. samples	N. PCR reactions	PCR-mix-1	RT-PCR-mix-2	RT-G-mix-2	Polymerase	M-MLV Revertase
4	7	80	40	2.0	4.0	2.0
6	9	100	50	2.5	5.0	2.5
8	11	120	60	3.0	6.0	3.0
10	13	140	70	3.5	7.0	3.5
12	15	160	80	4.0	8.0	4.0
14	17	180	90	4.5	9.0	4.5
16	19	200	100	5.0	10.0	5.0
18	21	220	110	5.5	11.0	5.5
20	23	240	120	6.0	12.0	6.0
22	25	260	130	6.5	13.0	6.5
34	37	380	190	9.5	19.0	9.5
46	49	500	250	12.5	25.0	12.5

Amplification

1. Create a temperature profile on your instrument as follows:

	***Rotor-type instruments*[1]**			***Plate-type instruments*[2]**		
Step	***Temperature, °C***	***Time***	***Cycles***	***Temperature, °C***	***Time***	***Cycles***
1	**50**	30 min	1	**50**	30 min	1
2	**95**	15 min	1	**95**	15 min	1
3	**95**	10 s	5	**95**	10 s	5
	56	35 s		**56**	40 s	
	72	15 s		**72**	20 s	
4	**95**	10 s	40	**95**	10 s	40
	54	35 s Fluorescence acquiring		**54**	40 s Fluorescence acquiring	
	72	15 s		**72**	20 s	

[1] *For example Rotor-Gene™ 6000/Q (Corbett Research, Qiagen)*
[2] *For example, SaCycler-96-5x™ (Sacace), CFX (BioRad)*

Fluorescent signal is detected in the channels for the FAM, JOE, ROX, Cy5 and Cy5.5* fluorophores.

* *only for 5-channels Real Time PCR instruments*

INSTRUMENT SETTINGS

Rotor-type instruments (RotorGene 6000, RotorGene Q)

Channel	Calibrate / Gain Optimisation	Threshold	More Settings/ Outlier Removal	Slope Correct	Eliminate Cycles before
FAM/Green	5Fl - 10Fl	0,03	10%	ON	5
JOE/Yellow	5Fl - 10Fl	0,03	10%	ON	5
ROX/Orange	5Fl - 10Fl	0,03	10%	ON	5
Cy5/Red	5Fl - 10Fl	0,03	15%	ON	5
Cy5.5/Crimson	5Fl - 10Fl	0,03	5%	ON	5

Plate- or modular type instruments (CFX™ (BioRad); SaCycler-96-5x™ (Sacace), For result analysis, set the threshold line at a level where curves of fluorescence are linear.

DATA ANALYSIS

The *DENGUE* genotype 1 cDNA is detected in the FAM/Green channel.

The *DENGUE* genotype 2 cDNA is detected in the JOE/HEX/Yellow channel

The *DENGUE* genotype 3 cDNA is detected in the Rox/Texas Red/Orange channel.

The *DENGUE* genotype 4 cDNA is detected in the Cy5/Red channel

The signal of the IC is detected in the Cy5.5/Crimson/ Quasar705 channel*

* *only for 5-channels Real Time PCR instruments. On 4-channels RT PCR instrument kit can be used without IC detection.*

The results are interpreted by the real-time PCR instrument software by the crossing or not crossing of the threshold line by the fluorescence curve (in the middle of the linear section of the fluorescence curve for the positive control (C+) in logarithmic coordinates).

The result of amplification is considered **positive** if the fluorescence curve is characteristic of real-time PCR (S-shaped) and crosses the threshold line once in the significant fluorescence increase section and if the Ct value detected in the channel is below the threshold value specified in the below table.

The result of amplification is considered **negative** if the fluorescence curve is not S-shaped and if it does not cross the threshold line (the Ct value is absent).

RESULTS INTERPRETATION

Sample	Rotor-Gene 6000/Q					CFX96, SaCycler				
	FAM/ Green	JOE/ Yellow	ROX/ Orange	Cy5/ Red	Cy5.5/ Crimson	FAM	HEX	ROX	Cy5	Cy5.5/ Quasar705
	DV 1	DV 2	DV 3	DV 4	IC	DV 1	DV 2	DV 3	DV 4	IC
NCA	-	-	-	-	-	-	-	-	-	-
C–	-	-	-	-	**<29**	-	-	-	-	**<31**
Pos C+	**<25**	**<25**	**<25**	**<25**	**<25**	**<28**	**<28**	**<28**	**<28**	**<28**
Clinical samples	**<38**	**<38**	**<38**	**<38**	**<29** (plasma) **<31** (tissue, mosquitoes)	**<38**	**<38**	**<38**	**<38**	**<31** (plasma) **<31** (tissue, mosquitoes)

Sample contains *DENGUE* genotype 1 RNA if the Ct value detected in the FAM channel is less than 38.

Sample contains *DENGUE* genotype 2 RNA if the Ct value detected in the JOE/HEX channel is less than 38.

Sample contains *DENGUE* genotype 3 RNA if the Ct value detected in the ROX channel is less than 38.

Sample contains *DENGUE* genotype 4 RNA if the Ct value detected in the Cy5 channel is less than 38.

The result is **invalid** if *Ct* value is not determined (absent) in the channel for FAM, JOE, ROX, Cy5 fluorophores, whereas the *Ct* value in the channel for the Cy5.5 fluorophore is not determined (absent) or greater than the specified boundary Ct value. In such cases, PCR analysis should be repeated starting from RNA extraction stage. If the same result is obtained in the second run, re-sampling of material is recommended

Results are accepted as significant only if both positive and negative controls of RNA extraction and the negative controls of amplification passed correctly (see above the table for controls).

Control	**Stage for control**	***Ct* value in the channel for fluorophore**				
		FAM	**JOE**	**ROX**	**Cy5**	**Cy5.5**
NCE	RNA extraction	NEG	NEG	NEG	NEG	POS
NCA	PCR	NEG	NEG	NEG	NEG	NEG
C+	PCR	POS	POS	POS	POS	POS

QUALITY CONTROL PROCEDURE

A defined quantity of Internal Control (IC) is introduced into each sample and control at the beginning of sample preparation procedure in order to control the extraction process of each individual sample and to identify possible reaction inhibition.

A negative control of extraction (NCE), negative amplification control (NCA), positive amplification control (C+) are required for every run to verify that the specimen preparation, the amplification and the detection steps are performed correctly.

If the controls are out of their expected range (see table Results for Controls), all of the specimens and controls from that run must be processed beginning from the sample preparation step.

SPECIFICATIONS

Sensitivity

The analytical sensitivity of **DENGUE Real-TM Genotype** PCR kit is specified in the table below.

Biological material	Pathogen agent	Sensitivity, copies/ml
Tissue, mosquito suspension	*Dengue virus* type 1-4	10^3
Blood plasma, blood serum	*Dengue virus* type 1-4	5×10^2

The claimed analytical features of **DENGUE Real-TM Genotype** PCR kit are guaranteed only when additional reagents kits “Magno-Virus”, “Ribo-Virus” or “DNA/RNA-prep” are used.

Specificity

– The analytical specificity of **DENGUE Real-TM Genotype** PCR kit is ensured by selection of specific primers and probes as well as by selection of strict reaction conditions. The primers and probes were checked for possible homologies to all sequences deposited in gene banks by sequence comparison analysis as well as with genomic DNA/RNA of the following organisms and viruses: hepatitis A virus; hepatitis B virus; hepatitis D virus; human immunodeficiency virus; cytomegalovirus; Epstein-Barr virus; herpes simplex virus types 1 and 2; chicken pox virus; human herpes virus types 6 and 8; parvovirus B19; West Nile encephalitis; adenovirus types 2, 3, and 7. flavivirus (TBEV, Japanese B encephalitis virus, Omsk hemorrhagic fever); rickettsiae of spotted fever group (Rickettsia conorii ssp. caspia, R.hejlonjiangensis); Coxiella burnetii; Bartonell quintana; Hantaviruses: Puumala, Dobrava; Leptospira interrogans, L.kirshneri, L.borgpetersenii.

Cross-reactions for the above-mentioned organisms and viruses have not been detected.

TROUBLESHOOTING

- The absence of positive signal in C+ in all channels may indicate incorrect amplification program or other errors made during PCR amplification. In this case, PCR should be carried out once again.
- Detection of any Ct value in C– suggests contamination of reagents or samples. In this case, it is necessary to repeat the analysis of all tests starting from the isolation stage and to take measures for detecting and eliminating the source of contamination.

KEY TO SYMBOLS USED

REF	List Number		Caution!
LOT	Lot Number		Contains sufficient for <n> tests
IVD	For *in Vitro* Diagnostic Use	VER	Version
	Store at	**NCA**	Negative Control of Amplification
	Manufacturer	**NCE**	Negative control of Extraction
	Consult instructions for use	**C+**	Positive Control of Amplification
	Expiration Date	**IC**	Internal Control

* SaCycler™ is a registered trademark of Sacace Biotechnologies
* CFX™ are trademarks of Bio-Rad Laboratories
* Rotor-Gene™ Technology is a registered trademark of Qiagen

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