For *in Vitro* Diagnostic Use

Parvovirus B19 Real-TM Quant

Handbook

Real Time PCR Kit for quantitative detection of *Parvovirus B19*

REF V49-50FRT

REF TV49-50FRT

Σ **50**

NAME

Parvovirus B19 Real – TM Quant

INTRODUCTION

Human Parvovirus B19 was first identified in 1975. B19 is widespread, and manifestations of infection vary with the immunologic and hematologic status of the host. In healthy immunocompetent individuals B19 is the cause of erythema infectiosum and, particularly in adults, acute symmetric polyarthropathy. Due to the tropism of B19 to erythroid progenitor cells, infection in individuals with an underlying hemolytic disorder causes transient aplastic crisis. In the immunocompromised host persistent B19 infection is manifested as pure red cell aplasia and chronic anemia. Likewise, the immature immune response of the fetus may render it susceptible to infection, leading to fetal death in utero, hydrops fetalis, or development of congenital anemia.

Diagnosis is primarily based on detection of specific antibodies by enzyme-linked immunosorbent assay or detection of viral DNA by PCR. Viremia in persistent and recurrent infection may range from very low to high titers and may be associated with chronic clinical manifestations, such as chronic anemia. Quantitative B19-DNA PCR assay can help guide the choice of treatment in persistent infections (i.e., intravenous immunoglobulin (IVIG) treatment vs immunosuppression reduction).

The high levels of sensitivity, specificity, and rapidity provided by the PCR technology for the detection and quantitation of B19 DNA represent a significant improvement for the laboratory diagnosis of B19 infection.

INTENDED USE

The **Parvovirus B19 Real-TM** is a Real-Time test for the quantitative detection of *Parvovirus B19* in the biological materials. DNA is extracted from samples, amplified using real time amplification with fluorescent reporter dye probes specific for *Parvovirus B19* and Internal Control (IC). Test contains an IC which serves as an amplification control for each individually processed specimen and to identify possible reaction inhibition.

MATERIALS PROVIDED

Module No.1: Real Time PCR kit (V49-50FRT)

Part N° 2 – **"Parvovirus B19 Real-TM":** Real Time amplification

- **PCR-mix-1 Parvovirus B19/Glob,** 0,6 ml;
- **PCR-buffer-FRT**,0,3 ml;
- **TaqF Polymerase,** 0,03 ml;
- ***B19 & IC* C+,** 0,1 ml;
- **Standards:**
  - QS1 B19, 0,2 ml;
  - QS2 B19, 0,2 ml;
- **Negative Control C-***, 1,2 ml
- **Internal Control IC****,** 1,0 ml;
- **DNA-buffer**, 0,5 ml;

Contains reagents for 55 tests.

Module No.2: Complete Real Time PCR test with DNA purification kit (TV49-50FRT)

Part N° 1 – **"DNA-Sorb-B":** Sample preparation

- **Lysis Solution**, 15 ml;
- **Sorbent,** 1,25 ml;
- **Washing Solution** 1, 15 ml;
- **Washing Solution** 2, 50 ml;
- **DNA-eluent**, 5 ml;

Contains reagents for 50 tests.

Part N° 2 – **"Parvovirus B19 Real-TM":** Real Time amplification

- **PCR-mix-1 Parvovirus B19/Glob,** 0,6 ml;
- **PCR-buffer-FRT**,0,3 ml;
- **TaqF Polymerase,** 0,03 ml;
- ***B19 & IC* C+,** 0,1 ml;
- **Standards:**
  - QS1 B19, 0,2 ml;
  - QS2 B19, 0,2 ml;
- **Negative Control C-***, 1,2 ml
- **Internal Control IC****,** 1,0 ml;
- **DNA-buffer**, 0,5 ml;

Contains reagents for 55 tests.

** must be used in the isolation procedure as Negative Control of Extraction.*
***add 10 µl of Internal Control during the DNA isolation directly to the sample/lysis mixture (see DNA-Sorb-B REF K-1-1/B protocol).*

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation:

- DNA extraction kit (Module No. 1)
- Biological cabinet
- Desktop microcentrifuge for "eppendorf" type tubes
- Dry heat block
- Vortex mixer
- Pipettes
- Sterile pipette tips with filters
- 1,5 ml polypropylene sterile tubes
- Biohazard waste container
- Refrigerator, Freezer

Zone 2: Real Time amplification:

- Real Time Thermal cycler
- Reaction tubes
- Workstation
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Vortex mixer
- Freezer, refrigerator

STORAGE INSTRUCTIONS

Parvovirus B19 Real-TM must be stored at 2-8°C, **DNA-Sorb-B** must be stored at 2-8°C. **PCR-mix-1 Parvovirus B19** and **TaqF Polymerase** must be stored at – 20°C. The kit can be shipped at 2-8°C for 3-4 days but should be stored at 2-8°C and -20°C immediately on receipt.

STABILITY

Parvovirus B19 Real-TM Test 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

***In Vitro* Diagnostic Medical Device**

For *In Vitro* Diagnostic Use Only

The user should always pay attention to the following:

- ⚠ Lysis Solution contains guanidine thiocyanate*. Guanidine thiocyanate is harmful if inhaled, or comes into contact with skin or if swallowed. Contact with acid releases toxic gas. (Xn; R: 20/21/22-36/37/38; S: 36/37/39).
- 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.

**** Only for Module No.2***

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

Parvovirus B19 Real-TM can analyze DNA extracted from:

1) *Plasma:*
   - 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.
2) *amniotic liquid* stored in “Eppendorf” tube. Centrifuge at 10000g/min for 10 min. Discard the supernatant and leave about 100 µl of solution for DNA extraction.
3) *sputum:* add 1 volume of sputum to 1 volumes of saline water and vortex vigorously. Centrifuge at 10000g/min for 10 min. Discard the supernatant and leave about 100 µl of solution for DNA extraction.
4) *swabs:* insert the swab into the nuclease-free 1,5 ml tube and add 0,2 ml of Transport medium. Vigorously agitate swabs in medium for 15-20 sec.

It is recommended to process samples immediately after collection. Store samples at 2–8 °C for no longer than 24 hours, or freeze at –20/80°C. Transportation of clinical specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.

DNA 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:

⇒ **DNA-Sorb-B** (Sacace, REF K-1-1/B);

⇒ **Ribo Virus 50**– spin column extraction kit (Sacace, REF K-2-C);

⇒ **SaMag Viral Nucleic Acids Extraction Kit** (Sacace, REF SM003): for plasma.

Please carry out the DNA extraction according to the manufacturer's instructions. Add 10 µl of Internal Control during DNA isolation procedure directly to the sample/lysis mixture.

SPECIMEN AND REAGENT PREPARATION (reagents supplied with the module no.2)

1. **Lysis Solution** and **Washing Solution** (in case of their storage at +2-8°C) should be warmed up to 60°C until disappearance of ice crystals.
2. Prepare required quantity of 1.5 ml polypropylene tubes.
3. Add to each tube **300 µl** of **Lysis Solution** and **10 µl** of **Internal Control** IC.
4. Add **100 µl** of **Samples** to the appropriate tube.
5. Prepare Controls as follows:
    - add **100 µl** of **C–** (**Negative Control**) to labeled C*neg*.
    - add **90 µl of C– (Negative Control)** and **10 µl** of **Pos B19 & Human DNA C+** to the tube labeled Cpos
6. Vortex the tubes, incubate 5 min at 65°C and centrifuge for 5 sec.
7. Vortex vigorously **Sorbent** and add **25 µl** to each tube.
8. Vortex for 5-7 sec and incubate all tubes for 10 min at room temperature. Vortex periodically
9. Centrifuge all tubes for 1 min at 5000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between tubes.
10. Add **300 µl** of **Washing Solution 1** to each tube. Vortex vigorously and centrifuge for 1 min at 5000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between tubes.
11. Add **500 µl** of **Washing Solution 2** to each tube. Vortex vigorously and centrifuge for 1 min at 10000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between tubes.
12. Repeat step 11.
13. Incubate all tubes with open cap for 5 min at 65°C.
16. Resuspend the pellet in **50 µl** of **DNA-eluent.** Incubate for 5 min at 65°C and vortex periodically.
17. Centrifuge the tubes for 2 min at maximum speed (12000-16000 g). The supernatant contains DNA ready for amplification. The amplification can be performed on the same day of extraction.

PROTOCOL (Reaction volume 25 µl):

1. Prepare one new tube and add for each sample (N) **10*N µl** of **PCR-mix-1 Parvovirus B19/Glob**, **5*N** of **PCR-buffer-FRT** and **0,5*N µl** of **TaqF DNA Polymerase.** Vortex and centrifuge for 2-3 sec
2. Add **15 µl** of **Reaction Mix** and **10 µl** of **extracted DNA*** sample to appropriate tube.

(**Re-centrifuge all the tubes with extracted DNA for 2 min at maximum speed (12000-16000 g) and take carefully supernatant. N.B. don't disturb the pellet, sorbent inhibit reaction!).*

3. For qualitative analysis prepare 2 controls:
   - add **10 µl** of **DNA-buffer** to the tube labeled Amplification Negative Control;
   - add **10 µl** of ***B19 & IC* C+** to the tube labeled *C+*
4. For quantitative analysis prepare 4 additional tubes and perform QS1 and QS2 standards twice.

**QS1 and QS2 values are specific for each lot and are reported in the Quant Data Card provided in the kit.*

The results are interpreted through the presence of crossing of fluorescence curve with the threshold line.

IC is detected on the FAM (Green) channel, *Parvovirus B19* on the JOE (Yellow)/HEX/ Cy3 channel.

Create a temperature profile on your Real-time instrument as follows:

	Rotor-type Instruments[1]			**Plate- or modular type Instruments[2]**		
Step	***Temperature, °C***	***Time***	**Repeats**	***Temperature, °C***	***Time***	**Repeats**
1	**95**	15 min	1	**95**	15 min	1
2	**95**	5 s	5	**95**	5 s	5
	60	20 s		**60**	20 s	
	72	15 s		**72**	15 s	
3	**95**	5 s	40	**95**	5 s	40
	60	20 s *fluorescent signal detection*		**60**	30 s *fluorescent signal detection*	
	72	15 s		**72**	15 s	

[1] For example Rotor-Gene™ 3000/6000/Q (Corbett Research, Qiagen)
[2] For example, SaCycler-96™ (Sacace), CFX/iQ5™ (BioRad); Mx3005P™ (Agilent), ABI® 7300/7500/StepOne Real Time PCR (Applied Biosystems), SmartCycler® (Cepheid), LineGeneK® (Bioer)

Fluorescence is detected at the 2nd step of Cycling 2 stage (60 °C) in FAM/Green and JOE/Yellow/Hex/Cy3 fluorescence channels.

NSTRUMENT SETTINGS

Rotor-type instruments

Channel	Calibrate/Gain Optimisation…	Threshold	More Settings/ Outlier Removal	Slope Correct
FAM/Green	*from 3 Fl to 8 Fl*	*0.03*	*10 %*	*On*
JOE/Yellow	*from 3 Fl to 8 Fl*	*0.03*	*10 %*	*On*

Plate-type instruments

The threshold line should cross only sigmoid curves of signal accumulation of positive samples and should not cross the baseline; otherwise, the threshold level should be raised. Set the threshold at a level where fluorescence curves are linear and do not cross curves of the negative samples.

RESULTS INTERPRETATION

The Internal Control (IC) is detected on the FAM channel and Parvovirus B19 on the Joe/HEX/Cy3 channel.

Boundary value of the cycle threshold, Ct

Sample	Channel for fluorophore	Ct boundary value	
		Rotor-type instruments	Plate-type instruments
C+	FAM/Green	28	29
	JOE/Yellow/Hex/Cy3	27	30
C-	FAM/Green	27	28
Clinical samples	FAM/Green	27	28
	JOE/Yellow/Hex/Cy3	35	40

Qualitative analysis

Results are accepted as relevant if positive and negative controls of amplification and extraction are passed.

Results for controls

Control	Stage for control	Ct FAM (Green)	Ct JOE(Yellow)/HEX/Cy3	Interpretation
NCE	DNA isolation	POS	NEG	Valid result
C+	DNA isolation,	POS	POS	Valid result
NCA	PCR	NEG	NEG	Valid result
QS2	PCR	POS	POS	Valid result

- The sample is considered to be positive for *Parvovirus B19* if in the channel JOE(Yellow)/HEX/Cy3 the value of **Ct** is different from zero (see boundary value table);
- Specimens with valid Ct (see boundary value table) in the channel FAM (Green) and absent fluorescence signal in the channel JOE(Yellow)/HEX/Cy3 are interpreted as negative.
- The result is **invalid** if the Ct value of a sample in the JOE(Yellow)/HEX/Cy3 channel is absent while the Ct value in the FAM (Green) channel is either absent or greater than the specified boundary value (see boundary value table). It is necessary to repeat the PCR analysis of such samples.

Quantitative analysis

For each control and patient specimen, calculate the concentration of Parvovirus B19 using the following formula:

$$\frac{\text{Parvovirus B19 DNA copies/specimen (Joe/Yellow/HEX/Cy3 channel)}}{\text{IC DNA copies/specimen (FAM/Green channel)}} \times \text{coefficient*} = \text{copies DNA B19/mL}$$

*coefficient is specific for each lot and reported in the Parvovirus B19 Quant Data Card provided in the kit.

PERFORMANCE CHARACTERISTICS

Analytical specificity

The analytical specificity of the primers and probes was validated with negative samples. They did not generate any signal with the specific *Parvovirus B19* primers and probes. The specificity of the kit **Parvovirus B19 Real – TM Quant** was 100%. The potential cross-reactivity of the kit **Parvovirus B19 Real – TM Quant** was tested against the group control. It was not observed any cross-reactivity with other pathogens.

Analytical sensitivity

The kit **Parvovirus B19 Real – TM Quant** allows to detect *Parvovirus B19* DNA in 100% of the tests with a sensitivity of not less than 200 copies/ml. The detection was carried out on the control standard and its dilutions by negative sample.

Parvovirus B19 Real – TM Quant is linear from 800 to 10.000.000 copies/ml. Test results greater than 10.000.000 copies/ml are above the upper limit of quantitation of the test and should be reported as "greater than 10.000.000 copies/ml". If quantitation results are desired for such samples, the specimen should be diluted 1:10 with negative serum and retested. Test results less than 800 copies/ml are below the lower limit of quantitation of the test and should be reported as "less than 800 copies/ml".

Target region: VP1

TROUBLESHOOTING

1. Weak or absent signal of the IC (Fam (Green) channel): retesting of the sample is required.
    - The PCR was inhibited.
        - ⇒ Make sure that you use a recommended DNA extraction method and follow the manufacturer's instructions.
        - ⇒ If the DNA-Sorb isolation kit is used as a DNA extraction kit, re-centrifuge all the tubes before pipetting the extracted DNA for 2 min at maximum speed (12000-16000 g) and take carefully supernatant. Don't disturb the pellet, sorbent inhibit reaction.
    - The reagents storage conditions didn't comply with the instructions.
        - ⇒ Check the storage conditions
    - The PCR conditions didn't comply with the instructions.
        - ⇒ Check the PCR conditions and for the IC detection select the fluorescence channel reported in the protocol.
    - The IC was not added to the sample during the pipetting of reagents.
        - ⇒ Make attention during the RNA extraction procedure.
2. Weak (Ct > 37) signal on the Joe (Yellow)/Cy3/HEX channel: retesting of the sample is required.
3. Joe (Yellow)/Cy3/HEX signal with Negative Control of extraction.
    - Contamination during DNA extraction procedure. All samples results are invalid.
        - ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol.
        - ⇒ Use only filter tips during the extraction procedure. Change tips among tubes.
        - ⇒ Repeat the DNA extraction with the new set of reagents.
4. Any signal with Negative PCR Control.
    - Contamination during PCR preparation procedure. All samples results are invalid.
        - ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol or special DNA decontamination reagents.
        - ⇒ Pipette the Positive controls at the end.
        - ⇒ Repeat the PCR preparation with the new set of reagents.

KEY TO SYMBOLS USED

Symbol	Meaning	Symbol	Meaning
REF	List Number	!	Caution!
LOT	Lot Number	Σ	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™ and iQ5™ are registered trademarks of Bio-Rad Laboratories
* Rotor-Gene™ is a registered trademark of Qiagen
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* LineGeneK® is a registered trademark of Bioer
* SmartCycler® is a registered trademark of Cepheid

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