ARGENE

Parvovirus B19 R-gene®
REF 69-019
REF 69-019B

CE
IVD

COMPOSITION

REF 69-019	**DNA EXTRACTION KIT**	**Ref.: 67-000**
	Real-Time detection and quantification kit – Parvovirus B19 R-gene®	**Ref.: 69-019B**

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1. Intended use

The Parvovirus B19 R-gene® kit is used to detect and quantify the genome of Parvovirus B19 genotypes in whole blood, blood plasma and serum, using Real-Time PCR after extraction of viral DNA. The viral load is measured using a range of 4 quantification standards provided in the kit.

Viral load is measured to monitor the evolution of chronic viral infections and helps in introducing and monitoring appropriate treatment.
The Parvovirus B19 R-gene® kit is also used to detect Parvovirus B19 genotypes in bone marrow and medullary plasma.

Combined with other methods of biological investigation (medical imaging, biochemical, immunological analysis, etc.), the results obtained with the Parvovirus B19 R-gene® kit are used to diagnose Parvovirus B19 infections and monitor their evolutions.
This kit cannot be used for screening blood from donors.

2. Presentation of the kit

Parvovirus B19 is a member of the *Parvoviridae* family and the *Erythrovirus* genus due to its structure and its capacity to infect erythroid precursor cells. It is a unenveloped, single-stranded DNA virus of about 5.5 kb, divided into three genotypes. Genotype 1 is the most common genotype and it is found all over the world; genotypes 2 and 3 are more geographically restricted (Western Europe, USA, Brazil and Africa) and their prevalence is less pronounced.

The seroprevalence of this virus varies with age and country. The virus is disseminated by air mainly, but blood and transplacental passage is also possible. The primary infection generally takes place during childhood. Although it is mostly asymptomatic, this primary infection can be revealed in the form of a benign infantile rash called epidemic megalerythema or fifth disease. The infection can lead to serious consequences for vulnerable patients (pregnant women, patients suffering from congenital anaemia) or immunodepressed (transplant patients, AIDS patients, etc.).

In immunocompetent patients: The primary infection is often asymptomatic. Parvovirus B19 is a pathogen responsible for epidemic megalerythema (or fifth disease) observed in children during primo-infection, and is expressed by a benign infantile skin rash. Epidemics generally take place at school and at certain times of the year, mainly in spring and winter. This virus is also the cause of arthropathy, the most common symptom in infected adults. It is also associated with heart disease (myocarditis) and neurological diseases.

In immunodepressed patients: This virus may be responsible for severe chronic anaemia with destruction of erythroblasts in the bone marrow.

In pregnant women: a primary infection with Parvovirus B19 in a pregnant woman can lead to fœtal anaemia, which can cause hydrops foetalis, spontaneous abortion or foetal death *in utero*.

In people suffering from constitutional haemolytic anaemia: Parvovirus B19 can cause acute attacks of deglobulisation.

The Parvovirus B19 R-gene® kit is used to detect and quantify the 3 Parvovirus B19 genotypes in whole blood, blood plasma and serum, and to detect the 3 Parvovirus B19 genotypes in whole bone marrow and medullary plasma, using real-time amplification after extraction of the viral DNA. Many automatic and manual purification devices have been validated with the kit. Extracts can be amplified on most Real-Time PCR platforms available in laboratories. Simple and complete, this kit fits in perfectly with laboratory routine.

The results obtained are validated by the different tests in the kit, including an extraction + inhibition control.

An international Parvovirus B19 standard issued by the World Health Organization (WHO), supplied by the NIBSC (ref.: 99/802), is available. It is used as a reference material for calibrating and quantifying Parvovirus B19 viral loads. The results obtained can thus be standardised, whatever the type of specimen and combinations of extraction and amplification platforms used. The use of this international standard will allow comparison of the viral load of a monitored patient between laboratories, using two different methods.

The results obtained with the Parvovirus B19 R-gene® kit, expressed as copies/mL by the real-time PCR platform can then be converted into International Units/mL (UI/mL) by the user, via the conversion factor described in the paragraph on "Results Validation and Interpretation" in this technical information sheet.

Thanks to a general amplification program with the entire range of R-gene® products, the sample analysis can be simultaneously analyzed with the following other targets : BKV with BK Virus R-gene® kit (ref.: 69-013B), HSV1, HSV2, VZV with HSV1 HSV2 VZV R-gene® kit (ref.: 69-004B), CMV with CMV R-gene® kit (ref.: 69-003B), CMV, HHV-6, HHV-7 and HHV-8 with CMV HHV6,7,8 R-gene® kit (ref.: 69-100B), Adénovirus with Adenovirus R-gene® kit (ref.: 69-010B) and EBV with EBV R-gene® (ref.: 69-002B).

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3. Principle of the test

3.1. Sample type

- The Parvovirus B19 R-gene® kit is used to detect and quantify the Parvovirus B19 genome in samples of whole blood, blood plasma and serum using the quantification range supplied in the kit. The Parvovirus B19 R-gene® kit is used to detect the Parvovirus B19 genome in samples of whole bone marrow and medullary plasma.
- The quantitative results are expressed as copies/mL of the sample. The results are validated by the extraction test, inhibition test and positive and negative controls provided in the Parvovirus B19 R-gene® kit.

3.2. DNA Extraction

- The extraction methods tested and validated with the Parvovirus B19 R-gene® kit, ref.: 69-019B, are the following:
  - QIAamp® DNA Blood Mini Kit
  - DNA EXTRACTION KIT (supplied under reference 67-000 of ref.:69-019)
  - NucliSENS® easyMAG®
  - MagNA Pure Compact Instrument
  - MagNA Pure LC Instrument
  - EZ1 Advanced XL
- The target DNA, present in the sample and in the extraction + inhibition control (**IC2**), is extracted using one of the extraction methods above.
- The technique used by the DNA extraction kit (ref.: 67-000) associates the selective binding properties of silica gels with a microcentrifugation speed. The sample and internal control (**IC2**) are first lysed with protease in order to optimize the DNA binding capacities on the membrane. The use of the silica column allows, after DNA coating, efficient washing of the sample to eliminate contaminants. After elution, DNA is suitable for direct use in amplification techniques.

3.3. Real time amplification and quantification

- Amplification is performed using the 5' nuclease TaqMan® technology (patent nº: 5210015, 5487972) also called hydrolysis probes. The ready-to-use amplification mixture includes: primers, dNTPs, amplification buffer, Taq Polymerase, specific Parvovirus B19 primers and probe as well as the primers and probe specific for the internal control (**IC2**) which also must be subjected to the entire extraction procedure (including lysis).

- The following range of Real Time PCR platforms are validated with the Parvovirus B19 R-gene®, ref.: 69-019B:
  - LightCycler® 2.0
  - LightCycler® 480 System II
  - ABI 7500
  - ABI 7500 Fast
  - ABI StepOne
  - Dx Real-Time System (Bio-Rad)
  - CFX96 (Bio-Rad)
  - Rotor-Gene® Q
  - Rotor-Gene® 6000
  - SmartCycler 2.0

- Extracted samples are amplified and quantified at the same time.
- The amplification stage is carried out with primers used to amplify a sequence with good efficacy. The amplified fragment for all the Parvovirus B19 genotypes, is 131 pub and is found in gene NS1 coding for a 77 kea non-structural protein (NS1) (regulatory, pleiotropic protein).
- A range of 4 points (**QS1, QS2, QS3 and QS4**), between 5 000 copies and 5 copies/µL of standard DNA, i.e. 50 000 to 50 copies/amplification, is used to produce the standard curve on the basis of which the samples will be quantified.
- Point QS3 in the range, containing 50 copies/µL of standard DNA or 500 copies of plasmid by PCR, is used for quantification using a standard external curve for thermocyclers used to import the range (see: explanations in the "Standard range and controls The quantification standard (**QS3**)" section). This possibility is valid on condition that a standard curve has first been produced with the 4 points in the range provided, validated and registered as an external standard curve. This method is used to avoid carrying out all 4 points of the standard range for each experiment (under the conditions defined in the "The Quantification standard (**QS3**)" section). It is also used as a positive control for qualitative detection of Parvovirus B19.
- The sensitivity control (**SC**) consists of a plasmid at a concentration of 1 copy/µL of standard plasmid DNA, i.e. 10 copies/PCR. This control (**SC**) is used to check the performance of the assay over time.
- An extraction and inhibition control (**IC2**),is included in the Parvovirus B19 R-gene® kit ref. : 69-010B in order to check, starting from the lysis step, if the sample has been well extracted and does not contain amplification inhibitors.

WARNING: **The performances described have been validated and are guaranteed using the technical extraction/amplification unit combinations recommended in the technical information sheet (see "Performances" chapter).**

To monitor the evolution of viral load expressed as copies/mL in the samples of whole blood, plasma and serum from one patient test after test, it is essential for successive sample analyses to be taken strictly according to the same protocol and using the same combination of extraction/amplification units.

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4. Content of the kit and storage

4.1. DNA EXTRACTION kit **67-000**

Number of extractions: 50

A	QIAamp® Mini column	5 x 10
B	Collection tubes (2 mL)	2 x 50
C	AL buffer Xn - HARMFUL	12 mL
D	AW1 buffer (concentrated) Xn - HARMFUL	19 mL
E	AW2 buffer (concentrated)	13 mL
F	AE buffer	12 mL
G	QIAGEN protease Xn - HARMFUL	24 mg
H	Protease solvent	1,2 mL

Package Insert: *1 Package Insert provided in the kit or downloadable from* www.biomerieux.com/techlib.

- The kit can be stored before and after opening at +2℃/+8℃ until the expiration date written on the box. Storage at higher temperatures should be avoided. The reconstituted QIAGEN protease can be stored in aliquots at -18℃/-22℃ to avoid successive freezing.

4.2. Detection and Quantification Kit Parvovirus B19 R-gene® **69-019B**

Number of tests: 90

W0	Water for extraction (PCR quality)	2 x 1,8 mL
IC2	Internal Control 2	1 mL
R0	Water for amplification (PCR quality)	300 µL
QS1	Quantification standard 1 Parvovirus B19 (5 000 copies / µL)	300 µL
QS2	Quantification standard 2 Parvovirus B19 (500 copies / µL)	300 µL
QS3	Quantification standard 3 Parvovirus B19 (50 copies / µL)	300 µL
QS4	Quantification standard 4 Parvovirus B19 (5 copies / µL)	300 µL
SC	Sensitivity control Parvovirus B19 (1 copie / µL)	300 µL
R19	Parvovirus B19 and **IC2** Amplification premix	3 x 450 µL

Package Insert: *1 Package Insert provided in the kit or downloadable from* www.biomerieux.com/techlib.

- On reception, the quantification kit (ref.: 69-019B) must be stored, before and after opening, at -18℃/-22℃ and away from light, until its expiry date.
- Before and after opening of the kit (ref.: 69-019B), Internal control 2 (**IC2**), reagent (**W0**), quantification standards (**QS1**, **QS2**, **QS3**, **QS4**) and sensitivity control (**SC**) must be stored in the extraction room at -18℃/-2 2℃.
- The reagents (**R19**) and (**R0**) must be stored in the room reserved for the preparation of the premix at -18℃/-22℃.
- Each premix cannot undergo more than 10 freezing/defrosting cycles.
- Replace the amplification premixes (**R19**), quantification standards (**QS**) and sensitivity control (**SC**) at -18℃/-22℃ immediately after use.

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5. Material and reagents required but not supplied

5.1. For sample extraction

5.1.1. With DNA EXTRACTION KIT (ref.: 67-000)

- Éthanol 96-100%.
- Centrifuge (6 000 g / 12 000g).
- Vortex.
- Polypropylen test tubes for microcentrifugation (1,5 mL, 2 mL).
- Water bath +56°C.
- Micropipettes with plugged tips.
- Single use gloves.

5.1.2. Other extraction methods validated

- Follow the manufacturer's instructions.

5.2. For the detection and quantification Parvovirus R-gene® kit, 69-019B

- Micropipettes (P20) with plugged tips de 20 µL.
- Thermocyclers validated with Parvovirus B19 R-gene®.
- Centrifuge appropriate for the amplification instrument.
- Single use gloves.
- Capillaries, tubes or microplates for real time PCR platforms validated for Parvovirus B19 R-gene®.
- Cooling block suitable for the thermocycler of choice.
- U.V light.
- Workstation or plexiglass screen for samples and premix distribution.
- Colour Compensation r-gene® (ref.: 71-103) for result interpretation on LightCycler® 2.0.

6. Reagents reconstitution

This stage is part of the framework for using the "DNA EXTRACTION KIT " manual ref.: 67-000.

6.1. Protease stock solution preparation

- Add 1.2 mL of protease solvent (**H**) to the 24 mg of lyophilized protease (**G**).
- Store aliquots at -18°C/-22°C (repeated freezing a nd thawing must be avoided).

6.2. AL buffer (C) preparation)

- Mix AL buffer (**C**) thoroughly by shaking before use.
- Do not store protease mixed with AL buffer (**C**).
- If precipitate is observed in AL buffer (**C**), warm at +70°C to dissolve it.
- AL buffer (**C**) is stable when stored tightly closed at +2°C/+8°C .

6.3. AW1 buffer (D) preparation

- AW1 buffer (**D**) is supplied as a concentrate.
- Prior to first time use, add a volume of 25 mL of ethanol (96-100%) to the 19 mL of concentrated buffer.
- AW1 (**D**) buffer is stable when stored tightly closed at +2°C/+8°C.

6.4. AW2 buffer (E) preparation

- AW2 buffer (**E**) is supplied as a concentrate.
- Prior to first time use, add a volume of 30 mL of ethanol (96-100%) to the 13 mL of concentrated buffer.
- AW2 (**E**) buffer is stable when stored tightly closed at +2°C/+8°C.

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7. Warnings and precautions

- This kit is intented for in vitro use only. The kit must be handled by a qualified staff member, in accordance with Good Laboratory Practice and handling instructions for molecular biology.
- Read all the instructions before starting the manipulation.

7.1 General warnings and precautions

- Wear protective clothing, i.e: Disposable gloves, lab coat, safety goggles, mask.
- Avoid contact between the reagents and the skin. Wash immediately with copious amounts of water if contact occurs.
- Samples must be prepared under a biological safety hood.
- Never pipet by mouth.
- Do not smoke, eat or drink in dedicated work areas.
- Handle and dispose all specimens and materials as potentially infectious. After use: material, reagents and waste must be handled as potentially infectious.

7.2 Warnings and precautions for molecular biology

- Amplification procedures require highly skilled techniques to avoid risk of sample contamination:
  - Use separate working places for sample preparation and amplification reactions. Movement in the laboratory must be in one direction only from the reagent preparation area to the amplification area. Allocate a set of lab coats and pipettes to each area. Never introduce an amplified product in reagent and/or sample preparation areas.
  - Pipettes used to handle samples are reserved for this purpose only. These pipettes must be positive displacement pipettes or pipettes equipped with filter tips. All tips must be sterile.
  - The pipettes used to aliquot reagents must be reserved only for this purpose. The necessary reagents for amplification are aliquoted in order to be used during one single experiment.
  - Tubes from different specimens and amplification premix must never be opened at the same time.
  - Used samples must be exclusively reserved for this analysis.

- Do not use reagents after expiration date printed on the labels.
- Do not substitute reagents from kits with different batch numbers or from other manufacturers.
- The reagents must be fully defrosted to room temperature before testing.
- The use of a metal cold block (+2°C/+8°C) is recom mended for the manipulation of the reagents and the samples.
- Always perform preventive maintenance for workstations, for automated extraction, amplification, and centrifuge systems, according to the manufacturer's recommendations.

For more detailed information, see the product safety data sheet which can be downloaded from www.biomerieux.com/techlib.

7.3 Reagent specific warnings and precautions

- AL (**C**) buffer and AW1 buffer (**D**) contain guanidinium chloride (chaotropic salt).
  - R22: Harmful if swallowed.
  - R36/38: Irritating for eyes and skin.
  - S13: Keep away from food, drinks and animal feeding supplies.
  - S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
  - S36: Wear suitable protective clothing.
  - S46: If swallowed, seek medical advice immediately and show the container or label.

  This component must not be used with disinfecting agents that contain bleach.

- The AWR buffer (**E**) and protease diluent (**H**) in kit 67-000 contain 0.04 % of sodium azide as preservative.

- Protease (**G**) contains subtilisin.
  - R37/38: Irritating to respiratory system and skin.
  - R41: Risk of serious damage to eyes.
  - R42: May cause sensitisation by inhalation.
  - S22: Do not breathe dust.
  - S24: Avoid contact with skin.
  - S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
  - S36/37/39: Wear suitable protective clothing, gloves and eye/face protection.
  - S46: If swallowed, seek medical advice immediately and show the container or label.

- Avoid contact between the reagents and the skin. Wash immediately with copious amounts of water if contact occurs. Wear gloves when handling the reagents.

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8. Internal quantification standards and controls

GLOSSARY :

530 nm *= the reading channel "FAM" or "Green" or other depending on the real-time PCR platforms. For reasons of simplification, only "**530 nm**" is used.*

560 nm *= the reading channel "VIC", "Hex" or other according to the real-time PCR platforms. For reasons of simplification, only "**560 nm**" is used.*

CT = Crossing Threshold for most real-time PCR platforms or CP (Crossing Point) for devices in the LightCycler® range. For reasons of simplification, only CT is used.

WARNING: **Be sure to respect the order dof adding reagent / samples (see paragraph "Amplification preparation").**

8.1. The internal quantification standards (QS1, QS2, QS3, QS4)

- The use of the internal quantification standard range is imperative for sample quantification.
- The quantification standards are used to produce a standard curve in the software provided with the thermocycler.
- The quantification standards range from 5 000 copies/µL (**QS1**) to 5 copies/µL (**QS4**).
- The quantification standards must be designated as « standard »and their values must be entered when samples are defined in the table of data analysis software.
- The QS signal is detected at **530 nm**.

8.2. The quantification standard (QS3)

- The quantification standard (**QS3**) allows the importation of the standard curve created in the first run.
- The importation of the standard curve is a quantification method that must only be used from run to run with reagents of the SAME batch. The period between the run defining the standard curve with all four quantification standards and the run using the imported standard curve should not be longer than 3 months.

Note: Check the possibility of importing a range on thermocyclers validated with the Parvovirus B19 R-gene® kit.

- The signal is detected at **530 nm**.

Note: In the event of purely qualitative detection, the QS3 serves as a positive control that verifies that the amplification step has been carried out correctly

8.3. Sensitivity control (SC)

- The sensitivity control (**SC**) validates the performance of the assay and should be considered as a run-control.
- The sensitivity control (**SC**) is amplified with **R19** amplification premix.
- Systematically tested, the sensitivity control (**SC**) is the equivalent of a weak positive sample. For this reason, it may occasionally turn out to be negative.
- The signal is detected at **530 nm**.

8.4. Extraction + Inhibition controls

8.4.1. The sample extraction + inhibition control (IC2sample)

- This control consists of an internal control (**IC2**) that must be added to the samples prior to extraction and checks both the efficacy of the extraction and detects the presence of possible inhibitors.
- The signal is detected at **560 nm**.

8.4.2. The reference extraction + inhibition control (IC2W0)

- This control consists of an internal control (**IC2**) that must be added to the negative extraction control (**W0**) prior to extraction and must be amplified at the same time as the patient samples in order to obtain a reference (CT) (**IC2W0**). The results must be compared with the extraction + inhibition control of patient samples (**IC2sample**).
- The signal is detected at **560 nm**.

⇒ Comparison of CT (Crossing Threshold) values of both **IC2W0** and IC2sample controls at **560 nm** evaluates the efficacy of the extraction and detects the presence of possible inhibitors.

8.5. The negative controls

8.5.1. The negative extraction + amplification control (IC2W0)

- This is the same tube as was described in 8.4.2 (Reference extraction+inhibition test) chapter but, once the reading has been made at **530 nm**, it constitutes a negative control used to check the absence of contamination on extraction and amplification.
- The signal is detected at **530 nm**.

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8.5.2. The negative amplification control

- This is the same tube as was described in the "Reference extraction + inhibition control" chapter but, once the reading has been made at **530 nm**, it constituted a negative control used to check the absence of contamination on extraction and amplification.
- This control is optional.

⇒ The signal is detected at **530 nm**. The comparison of the CT value at **530 nm** of the negative amplification control **R0** and **IC2W0** identifies a possible contamination.

8.6 WHO Parvovirus B19 INTERNATIONAL STANDARD (not supplied)

- The WHO expert committee has drafted references for the use of biological substances for the prevention, treatment and diagnosis of human diseases. WHO international standards are recognised as being the reference in this field and constitute the International Unit (IU).
- The results of the viral load obtained with the Parvovirus B19 R-gene® kit, expressed in copies/mL by the real-time PCR software, can be converted into International Units/mL by the user, via a conversion factor. This conversion factor depends on the type of sample being tested and the combination of extraction and amplification platforms used. (see paragraph "Results Validation and Interpretation").

9. Sample treatment and transport

Samples must be collected and transported following instructions of the laboratory

9.1. Sample transport

- For samples to be transported, check your local legislation for hazardous and infectious material transport.
- Samples must be transported and treated by the laboratory in the shortest possible time (preferably within 24 hours).

9.2. Sample treatment

9.2.1. Blood samples

WARNING: **The use of heparinized tubes does not suit genic amplification analysis. Blood collection tubes contain citrate which may be responsible for decreasing signal during the detection of amplified products.**

- Blood must be collected in EDTA tubes.
- Before the extraction step, homogenize blood sample by over turning the sample tubes for 10 minutes with an automatic shaker.
- Aliquot each blood sample in small volumes under a biological safety cabinet.
- Period between blood collection and arrival at the laboratory must not exceed 24 hours.
- Blood samples must be sent to the laboratory at room temperature (+18℃/+25℃).
- The samples can be stored at -18℃/-22℃ for up to 12 weeks and at -78℃/-82℃ for a longer period.

9.2.2. Serum or plasma samples

- Blood must be collected in a dry tube or a tube containing EDTA.
- Centrifuge tube at 1200 x g for 10 minutes at 20℃ . A maximum of 2 mL (200 µL minimum) serum and plasma is decanted under a biological safety cabinet in cryotubes.
- Serum and plasma must be sent to the laboratory preferably at room temperature (+18℃/+25℃) or at +2 ℃/+6 ℃. Serum and plasma not directly treated upon arrival must be store at +2℃/+6 ℃ for one week maximum. In case thi s delay exceeds one week, store serum and plasma at -18℃/-22℃.
- If serum and plasma samples are sent to the laboratory on dry-ice, they must be stored afterwards at -18℃/-22℃ or preferably at-78℃/-82℃.

9.2.3. Whole bone marrow samples

- The samples are taken in EDTA tubes.
- Aliquot the sample in a microbiology safety cabinet.
- The samples are transferred to the laboratory as quickly as possible (preferably within less than 24h) after sampling.
- The samples are transported to the laboratory at ambient temperature (+18℃/+25℃).
- If the whole bone marrow samples are not processed on arrival at the laboratory, they will be stored at -18℃/-22℃ or -78℃/-82℃ preferably.

9.2.4. Samples of medullary plasma

- Bone marrow samples are taken in a tube with EDTA.
- The samples are transferred to the laboratory as quickly as possible (preferably within less than 24h) after sampling.
- The tubes of bone marrow are centrifuged at 2800 x g for 10 minutes to obtain medullary plasma.
- Aliquot the sample in a microbiology safety cabinet.
- If the medullary plasma samples are not processed on arrival at the laboratory, they will be stored at -18℃/-22℃.

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10. Extraction protocol

WARNING: **Before starting the extraction procedure, make sure samples and reagent IC2 have been homogenized.**

In the room reserved for sample extraction

10.1. DNA Extraction Kit (ref.: 67-000 + IC2+ W0)

The manual DNA Extraction Kit is only valid for an extraction of whole blood samples.

- Equilibrate samples, IC2 and **W0** to room temperature +18°C/+25°C.
- Equilibrate AE buffer (**F**) to room temperature +18°C/+25°C.
- Make sure that AL buffer (**C**), AW1 buffer (**D**), AW2 buffer (**E**), and reconstituted protease solution have been prepared according the instructions given in section "Reagents reconstitution".
- Redissolve any precipitate in AL buffer (**C**) by heating it at +70°C if necessary. Cool to room temperature before use
- All centrifugation steps must be carried out at room temperature.

10.1.1. Lysis

- Prepare and identify (on the lid) an equal number of 1.5 mL microcentrifuge tubes to samples being analyzed. Add **ONE** tube dedicated to the extraction of the mix (**W0**+IC2).
- Heat the water bath to +56°C.
- Pipette 200 µL of AL buffer (**C**) into each 1.5 mL microcentrifuge tube previously identified.
- Add 20 µL of protease.
- Add 10 µL of internal control (IC2).
- Add 200 µL of W0 in the tube identified for the mix (**W0**+IC2).
- Add 200 µL of sample in the tubes identified for sample extraction. PBS may be added to the sample if the sample volume is less than 200 µL. In this case the result obtained will be only qualitative.
- Mix by pulse-vortexing for 15 seconds. To ensure efficient lysis, it is essential that the sample is mixed thoroughly to yield a homogeneous solution.
- Incubate at +56°C for 10 minutes.
- Lysis is completed after a 10 minute incubation. Longer incubation times have no effect on the yield or quality of the purified DNA. Potentially infectious agents can be inactivated by incubating the sample at +95°C for 15 minutes after the lysis step. However, extending this incubation time gives rise to a degradation of DNA.
- Centrifuge the tubes briefly to remove any droplets present in the cap.

10.1.2. Column loading

- Add 200 µL of 96-100% ethanol to the sample, and mix by pulse-vortexing for 15 seconds.
- Centrifuge the tubes briefly to remove any droplets present in the cap.
- Prepare and identify the same number of spin columns as the samples to be tested. Carefully apply the above mixture to the spin column (in a 2 mL collection tube) without wetting the rim.
- Close each spin column in order to avoid aerosol formation during centrifugation and centrifuge at 6 000xg for 1 minute. If the lysate has not completely passed through the column after centrifugation, centrifuge again at higher speed until the spin column is empty.
- Place the spin column into a clean 2 mL collection tube (provided), and discard the tube containing the filtrate.

10.1.3. Washing

- Carefully open the spin column and add 500 µL of AW1 buffer (**D**) without wetting the rim. Close the cap and centrifuge at 6 000xg for 1 minute.
- Place the spin column in a clean 2 mL collection tube (provided), and discard the tube containing the filtrate.
- Carefully open the spin column and add 500 µL of AW2 buffer (**E**) without wetting the rim.
- Close the cap and centrifuge at full speed (12 000xg) for 3 minutes.
- Place the columns on a clean 2 mL tube (not provided) and eliminate tubes containing filtrates.
- Centrifuge for 1 minute at full speed (12 000 x g) prior to elution. This step eliminates any trace of AW2 buffer (**E**).
- Place each column on a clean, identified 1.5 mL tube (not provided) and eliminate tubes containing filtrates.

10.1.4. Elution

- Carefully open the spin column.
- Add 100 µL of AE elution buffer (**F**) balanced at ambient temperature for the blood samples.
- Close the columns and incubate for 5 minutes at ambient temperature.
- Centrifuge at 6 000 x g for 1 minute.
- The DNA extracted is in the eluate.
- The DNA extract is stable for one year if stored at -18°C/-22°C.

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10.2. Extraction instruments and/or kits validated with Parvovirus B19 R-gene®

- These extraction instruments must be regularly maintained as recommended by the manufacturer by a qualified and trained staff.

Instrument	Kit	Sample Volumes + IC2	Sample Type	Protocol	Elution Volume
MagNA Pure Compact Roche Diagnostics ref.: 03 731 146 001	MagNA Pure Compact Nucleic Acid Isolation Kit I ref.:03 730 964 001 32 isolations	200 µL of sample + 10 µL IC2	Blood plasma	Total_NA_Plasma_100_400	50 µL
			Whole blood	DNA_Blood_100_400	100 µL
MagNA Pure LC Roche Diagnostics ref.:12 236 931 001	MagNA Pure LC DNA Isolation Kit I ref.:03 003 990 001 192 isolations		Whole blood	DNA I Blood Cells High Performance	100 µL
		200 µL of pretreated sample(1) + 10 µL IC2	Whole bone marrow		
NucliSENS® easyMAG® bioMerieux ref.: 280110	NucliSens® easyMAG® reagents	200 µL of sample + 10 µL IC2	Whole blood	"Viral **Whole Blood Extraction**" protocol The manufacturer's Specific B protocol with 140 µL of silica(2)	50 µL
			Whole blood	Manufacturer's Specific B protocol with 2 mL of Lysis Buffer + 140 µL silica	
			Serum / Blood plasma / Medullary plasma	The manufacturer's Specific B protocol with 50 µL of silica	
		400 µL of sample + 10 µL IC2	Blood plasma	The manufacturer's Specific B protocol with 50 µL of silica	100 µL
EZ1 Advanced XL	EZ1 DSP virus kit ref.:62724	200 µL of sample + 10 µL IC2+ 200 µL ATL(3)	Whole blood	EZ1 DSP virus	90 µL
		200 µL of sample + 10 µL IC2	Blood plasma		60 µL

(1) Pretreatment of whole bone marrow samples, rich in inhibitors, is recommended: a 1/3 dilution in RPMI culture medium before extraction using the MagNa Pure LC (Roche Diagnostics) automatic unit is recommended.

(2) A premix consisting of IC2, Lysis buffer and Silica must be prepared extemporaneously and added to the samples previously distributed in shuttles containing 2 mL of Lysis buffer. For n samples, mix 600µL Lysis buffer x (n+1) + 10µL IC2 x (n+1) + 140µL Silica x (n+1). Add 740µL of the mixture to each sample.

(3) Pretreatment with ATL buffer (QIAGEN - Tissue Lysis Buffer - Ref: 939011) at the time of extraction by the EZ1 Advanced XL automatic unit, is essential for whole blood samples. This reagent is not supplied in the QIAGEN - EZ1 DSP virus kit - Ref 62724

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11. Detection and Real-time quantification protocol

*Note: With a view to simplify the protocol, the device dedicated to holding the amplification reaction mix is referred to as a "**tube**".*

WARNING: **To monitor the evolution of viral load expressed as copies/mL in the samples of whole blood, blood plasma and serum from one patient test after test, it is essential for successive sample analyses to be taken strictly according to the same protocol and using the same combination of extraction /amplification units.**

- To determine the number of tubes, check if the experiment requires the creation of a standard curve (see section "The quantification standard QS3 ").
- Schedule:

- 1 tube per tested sample.
- 1 or 4 tube(s) for the standard range imported/created for quantitative detection or 1 tube for QS3 as a positive control for qualitative detection only.
- 1 tube for adenovirus sensitivity control (**SC**).
- 1 tube for reference extraction + inhibition control (**IC2W0**) also used as negative control for extraction and amplification.

Notes:

- For the Bio-Rad amplification units, use the transparent plates (ref.: HSP9601) with optical stoppers (ref.: TCS0803).
- For the LightCycler® 480 System II amplification unit, use the white LightCycler® plates 480 Multiwell Plate 96 (ref.: 04729692001).

11.1. Program

- Regardless which real time PCR platform is used, the amplification program remains the same. The amplification program is described in the table below:

Steps		Time	Temperature	Cycles	Fluorescence acquisition				
					LC2.0 LC480	ABI7500 Fast ABI StepOne® ABI 7500	Dx Real-Time System / CFX 96	Rotor-Gene® Q Rotor-Gene® 6000	SmartCycler 2.0
Polymerase Activation		15 min.	95°C	1	-	-	-	-	-
Amplification	Denaturation	10 sec.	95°C	45	-	-	-	-	-
	Hybridization Elongation	40 sec.	60°C		**530 - 560**	**FAM - VIC**	**FAM - HEX**	**Green - Yellow**	**FAM – Cy3**
					End of the elongation				

WARNING: **For simultaneous detection of the Parvovirus B19 pathogen on the ABI 7500 Fast with the Parvovirus B19 R-gene® kit and one or more pathogen(s) in the MWS r-gene® range, from a sample of whole blood or plasma extracted on the NucliSENS® easyMAG® according to the Specific B 200/50 protocol, use the written amplification program in the technical information sheet for one of the MWS r-gene® kits without ROX activation as passive reference.**

Note 1: Temperature transition rate/slope is pre-set at 20°C/sec or 100%.

Note 2: On LightCycler® instruments, add a cooling step: 30 sec/40°C / 1 cy cle at the end of the PCR.

Note 3: On LightCycler®, adjust the «SEEK TEMPERATURE» parameter to 60°C during programming.

*Note 4: On the LightCycler® 2, it is **ESSENTIAL** to use a colour compensation file to interpret the results.*
Make sure that this is still valid (see appropriate technical information sheet) and has been created and recorded in the LightCycler(2.0 management software, using the r-gene® Colour Compensation reagent (ref.:71-103).

Note 5: On LightCycler® 480, there are two optical systems: only "System II" is compatible with the Parvovirus B19 R-gene® kit. "System II" includes automatic colour compensation in its software.

Note 6: On Applied Biosystems® select « NONE » in « PASSIVE REFERENCE » when programming.

Note 7: On Rotor-gene®, calibrate the signal by clicking on "GAIN OPTIMISATION".

Programming and analysis assistance sheets, per device type, downloadable at www.biomerieux.com/techlib

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11.2. Amplification preparation

In the room reserved for amplification

- Before starting the experiment:
  - The reagents must be fully defrosted to room temperature before testing.
  - Mix each reagent (to a vortex for 2 seconds or through successive pipetting) and centrifuge briefly.
  - Homogenize thawed reagents by vortexing or by pipetting, then briefly centrifuge.
  - Make sure the cooling block was decontaminated by exposure under U.V. light for 30 min.
  - Make sure the cooling block was correctly pre-cooled at +2°C/+8°C.

WARNING: **- In order to keep contamination to a minimum, close the tubes as you go along.**
- Replace the amplification premix (R19), quantification standards (QS) and sensitivity test (SC) at -18°C/-22°C immediately after use
- Each premix (R19) cannot undergo more than 10 freezing/defrosting cycles

- Collect 15 µL of the amplification premix by gently homogenizing with the pipet in order to distribute the same volume in all tubes.
- Distribute 15 µL volume of amplification premix in all tubes.

WARNING: The order for adding samples/reagents as shown below must be followed

- *Optional: Add 10 µL of **R0** to the dedicated tube.*
- Add 10 µL of each extracted sample in the corresponding tubes.
- Add 10 µL of the sensitivity control (SC) in the corresponding tube (see chapter "Controls").
- Add 10 µL of each standard (from **QS4** to **QS1**) in the corresponding tubes (see chapter "Controls").
- Add 10 µL of extracted mix **IC2+W0** in the corresponding tube. This tube is the **IC2W0** control (see chapter "Controls").
- Centrifuge the tubes with the corresponding device and transfer them to the thermocycler.

11.3. Running the Parvovirus B19 R-gene® program

- Run the amplification program (stored according to the instructions described in section "Program").
- Designate the samples and controls.
- For Parvovirus B19 quantification enter the following values for the quantification standards in copies/mL

	Quantification (whole blood, blood plasma, serum)				
	Sample volume 200 µL	Sample volume 200 µL	Sample volume 200 µL	Sample volume 200 µL	Sample volume 200 µL
	Elution in 50 µL (copies/mL)	Elution in 60 µL (copies/mL)	Elution in 100 µL (copies/mL)	Elution in 90 µL (copies/mL)	Elution in 100 µL (copies/mL))
QS1	1 250 000	1 500 000	1 250 000	2 250 000	2 500 000
QS2	125 000	150 000	125 000	225 000	250 000
QS3	12 500	15 000	12 500	22 500	25 000
QS4	1 250	1 500	1 250	2 250	2 500

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12. Data Analysis

Programming and analysis assistance sheets, per device type, downloadable at www.biomerieux.com/techlib

GLOSSARY :

530 nm *= the reading channel "FAM" or "Green" or other depending on the real-time PCR platforms. For reasons of simplification, only "**530 nm**" is used.*

560 nm *= the reading channel "VIC", "Hex" or other according to the real-time PCR platforms. For reasons of simplification, only "**560 nm**" is used.*

CT = Crossing Threshold for most real-time PCR platforms or CP (Crossing Point) for devices in the LightCycler® range. For reasons of simplification, only CT is used.

12.1. With LightCycler® 2.0

- Analysis of the viral target is carried out in the ABSOLUTE QUANTIFICATION mode at 530 nm.
- Analysis of the extraction + inhibition control is carried out in the ABSOLUTE QUANTIFICATION mode at 560nm after activation of the colour compensation file (COLOUR COMPENSATION tab.) (Colour Compensation r-gene® ref: 71-103).
- Apply the FIT POINTS method.
- Move the threshold line (red horizontal cursor line) to a position where it crosses the fluorescence curves of all the samples in their linear part, above the baseline noise.
  Note : If the position of the crossing line is not sufficient for crossing all the sample curves in their linear part, repeat the step as frequently as necessary to obtain the CP for each sample.
- For each sample, a CP CROSSING POINT is calculated at 530 nm.
- To quantify the samples, select AUTOMATED F'' MAX mode (second derivative method).
- The concentration calculated for Parvovirus B19 appears in the CONC COLUMN (COPIES/mL).
- The extraction + inhibition controls are analysed by comparing the calculated CP value for each extraction+ inhibition control (**IC2sample**) to the CP value obtained with the reference extraction + inhibition test (**IC2W0**) at 560 nm.

12.2. With LightCycler® 480

- Switch on the LC480 (System II) FAM – HEX automatic compensation.
- The viral target is analysed in ABSOLUTE QUANTIFICATION mode at 530nm (FAM).
- The extraction + inhibition control is analysed in Absolute Quantification mode at 560 nm (HEX).
- Apply the FIT POINTS method.
- Move the threshold line (red horizontal cursor line) to a position where it crosses the fluorescence curves of all the samples in their linear part, above the baseline noise.
  Note: If the position of the crossing line is not sufficient for crossing all the sample curves in their linear part, repeat the step as frequently as necessary to obtain the CP for each sample.
- For each positive sample, a CROSSING POINT (CP) is calculated at 530 nm.
- To quantify the samples, apply the method, choose the mode ABS QUANT / 2ND DERIVATE MAX (second derivate method).
- The concentration calculated for Parvovirus B19 appears in the CONC COLUMN (COPIES/mL).
- The extraction + inhibition controls are analysed by comparing the calculated CP value for each extraction+ inhibition control (**IC2sample**) to the CP value obtained with the reference extraction + inhibition test (**IC2W0**) at 560 nm (HEX).

12.3. With ABI 7500, ABI7500 Fast and ABI StepOne®

- Make sure that NONE is selected in the PASSIVE REFERENCE field because the **R19** premix does not contain any passive reference fluorochrome.
- Analysis of the samples is carried out after having selected the FAM R-GENE detector in the DETECTOR field.
- Adjust the Manual Baseline (red horizontal cursor line) manually to a position where it crosses the fluorescence curves of all the samples in their linear part. This step is performed to identify the positive samples which correspond to a calculated CT value. Negative samples are defined as UNDETERMINED displayed in the CT column by the SDS software.
- Analysis of the extraction + inhibition controls (**IC2sample** and **IC2W0**) is carried out in the same way after having selected the VIC R-GENE detector in the DETECTOR field.
- To quantify the samples, return to linear mode.
- The concentration calculated for Parvovirus B19 appears in the report drafted and printed at the end of each experiment.
- The extraction + inhibition controls are analysed by comparing the calculated CT value for each extraction + inhibition control (**IC2sample**) to the CT value of the reference extraction + inhibition test (**IC2W0**) at 560 nm.

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12.4. With Dx Real-Time System and CFX 96 (Bio-Rad)

- Analysis of the viral target: select the QUANTITATION tab leaving the FAM button checked.
- If necessary, manually adjust the threshold line so as to cross each amplification curve at the end of the exponential phase. This step aims to identify positive samples for which a CT is calculated. Negative samples are indicated by N/A in the Ct column. For each positive sample concentration calculated in column STARTING QUANTITY (SQ) tab QUANTITATION and QUANTITATION DATA.
- The analysis of the extraction + inhibition controls (**IC2sample** and **IC2W0**) are performed in the same way after selecting the HEX detector.

12.5. With Rotor-Gene® Q and Rotor-Gene® 6000

- The viral target is analysed in CYCLING A GREEN mode at 530 nm.
- Analysis of the extraction + inhibition control is carried out in the CYCLING A YELLOW mode at 560 nm.
- The threshold line must be adjusted in LINEAR SCALE mode after selecting DYNAMIC TUBES.
- The concentration calculated for each sample appears in the CALC CONC column (COPIES/ML) in the Quant. RESULTS CYCLING A GREEN window.
- The extraction + inhibition controls are analysed by comparing the calculated CT value for each extraction + inhibition control (**IC2sample**) to the CT value obtained with the reference extraction + inhibition test (**IC2W0**) at 560 nm.

12.6. With SmartCycler® 2.0

- The viral target is analysed in FAM mode at 530 nm.
- The extraction + inhibition test is analysed in CY3 mode at 560 nm.
- For each positive sample, a CROSSING THRESHOLD (CT) is calculated at 530 nm (FAM CT).
- The concentration calculated for each sample appears in the FAM Std/Res Green column.
- The extraction + inhibition controls are analysed by comparing the calculated CT value for each extraction + inhibition controls (**IC2sample**) to the CT value of the reference extraction + inhibition test (**IC2W0**) at 560 nm (CY3 CT).

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13. Validation and interpretation of results

13.1. Test validation

WARNING: **The test is only valid if all following conditions are fulfilled. If this is not the case, all samples and controls must be tested again.**

1ère CONDITION: **IC2W0** should not give a detectable signal at **530 nm**.

2ème CONDITION: **IC2W0** must be equal or lower than **32** cycles at **560 nm**.

3ème CONDITION: The CT value of QS3, and the slope or efficacy required for the standard range must be within the values listed in the following table:

Quantitative Detection (whole blood, blood plasma, serum)				Qualitative Detection (whole bone marrow, medullary plasma
Instruments	**CT QS3**	**Valuable Slope/Efficiency**		**CT QS3**
	Parvovirus B19	The standard curve is created with all 4 quantification standards for each experiment.	The standard curve is created with all 4 quantification standards for subsequent experiments.	**Parvovirus B19**
LightCycler® 2.0 / LigthCycler® 480	29 – 33 cycles	1.8 < Efficacity < 2.1	1.9 < Efficacity < 2.05	29 – 33 cycles
Rotor-Gene® Q / Rotor-Gene® 6000		0.8 < Efficacity < 1.1	0.9 < Efficacity < 1.05	
ABI 7500 / ABI 7500 Fast / ABI StepOne®		-3.917 < Slope < -3.103	*Not applicable*	
Dx Real-Time System CFX 96		-3.917 < Slope < -3.103		
SmartCycler ®2.0		-0.322< Slope <-0.230		
		-4.339< *Slope* * <-3.103		

**With SmartCycler®, the slope of the regression line CT = f(Log (Concentration) is obtained by right-clicking on the graph and by selecting " Transpose X and Y axes "*

=> If all conditions are fulfilled, the results obtained with the samples can be validated.

13.2. Sample reading:

- Each sample must be analysed one by one.
- There is a **530 nm** CT for all positive samples.
- The absence of CT at **530 nm** corresponds to a negative or incorrectly extracted sample.

***Note:* In the event of a very high viral load of Parvovirus B19 being found during the primary infection, the default parameters of the different thermocyclers must be revised to modify the baseline criteria and thus obtain a CT value and not a sample with a falsely "non-determined" CT. However, in all cases, the lack of inhibition and successful completion of the extraction stage at 560 nm must be verified according to the following elements.**

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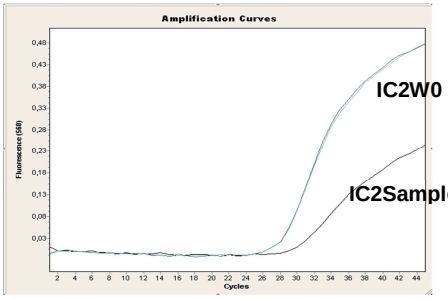
13.3. Interpretation of results

Extraction + Inhibition control (IC2sample)	CT [IC2sample]≤ CT [IC2W0] + **3** cycles		CT [IC2sample]> CT [IC2W0] + **3** cycles	
	NON INHIBITED SAMPLE and correctly extracted		**INHIBITED SAMPLE and/or poorly extracted**	
Sample	**Calculated CT**	**Non calculated CT**	**Calculated CT**	**Non calculated CT**
Parvovirus B19 **quantitative** Interpretation **(Whole blood, Blood plasma, Serum)**	Positive sample validated Quantification to be redone	Sample validated as negative	Positive sample validated Quantification to be redone	**Not Valid**
Parvovirus B19 **qualitative** Interpretation **(Whole bone marrow Medullary plasma)**	Sample validated as positive	Sample validated as negative	Sample validated as positive	**Not Valid**

WARNING:

In the case of a negative sample:
If the slope of the curve generates a fall of final flluorescence ≥50% compared to final fluorescence **IC2W0** (see figure opposite) a weak inhibition is possible. We suggest extracting and testing the sample again.



IMPORTANT NOTE :
- To interpret the results, it is recommended that the quantification result should be expressed as a logarithm base 10 (log_{10}).
- Within the context of patient follow-up, 2 quantifications were considered to be significantly different if their values expressed in log_{10} (copies/ml of sample) or log_{10} (UI/ml of sample) differed by at least 0.5 log_{10}.
If the results are given in log_{10}(copies/ml of sample), this interpretation is only applicable if the 2 quantifications are carried out using the same method and extraction protocol using the same real-time PCR unit.
- The results obtained with the Parvovirus B19 R-gene® kit must be compared with other clinical and biological results in order to make a patient diagnosis.

Purchasing this product gives the right to use it exclusively for in vitro human diagnosis, according to Roche patents. No patent or licence of any type other than the specific user right guaranteed on purchase is given in this document by bioMerieux.

13.4. Quantitative results expressed in International Units

Note: The conversion factor was determined on the basis of the 2nd Standard international Parvovirus B19 (NIBSC, ref.: 99/802).

- The results obtained in copies/mL can be converted into International Units (IU) / mL.
- For this, a conversion factor must be defined. This conversion factor depends on the type of sample being tested (blood plasma, whole blood or serum) and the combination of extraction and amplification platforms used. Therefore, each laboratory must define its own conversion factor.

- For example, studies performed at bioMerieux with the Parvovirus B19 R-gene® kit were used to determine the différent conversion factors for the following combinations of devices and samples:

	Blood plasma		**Whole blood** (1)	
Plateformes PCR / **Extraction**	**ABI7500 Fast**	**Dx Real-Time System**	**ABI7500 Fast**	**Dx Real-Time System**
NucliSENS® easyMAG® (protocol Specific B 200/50)	**1.059**	**1.111**	**5.224**	**4.817**

(1) Whole blood protocol used: Add the sample to 2mL of lysis buffer, then add 140µL of silica before transfer to the device.

***Example*:** For a sample of whole blood extracted with the NucliSENS® easyMAG® according to the Specific B 200/50 protocol and amplified on the ABI7500 Fast, the conversion factor for copies/mL in IU/mL is **5.224.**

Number of IU/mL: Number of cp/mL x 5.224
i.e. for a whole blood sample determined at 10 000 copies/mL, the corresponding quantification is 52 240 IU/mL.

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14. Trouble shooting

14.1. No signal or underestimated quantification in positive samples

POSSIBLES CAUSES	RECOMMENDATIONS
The amplification premix has been defrosted too many times. The amplification premix has remained at room temperature for too long or has been defrosted at too high a temperature.	• Please refer to the "Content of the kit and storage" section. • The amplification premixes (R19) must not be defrosted more than 10 times. • Check that the amplification premixes, the quantification standards and the sensitivity control have been returned to -18/-22°C immediately after use. • Make sure that the amplification premixes were defrosted at ambient temperature. • Use a cooling block when preparing and distributing the premixes.
Incorrect blood collection, transport and storage in the laboratory.	• See chapter "Sample treatment and transport" that defines the optimal conditions (temperature, time) for transport and storage. • Collect blood in EDTA tubes. • Check the delay between the sample collection and its analysis.
Transport and storage of Parvovirus B19 R-gene® - kit ref. : 69-019B conditions were not met.	• Follow instructions in section "Content of the kit and storage" regarding the storage of Parvovirus B19 R-gene® kit 69-019B at -18°C/-22°C and preferably in the d ark.
Problem in extraction step.	• Check if you carefully homogenized the samples before performing extraction. • Perform all washing steps and respect the incubation time when using the DNA Extraction kit ref.: 67-000. (See section "With DNA EXTRACTION KIT"). • Check if material and protocol used to extract sample. • Check if material and protocol used to extract sample correspond to material and protocol recommended for analysis with the Parvovirus B19 R-gene® kit 69-019B (See section "Sample extraction protocol"). • Always perform preventive maintenance of workstations for automated extraction, and centrifuge systems, according to the manufacturer's recommendations.
Pipetting error	• Check the calibration of your pipets. • Check the distributed volume of reagents and samples. • Carefully homogenize reagents and samples before their distribution in amplification tubes.
Programming error.	• Check all programming data (detection channel, mode, number of cycles, temperature and time). • Check all the steps regarding the entry of the samples. • Check the concentrations of the stored standards. • If you imported an external standard curve, check the validity of this importation.
Problem in amplification step	• Check the performances of the real time PCR platform as recommended by the manufacturer. • Always perform preventive maintenance of real time PCR platform, and centrifuge, systems according to the manufacturer's recommendations. • Check the attachment of the locking ring of the Rotor-Gene® carrousel.
Error in data analysis.	• Check the threshold line adjustment. • Check that the imported range is valid (see chapter "Internal quantification standards and controls").
Error in interpretating results.	• Check the validity of the results obtained in the experiments (check all the validation conditions as described in section "Validation and interpretation of results"). • With Applied Biosystems®: check if NONE is selected in PASSIVE REFERENCE FIELD • On LightCycler® 2.0: Make sure the results obtained were corrected using a valid colour compensation file created in advance. Compare the result of the extraction + inhibition control (**IC2sample**) of the suspected sample to the result of the reference extraction + inhibition control (**IC2W0**) (see chapter "Sample reading"). Dilute the extracted sample if necessary.

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14.2. Fluorescent signal on negative samples or overestimated quantification of clinical sample

POSSIBLES CAUSES	RECOMMENDATIONS
Contamination during experiment.	• Follow all recommendations in section “Warnings and precautions”. • Decontaminate the cooling block with U.V. light. • Respect the manufacturer’s recommendations for the decontamination of automated extraction workstation and real time PCR instrument. • Only a trained staff must handle the Parvovirus B19 R-gene® kit. • Use the **R0** reagent provided in the kit, in parallel with the samples extracted, to identify the contaminated stage.
Pipetting error.	• Check the calibration of your pipettes. • Check the distributed volumes of reagents and samples. • Carefully homogenize reagents and samples before their distribution in amplification tubes.
Programming error.	• Check all programming data (detection channel, mode, number of cycles, temperature and time). • Check all the steps regarding the entry of the samples. • Check the concentrations of the stored standards. • If you imported an external standard curve, check the validity of this importation.
Error in data analysis.	• Check the threshold line adjustment. • Check that the imported range is valid (see chapter “Internal quantification standards and controls”).
Error in results interpretation.	• Check the validity of the results obtained in the experiments (check all the validation conditions described in section “Validation and interpretation of results”). • With Applied Biosystems®: check if NONE is selected in PASSIVE REFERENCE FIELD. • On LightCycler® 2.0: Make sure the results obtained were corrected using a valid colour compensation file created in advance. Compare the result of the extraction + inhibition control (IC2sample) of the suspected sample to the result of the reference extraction + inhibition control (IC2W0) (see chapter “Sample reading”). Dilute the extracted sample if necessary.

14.3. The samples all seem inhibited.

POSSIBLES CAUSES	RECOMMENDATIONS
Inadequate extraction stage.	• Check that the samples have been properly homogenised before being extracted. • In the case of manual extraction using the DNA Extraction kit R-gene®. ref.: 67-000, perform the number of washes and incubation time stated in the “DNA Extraction Kit” section. • Check the materials and protocols used for extracting samples. • Kit performances are only validated for the extractions described in the “Sample extraction protocol” section. • Carefully monitor to ensure that the extraction devices are maintained in line with the manufacturer’s recommendations. • For coloured extracts and inhibited samples obtained on the NucliSENS® easyMAG® for the whole blood matrix, use the "**Whole Blood Viral Extraction**" protocol by preference
The IC2W0 does not result from the same run extraction.	• Make sure that every sample tested includes the same batch of IC2 as IC2W0. • Each extraction run should have its own IC2W0.

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15. Performances

WARNING: **The performances described have been validated and are guaranteed using the technical extraction/amplification platform combinations recommended in this chapter.**

All the technical studies described below took place using samples of Parvovirus B19 genotype 1 (essentially based on the 2nd WHO International Standard (NIBSC, ref: 99/802), the most commonly found genotype.
The whole blood samples tested in these studies were extracted with the NucliSENS® easyMAG® using the Specific B (200/50) protocol with addition of 2 mL of lysis buffer and 140 µL of silica per sample, before starting the extraction run.

15.1. Inter- and intra-experiment reproducibility

Precision studies were performed using the Parvovirus B19 R-gene® kit via a repeatability study (intra-test variation) and a reproducibility study (inter-test variation) on different samples of whole blood analysed 10 times on the Bio-Rad Dx RTS and/or the ABI 7500 Fast (Applied Biosystems®), after extraction by the NucliSENS® easyMAG® **using the Specific B protocol (200/50)**.

15.1.1. Intra-experiment reproducibility

5 whole blood samples at different concentrations of Parvovirus B19: 10LoD, 5LoD, 2LoD and 0.01LoD and 1 whole blood samples negative for Parvovirus B19 were tested. Each of these samples was extracted over 5 days by 2 different operators and amplified in duplicate on the Bio-Rad Dx Real-Time System and in triplicate on the ABI 7500 Fast. The following tables give the CT values at 530 nm and 560 nm and the quantifications in log_{10} cp/mL obtained on average for each of the whole blood samples positive for Parvovirus B19. The standard deviation and coefficient of variation were determined.

Results on Biorad Dx RTS (amplification in duplicate)

	Analysis at 530 nm		
	Ct mean at 530 nm	standard deviation	coefficient variation
10 LoD	35.94	0.5	1.39%
5 LoD	36.98	0.81	2.20%
2 LoD	38.18	0.78	2.04%
0.01 LoD	NA	NA	NA

	Results of quantification in log_{10} cp/mL		
	Mean log_{10} cp/mL	standard deviation	coefficient variation
10 LoD	2.65	0.19	7.07%
5 LoD	2.38	0.34	14.09%
2 LoD	1.97	0.25	12.61%
0.01 LoD	NA	NA	NA

	Analysis at 560 nm		
	Ct mean at 560 nm	standard deviation	coefficient variation
10 LoD	29.62	0.36	1.23%
5 LoD	29.57	0.38	1.27%
2 LoD	29.55	0.41	1.38%
0.01 LoD	29.64	0.43	1.65%

Results on ABI 7500 Fast (amplification in triplicate)

	Analysis at 530 nm		
	Ct mean at 530 nm	standard deviation	coefficient variation
10 LoD	35.9	0.75	2.08%
5 LoD	37.13	0.46	1.25%
2 LoD	38.15	0.51	1.34%
0.01 LoD	NA	NA	NA

	Results of quantification in log_{10} cp/mL		
	Mean log_{10} cp/mL	standard deviation	coefficient variation
10 LoD	2.74	0.17	6.08%
5 LoD	2.37	0.13	5.33%
2 LoD	2.06	0.18	8.88%
0.01 LoD	NA	NA	NA

	Analysis at 560 nm		
	Ct mean at 560 nm	standard deviation	coefficient variation
10 LoD	29.7	0.32	1.07%
5 LoD	29.86	0.31	1.02%
2 LoD	29.94	0.31	1.04%
0.01 LoD	30.05	0.3	1.00%

Inter-test reproducibility of the Parvovirus B19 R-gene® kit

⇨ These values demonstrated the good reproducibility of the Parvovirus B19 R-gene® kit (69-019B).

15.1.2. Intra-experimental reproducibility

5 whole blood samples at different concentrations of Parvovirus B19: 10LoD; 5LoD, 2LoD and 0.01LoD and 1 whole blood sample negative for Parvovirus B19 were tested.
Each sample was extracted in duplicate on NucliSENS® easyMAG® using the Specific B protocol (200/50), then pooled. Each extract was then amplified 10 times on theABI7500 Fast with the Parvovirus B19 R-gene® kit. The 10LoD, 5LoD and 2LoD samples were amplified 10 times out of 10 and the 0.01LoD sample was amplified once out of 10.
The following tables give the CT values at 530 nm and 560 nm and the quantifications in log_{10} cp/mL obtained on average for the 10 repetitions for each of the whole blood samples positive for Parvovirus B19. The standard deviation and coefficient of variation were determined.

	Analysis at 530 nm		
	Ct mean at 530 nm	standard deviation	coefficient variation
10 LoD	35.4	0.4	1.13%
5 LoD	36.34	0.48	1.32%
2 LoD	38.49	1.04	2.70%
0.01 LoD	NA	NA	NA

	Results of quantification in log_{10} cp/mL		
	Mean log_{10} cp/mL	standard deviation	coefficient variation
10 LoD	2.61	0.11	4.36%
5 LoD	2.37	0.14	5.79%
2 LoD	1.85	0.3	16%
0.01 LoD	NA	NA	NA

	Analysis at 560 nm		
	Ct mean at 560 nm	standard deviation	coefficient variation
10 LoD	30.04	0.17	0.56%
5 LoD	29.9	0.09	0.30%
2 LoD	30.07	0.08	0.27%
0.01 LoD	29.65	0.1	0.35%

Intra-test reproducibility of the Parvovirus B19 R-gene® kit

⇨ This study demonstrated the good repeatability of the Parvovirus B19 R-gene® kit (69-019B).

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15.2. Study of the linearity range

A clinical sample positive for Parvovirus B19 (genotype 1) previously tested and found to be positive at 11.5 log_{10} copies/mL was diluted before extraction, either in a whole blood matrix or a plasma matrix previously characterised negative for Parvovirus B19. Eleven 10% dilutions were prepared for each matrix. Extraction took place on the NucliSENS® easyMAG® using the Specific B protocol (200/50) and the extracts were amplified in duplicate on the ABI7500 Fast (Applied Biosystems®) using the R19 amplification premix in the Parvovirus B19 R-gene® kit

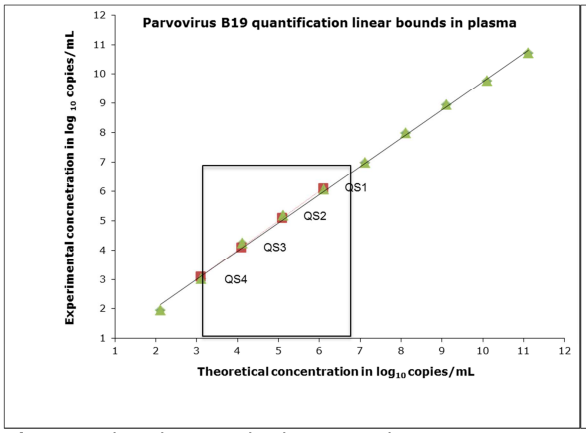


Figure 1: *Linearity range in plasma matrix*

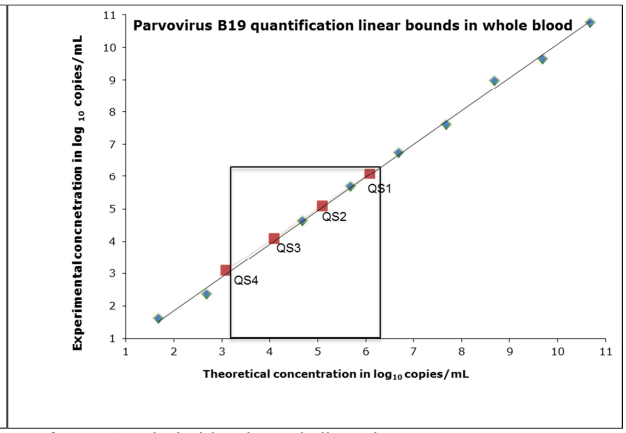


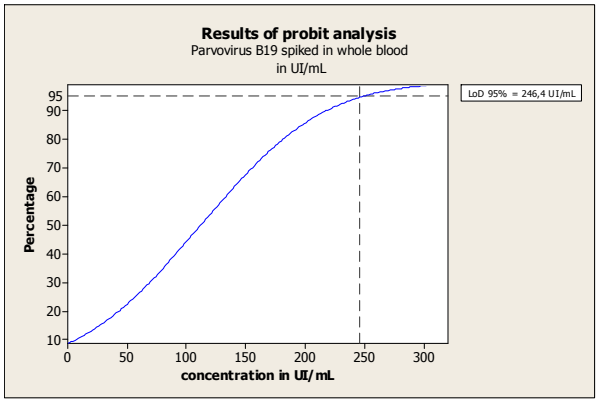
Figure 2: *Whole blood matrix linearity range*

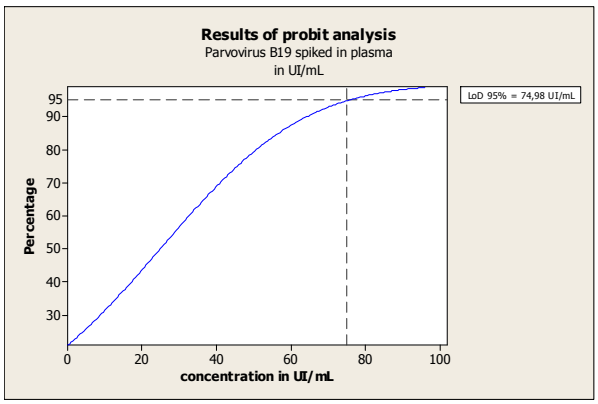
⇨ The figures show linearity:
* for the plasma matrix: between $5.05.10^{10}$ copies/mL (i.e. 10.7 log_{10} cp/mL) and 87 copies/mL i.e. 1.96 log_{10} cp/mL).
* for the whole blood matrix: between $6.1.10^{10}$ copies/mL (i.e. 10.7 log_{10} cp/mL) and 42 copies/mL i.e. 1.61 log_{10} cp/mL).

By applying the conversion factor determined at bioMerieux to the given matrix and the combination of NucliSENS® easyMAG® / ABI 7500 Fast, linearity was between $31.8.10^{10}$ IU/mL and 219.4 IU/mL for the whole blood matrix and $5.34.10^{10}$ IU/mL and 92.2 IU/mL for the blood plasma matrix.

15.3. Analytical sensitivity

The analytical sensitivity of the Parvovirus B19 R-gene® kit was determined on a range of dilutions from the 2nd WHO International Standard (NIBSC, ref:99/802). The series of dilutions was prepared in either a whole blood or plasma matrix, previously characterised as Parvovirus B negative. Each dilution was extracted 15 times using the NucliSENS® easyMAG® automatic extraction platform, using the Specific B protocol (200/50), then amplified with the Parvovirus B19 R-gene® kit on the ABI 7500 Fast (Applied Biosystems®). The curves shown opposite represent the probability analysis on the whole blood matrix on the one hand and the plasma matrix on the other





⇨ There is a 95% probability of detecting Parvovirus B19 at 246.4 IU/mL (IC 95%: 194.88- 377.65 IU/mL) i.e. 47 cp/mL (95% (IC): 37 - 72 cp/mL) in the whole blood matrix. In the plasma matrix, this 95% detection limit was 75 IU/mL (IC 95%: 55- 130 IU/mL) i.e. 70 cp/mL (95% (IC): 52 - 68 cp/mL).

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15.4. Analytical specificity

15.4.1. Exclusivity

The specificity of recognition of primers and probes selected specifically to detect Parvovirus B19 was carried out after the analysis of sequences (viral, bacterial and human samples) in the databanks.

It was also tested experimentally on the following pathogens, likely to be found in whole blood samples. The pathogens listed below were added to a whole blood matrix characterised as Parvovirus B19 negative. Extraction took place on the NucliSENS® easyMAG® using the Specific B protocol (200/50) and amplification on the ABI7500 Fast.

- Virus:
Herpesvirus: CMV, EBV, HHV6, HHV 7, HHV8, HSV1, HSV2, VZV
Polyomavirus: BKV, JCV
Bocavirus
Adenovirus: AdV3, AdV4, AdV5, AdV8, AdV11, AdV12, AdV40
Hepatitis A, B, C
HIV1
Parvovirus: Parvovirus 4 (PARV4) and Parvovirus 5 (PARV5)

- **Bacteria**: *Staphylococcus aureus, Streptococcus pyogenes, Streptococcus pneumoniae, E. coli, Pseudomonas aeruginosa, Proteus mirabilis, Klebsiella pneumoniae, Acinetobacter baumanii., Mycoplasma pneumoniae*

- **Yeasts**: *Candida albicans, Cryptococcus neoformans.*

⇨ No amplification was observed with any of these pathogens.

15.4.2. Inclusivity

The inclusivity of 3 parvovirus B19 genotypes was tested using the WHO 1st International Reference Panel (NIBSC, ref.: 09/110). This panel includes a sample of each of the 3 genotypes (1, 2 & 3) diluted in a plasma matrix.
These samples were diluted at 1/50 in the plasma matrix (previously characterised as Parvovirus B19 negative). Extraction took place on the NucliSENS® easyMAG® using the Specific B protocol (200/50) and amplification on the ABI7500 Fast with the Parvovirus B19 R-gene® kit.
The results are shown in the following table:

SAMPLES	CT values at 530 nm	concentration en log_{10} cp/mL	experimental concentration in log_{10} UI/mL (1)	WHO expected concentration in log_{10} UI/mL	delta log_{10} UI/mL
WHO B19V genotype 1 (1/50)	31.05	4.26	4.28	4.28	0
WHO B19V genotype 2 (1/50)	31.53	4.13	4.16	4.24	0.08
WHO B19V genotype 3 (1/50)	31.55	4.13	4.15	4.27	0.12
QS1	23.97	6.1			
QS2	28.13	5.1			
QS3	31.43	4.1			
QS4	35.54	3.1			

(1) *conversion to IU/mL by applying the conversion factor determined by bioMerieux (see paragraph "Expressing the results in International Units")*

The results show that the Parvovirus B19 R-gene® kit can be used to detect the 3 Parvovirus B19 genotypes. The results obtained in terms of quantification in log_{10} IU/mL (in the "experimental concentration" column) perfectly matched the expected results described in the technical information sheet for product NIBSC 09/110 (delta log_{10} IU/mL between the expected value and the experimental value between 0.00 and 0.12).

15.5. Carry Over / Cross-Contamination

The purpose of this study was to observe the potential number of contaminations of negative samples by strongly positive samples (10 000 LoD) during the extraction and amplification stages. A total of 110 samples of whole blood were extracted and amplified, including 55 positive (whole blood samples Parvovirus B19 negative added to Parvovirus B19 at a concentration equivalent to 10 000 LoD (i.e. 2.5.106 IU/mL) and 55 negative (whole blood samples Parvovirus B19 negative). Each of these samples was extracted alternating a positive sample / negative sample on the NucliSENS® easyMAG® using the Specific B protocol (200/50), then similarly amplified on the ABI7500 Fast. The 110 samples were tested on 5 different extraction and amplification runs: 11 negative and 11 positive samples per run.

The results obtained for this study are summarised in the following table:

	Run 1	Run 2	Run 3	Run 4	Run 5
Mean Ct at 530 nm B19 at 10 000 LoD	27.87	26.62	26.57	26.8	26.8
Mean Ct at 530 nm B19 negative	Neg	Neg	Neg	Neg	Neg
Mean Ct at 560 nm B19 at 10 000LoD	29.98	29.35	29.31	29.5	29.2
Mean Ct at 560 nm B19 negative	30.05	29.58	29.76	29.8	29.5
Number of contamination	**0/11**	**0/11**	**0/11**	**0/11**	**0/11**

⇨ According to this study, no cross-contamination of negative samples by positive samples was noted, even at concentrations equivalent to 10 000 LoD, when applying the protocol recommended in this technical information sheet.

15.6. Biological interference

To check whether the performance of the Parvovirus B19 R-gene® kit is affected by the presence of various pathogens which could be present in patient samples, a biological interference study was carried out. A panel of microorganisms (13 viruses, 4 bacteria and 2 yeasts) was tested. Each of these microorganisms was added at high concentration to a sample of Parvovirus B19 positive whole blood at a concentration equivalent to 3 LoD. Extraction took place on the NucliSENS® easyMAG® using the Specific B protocol (200/50). Each extract was amplified in triplicate on the ABI7500 Fast. This experiment was carried out over 2 runs to cover all the substances selected as potentially interfering.
The results are shown in the following table:

Pathogens	tested concentration	log_{10} cp/mL	delta log_{10} cp/ml	CT at 560 nm	delta CT at 560 nm with reference
Parvovirus B19 = REFERENCE (Run1)	3 loD	2.69	NA	29.78	NA
CMV (+B19 at 3 loD)	10^4 TCID50/mL	2.37	0.32	30.62	0.84
EBV (+B19 at 3 loD)	10^7 cp/mL	2.31	0.38	30.7	0.92
HHV6 (+B19 at 3 loD)	$>10^3$ TCID50/mL	2.32	0.37	30.57	0.79
HHV7 (+B19 at 3 loD)	10^4 cp/mL	2.45	0.24	29.98	0.2
HHV8 (+B19 at 3 loD)	10^4 cp/mL	2.31	0.38	30.72	0.94
HSV1 (+B19 at 3 loD)	10^4 TCID50/mL	2.39	0.3	30.53	0.75
HSV2 (+B19 at 3 loD)	10^4 TCID50/mL	2.58	0.11	30.08	0.3
VZV (+B19 at 3 loD)	10^3 TCID50/mL	2.43	0.26	30.4	0.62
BK polyomavirus (+B19 at 3 loD)	10^4 TCID50/mL	2.32	0.37	29.69	0.09
JC Polyomavirus (+B19 at 3 loD)	10^4 TCID50/mL	2.61	0.08	30.26	0.48
Bocavirus (+B19 at 3 loD)	10^6 cp/mL	2.55	0.14	30.25	0.47
ADV5 (+B19 at 3 loD)	10^3 TCID50/mL	2.68	0.01	29.35	0.43
Hépatite C (+B19 a 3 loD)	10^3 UI/mL	2.39	0.3	30.24	0.46
***Candida albicans* (+B19 at 3 loD)**	10^6 cfu/mL	2.47	0.22	29.84	0.06
***Cryptococcus neoformans* (+B19 at 3 loD)**	10^6 cfu/mL	2.4	0.29	30.09	0.31
Parvovirus B19 = REFERENCE (Run2)	3 loD	2.16	NA	30.31	NA
***Staphylococcus aureus* (+B19 at 3 loD)**	10^6 cfu/mL	2.46	0.3	29.79	0.52
***Mycoplasma pneumoniae* (+B19 at 3 loD)**	10^6 cfu/mL	2.44	0.28	29.84	0.47
***Streptococcus pneumoniae* (+B19 at 3 loD)**	10^6 cfu/mL	2.28	0.12	30.16	0.15
***Acinobacter baumanii* (+B19 à 3 loD)**	10^6 cfu/mL	2.54	0.38	29.66	0.65

⇨ These results show that none of the pathogens tested affect the performance of the Parvovirus B19 R-gene® kit: the differences in quantification in log_{10} cp/mL between the reference (Parvovirus B19 at 3 LoD) and the "co-infected" samples were between 0.01 and 0.38. Moreover, no inhibition of the "co-infected" samples was noted at 560 nm.

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15.7. Chemical interference

To study any eventual chemical interference with the Parvovirus B19 R-gene® kit, different substances corresponding to active molecules from the different medicinal products used in immunodepressed patients, were added to Parvovirus B19 positive samples of whole blood at 3 LoD. A reference corresponding to one sample of Parvovirus B19 positive whole blood at 3 LoD without added chemicals was tested in parallel. These samples were extracted using the NucliSENS® easyMAG® with the Specific B protocol (200/50) and the extracts were amplified in triplicate on the ABI7500 Fast (Applied Biosystems®) with the Parvovirus B19 R-gene® kit. This experiment was carried out over 2 runs to cover all the substances selected as potentially interfering.
The results summarised are shown in the following table:

Potentially Interfering substances	Tested Concentration	Log_{10} cp/mL	Mean Ct at 560 nm	Delta log_{10} cp/mL with REFERENCE	Delta CT with IC2 reference at 560 nm
Parvovirus B19 at 3 LoD = REFERENCE (Run 1)	**NA**	**2.44**	**30.07**	**NA**	**NA**
Azathioprine	3 µg / mL	2.57	30.17	0.13	0.10
Cyclosporine	5.4 µg / mL	2.66	30.15	0.22	0.08
Everolimus	12 µg/mL	2.54	30.22	0.10	0.15
Mycophenolate mofetil	78 µg/mL	2.54	29.6	0.10	0.47
Prednisone	36 µg/mL	2.11	30.12	0.33	0.05
Tacrolimus	0.207 µg/mL	2.01	30.26	0.43	0.19
Sulfamethoxazole	204 µg/mL	2.53	30.2	0.09	0.13
Trimethoprim	6 µg/mL	2.26	29.77	0.18	0.30
Fluconazole	42.3 µg/mL	2.11	30.36	0.33	0.29
Piperacillin	894 µg/mL	2.34	29.96	0.10	0.11
Tazobactam sodium	102 µg/mL	2.25	29.83	0.19	0.24
Clavulanate potassium	24 µg/mL	2.37	29.84	0.07	0.23
Ticarcillin Disodium	990 µg/mL	2.51	29.99	0.07	0.08
Parvovirus B19 at 3 LoD = REFERENCE (Run 2)	**NA**	**2.31**	**29.94**	**NA**	**NA**
Sirolimus	0.105 µg/mL	2.49	29.95	0.18	0.02
Cefotetan	711 µg/mL	2.33	30.01	0.02	0.07
Vancomycin	189 µg/mL	2.36	30.48	0.05	0.54

⇨ These results show that none of the chemicals tested affect the performance of the Parvovirus B19 R-gene® kit: the differences in quantification in log_{10} cp/mL between the reference (Parvovirus B19 at 3 LoD) and the samples containing chemical substances were between 0.02 and 0.43. Moreover, no inhibition of the samples was noted at 560 nm.
The results show that none of the substances at the concentrations tested affected the detection and quantification of Parvovirus B19.

15.8. Validation of the MWS r-gene® program with the Parvovirus B19 R-gene® kit

The samples from the Parvovirus B19 QCMD 2011 panel in plasma matrix and a Parvovirus B19 whole blood panel (10 LoD, 5 LoD, 2 LoD and 0.01 LoD) were extracted with the NucliSENS® easyMAG® using the Specific B protocol (200/50). These samples were amplified in parallel on the ABI7500 Fast using either the protocol from the Parvovirus B19 R-gene® kit (program "*DNA R-Gene"*) or the program from the MWS r-gene® kits. Amplification was carried out on the ABI 7500 Fast (Applied Biosystems®).
The results are shown in the following table:

		Comparison between Parvovirus B19 R-gene® and MWS r-gene® program		
	Samples	Delta Ct 530 nm	Delta Ct 560 nm	Delta log_{10} copies/mL
PLASMA	QCMD B19-11 n°1	0.42	0.19	0.20
	QCMD B19-11 n°2	0.18	0.28	0.05
	QCMD B19-11 n°3	0.26	0.01	0.06
	QCMD B19-11 n°4	0.52	0.09	0.29
	QCMD B19-11 n°5	0.2	0.81	0.12
	QCMD B19-11 n°6	neg	0.04	N/A
	QCMD B19-11 n°7	0.22	0.03	0.10
	QCMD B19-11 n°8	0.67	0.21	0.39
WHOLE BLOOD	B19 at 10LoD	0.1	0.32	0.19
	B19 at 5LoD	1.66	0.33	0.30
	B19 at 2LoD	1	0.29	0.09
	B19 at 0,01LoD	N/A	0.1	N/A

Amplification Program : "Parvovirus B19 R-gene® "

Taq Polymerase Activation	95°C	15 min.
Amplification 45 cycles	95°C	10 sec.
	60°C	40 sec.*

** reading 530 nm - 560 nm*

Amplification Program : " MWS r-gene® "

Transcriptase inverse	50°C	5 min.
Denaturation	95°C	15 min.
Amplification 45 cycles	95°C	10 sec.
	60°C	40 sec.*
	72°C	25 sec.

** reading 530 nm - 560 nm*

No significant difference was noted between the two amplification programs: the differences in quantification obtained for each sample tested (whole blood or plasma matrix) between the two programs were less than 0.5 log_{10} copies/mL.

⇨ The MWS r-gene® program is validated for amplification of the Parvovirus B19 genome on the ABI 7500 Fast (Applied BioSystems) with the Parvovirus B19 R-gene® kit using blood and plasma samples extracted by the NucliSENS® easyMAG® using the Specific B protocol (200/50).

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15.9. Etude de performance biologiques

The biological performance of the Parvovirus B19 R-gene® kit was established on three sites.

These studies were carried out to evaluate the overall positive and negative concordance of the kit, compared with a routine laboratory technique, on different types of samples collected during the laboratory's routine activity.

15.9.1. Study of biological performance on clinical samples of serum – Virology laboratory at the Trousseau Hospital (Paris-France)

The objective was to evaluate the biological performance of the Parvovirus B19 R-gene® (kit, ref.: 69-019B) on serum samples collected during the laboratory's routine activity.
The routine laboratory technique is a real-time quantitative PCR ("in house") method using primers targeting region NS1, a technique described in Servant A *et al*., Journal of Virology vol.76, No.18 :p 9124 -9134 (2002) (1).

The samples were extracted using the NucliSENS® easyMAG® with the Specific B protocol (200/50) then amplified on the Dx Real Time System (Bio-Rad) with the Parvovirus B19 R-gene® kit and on ABI 7500 with the in-house PCR.

The conversion factor was determined at the virology laboratory of the Trousseau University Hospital for both techniques and combinations of platforms based on the WHO 2nd IS, so as to express the results in copies/mL as IU/mL.

A total of 320 serum samples was tested.

Results of concordance obtained:

		PCR « IN HOUSE »		Total
		+	-	
Parvovirus B19 R-gene®	+	75	2[a]	77
	-	8[b]	235	243
	Total	83	237	320

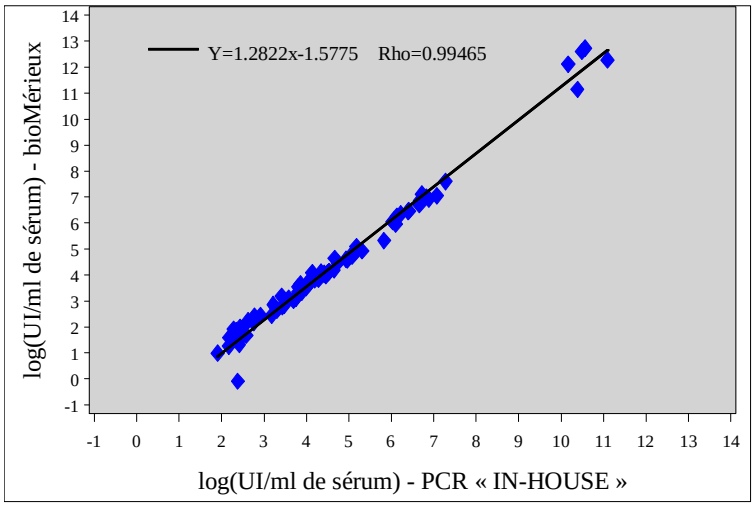
Positive concordance: 75 / 83 i.e. 90.4% [82.1; 95.0] %
Negative concordance: 235 / 237 i.e. 99.2% [97.0; 99.9] %
Global concordance: 310 / 320 i.e. 96.9% [94.3; 98.5] %

Of 320 samples tested, 10 gave discordant results.

a For the 2 samples positive for Parvovirus B19 R-gene® and negative with the in-house PCR, one of them could not be retested owing to lack of material and the other was found to be negative in second intention using a new extract with the Parvovirus B19 R-gene® kit, but this sample had a low viral load in first intention (2.21 $\log_{10}$ IU/mL).

b These 8 samples negative for Parvovirus B19 R-gene® and positive with the in-house PCR, displayed low viral loads between 1.42 and 2.16 $\log_{10}$ IU/mL.

Quantitative analysis of positive serum samples in the two techniques:



Rho: Spearman's correlation coefficient

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The quantitative analysis of results between the laboratory's Real-Time PCR technique and the Parvovirus B19 R-gene® Real-Time PCR was carried out on confirmed positive samples using both techniques. It showed good correlation (Rho correlation coefficient = 0.99) between the 2 techniques, even if the quantification difference was larger for high viral loads.

Quantification performance for the Parvovirus B19 R-gene® kit ref.: 69-019B on serum samples was therefore established.

15.9.2. Biological performance study on clinical samples of whole blood and whole bone marrow – Virology laboratory of the CHU Necker Enfants Malades (Necker Sick Children University Hospital) (Paris -France)

The objective was to evaluate the biological performance of the Parvovirus B19 R-gene® (kit, ref.: 69-019B) on whole blood and whole bone marrow samples collected during the laboratory's routine activity.

The laboratory's routine technique is a real-time qualitative PCR (in-house PCR) based on amplification of a fragment of gene NS1 using the protocol published by Knöll *et al*., Journal of Medical Virology, 67 : 259 – 266 (2002). [(2)].

The samples were extracted using the MagNA Pure LC (Roche Diagnostics) and DNA I Blood Cell High Performance protocol, then amplified on the CFX 96 (Bio-Rad) with the 2 PCRs. For the bone marrow samples, a predilution to 1/3 of the sample in RMPU culture medium was prepared before extraction; this type of matrix is very rich in inhibitors.

A total of 249 whole blood and 29 whole bone marrow samples was tested.

Results of the concordance obtained on the whole blood samples:

		PCR « IN HOUSE »		Total
		+	-	
Parvovirus B19 R-gene®	+	82	11[a]	93
	-	5[b]	151	156
	Total	87	162	249

Positive concordance: 82 / 87 i.e. 94.3% [87.2; 97.5] %
Negative concordance: 151 / 162 i.e. 93.2% [88.3; 96.2] %
Global concordance: 233 / 249 i.e. 93.6% [89.8; 96.0] %

Of 249 samples tested, 16 gave discordant results.

a Of the 11 samples positive for Parvovirus B19 R-gene® and negative with the in-house PCR, 10 had low CT values at 530 nm of between 37.25 and 41.01 cycles. A sample which recorded a result of 34.50 cycles for CT in first intention, was confirmed positive with both kits in second intention using a new extraction.

b Of the 5 samples negative for Parvovirus B19 R-gene® and positive with the in-house PCR, 4 had low viral loads (CT values between 38.52 and 41.53 cycles). One sample recorded a CT of 26.09 cycles. This sample could not be retested in second intention owing to lack of material. The MagNA Pure LC extract was then sent back and tested internally (1/10 dilution) with another PCR technique (RealStar® Parvovirus B19 PCR Kit 1.0 from Altona Diagnostics GmbH) and gave a negative result.

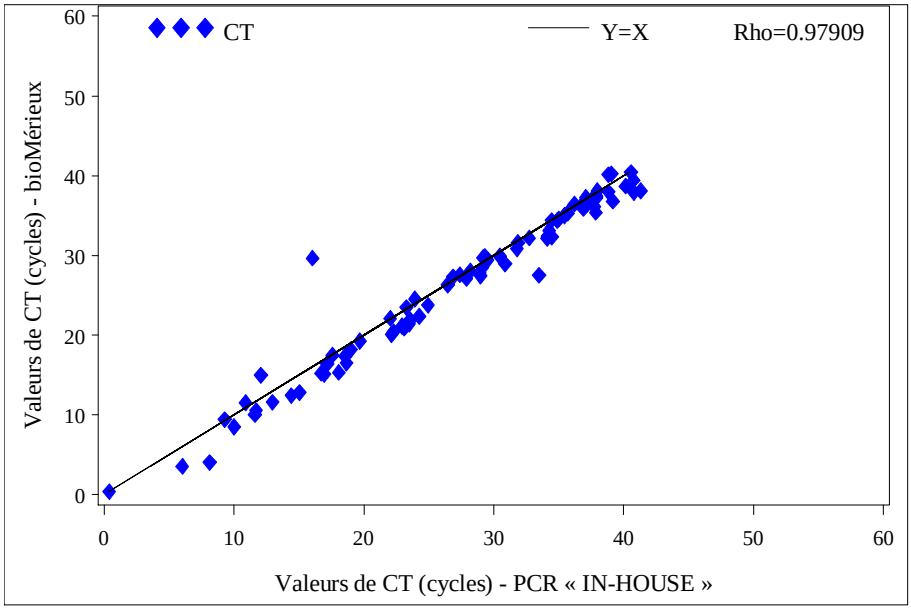
Results of the concordance obtained on the whole bone marrow samples:

		PCR « IN HOUSE »		Total
		+	-	
Parvovirus B19 R-gene®	+	14	5	19
	-	0	10	10
	Total	14	15	29

Positive concordance: 14 / 14 i.e. 100.0% [76.8; 100.0] %
Negative concordance: 10 / 15 i.e. 66.7% [41.7; 84.8] %
Global concordance: 24 / 29 i.e. 82.8% [65.5; 92.4] %

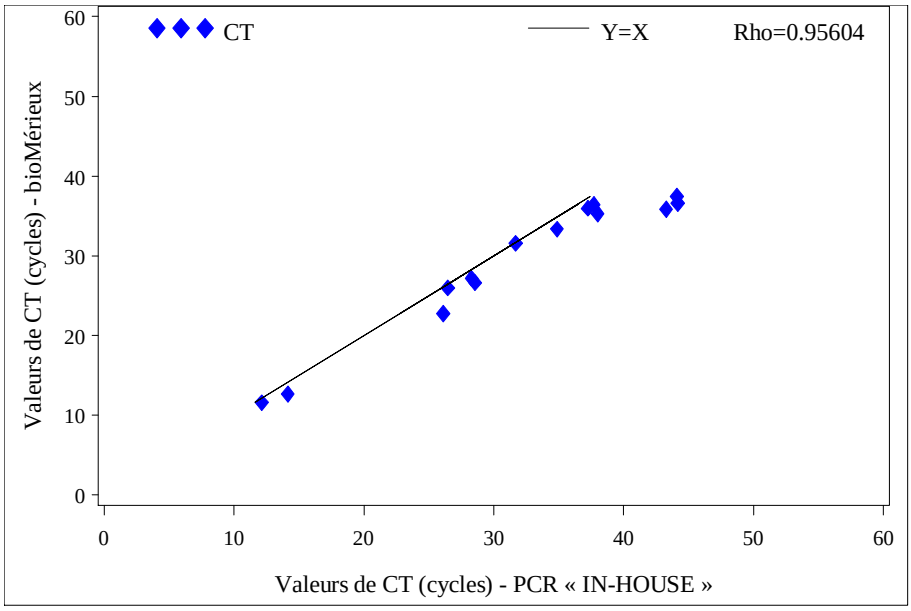
Of 29 samples tested, 5 gave discordant results. Three of these samples gave a low CT value (CT between 38.15 and 38.97 cycles).
For the other two samples (CT value 33.73 cycles and 36.2 cycles), a bidirectional sequence was carried out and showed the presence of sequences specific to human Parvovirus B19.

Semi-quantitative analysis (CT concordance) of the whole blood samples which were positive for both techniques:



Rho: Spearman's correlation coefficient

Semi-quantitative analysis (CT concordance) of the whole bone marrow samples which were positive for both techniques:



Rho: Spearman's correlation coefficient

The Spearman coefficients of correlation (Rho) at 0.98 for the whole blood matrix and 0.96 for the whole bone marrow matrix demonstrated good CT concordance between the two real-time PCR techniques (Parvovirus B19 R-gene® and the laboratory's in-house PCR) on both types of matrix (whole bone marrow and whole blood).

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15.9.3. Performance study of blood plasma and medullary plasma on clinical samples – Virology Laboratory at Lyon Civil Hospices (France)

The objective was to evaluate the biological performance of the Parvovirus B19 R-gene® (kit, ref.: 69-019B) on blood plasma and medullary plasma samples collected during the laboratory's routine activity.
The laboratory's routine technique is a commercial real-time quantitative PCR kit (Abbott, "Parvo B19 PCR Kit") which was used to amplify a fragment of 76 pb of the Parvovirus B19 genome. The results were expressed directly in IU/mL.
The conversion factor for the Parvovirus B19 R-gene® technique was determined at the laboratory on the combination of platforms linked to the study, in order to convert the results expressed as copies/mL in IU/mL.
The samples of blood and medullary plasma were extracted with the NucliSENS® easyMAG® using the Specific B protocol (200/50) then amplified on ABI 7500 for both techniques.

A total of 315 blood plasma samples and 42 medullary plasma samples was tested.

Results of the concordance obtained on the blood plasma samples:

		PCR ABBOTT		Total
		+	-	
Parvovirus B19 R-gene®	+	61	16[b]	77
	-	4[a]	234	238
	Total	65	250	315

Positive concordance: 61 / 65 i.e. 93.8% [85.2; 97.6] %
Negative concordance: 234 / 250 i.e. 93.6% [89.9; 96.0] %
Global concordance: 295 / 315 i.e. 93.7% [90.4; 95.9] %

Of 315 samples tested, 20 gave discordant results.
a Of these 4 samples negative for Parvovirus B19 R-gene® and positive with Abbott PCR (viral load between 1.60 and 4.06 $\log_{10}$ IU/mL), 3 were found to be positive in second intention using a new extract with the Parvovirus B19 R-gene® kit. A single sample remained negative in second intention with the Parvovirus B19 R-gene® kit (viral load 2.08 $\log_{10}$ IU/mL with the Abbott PCR).
b Of the 16 samples positive for Parvovirus B19 R-gene® and negative with Abbott PCR, 4 could not be retested in second intention owing to lack of material. Five others were confirmed Parvovirus B19 positive in second intention using a new extraction (viral loads between 0.82 and 2.93 $\log_{10}$ IU/mL). Bidirectional sequencing took place on the 2 samples with viral loads higher than 2 $\log_{10}$ IU/mL and this confirmed the presence of sequences specific to human Parvovirus B19. Finally, 7 samples were found to be negative for Parvovirus B19 in second intention with the Parvovirus B19 R-gene® kit, but these samples had low viral loads during the first intention test (0.57 and 1.41 $\log_{10}$ IU/mL).

Results of the concordance obtained on the medullary plasma samples:

		PCR ABBOTT		Total
		+	-	
Parvovirus B19 R-gene®	+	3	3	6
	-	1	35	36
	Total	4	38	42

Positive concordance: 3 / 4 i.e. 75.0% [30.1; 95.4] %
Negative concordance: 35 / 38 i.e. 92.1% [79.2; 97.3] %
Global concordance: 38 / 42 i.e. 90.5% [77.9; 96.2] %

Of 42 samples tested, 4 gave discordant results.
- 3 samples with low viral load (between 1.80 and 1.07 $\log_{10}$ IUmL) were found to be positive with the Parvovirus B19 R-gene® kit.
- 1 sample with a viral load of 2.65 $\log_{10}$ IU/mL was found to be positive with the Abbott PCR and negative with the Parvovirus B19 R-gene® kit. This sample could not be retested as there was not a large enough volume of sample.

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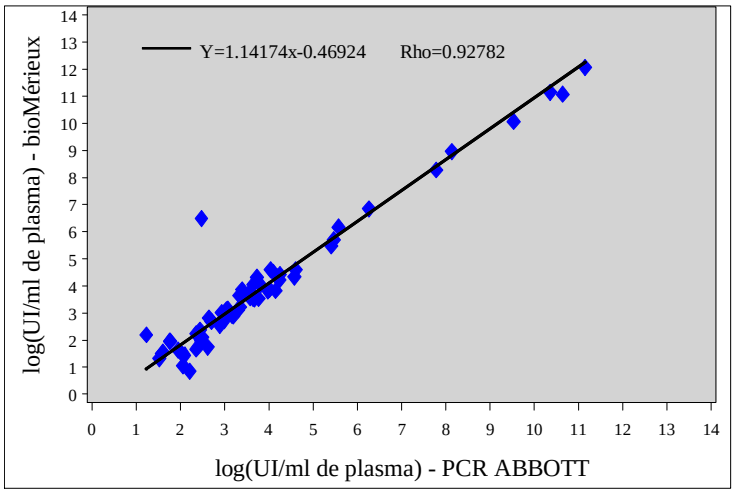
Quantitative analysis of positive blood plasma samples in the two techniques:



Rho: Spearman's correlation coefficient

The quantitative analysis of results for blood plasma between the Abbott Real-Time PCR technique and the Parvovirus B19 R-gene® Real-Time PCR was carried out on confirmed positive samples using both techniques.
It showed good correlation between the quantifications of the 2 techniques for blood plasma. Indeed, the mean difference in quantification between the two PCR techniques was around 0.1 log_{10} IU/mL. The quantification performance of the Parvovirus B19 R-gene® kit on blood plasma was therefore demonstrated.

16. Test Report on QCMD panel

16.1. Test report on QCMD 2012 panel

The 8 samples of the QCMD B19 2012 panel (plasma matrix) were tested with the Parvovirus B19 R-gene® kit. 200 µL of each sample was extracted with the NucliSENS® easyMAG® then amplified on ABI 7500 Fast and the Dx Real Time System (Bio-Rad) with the amplification premix **R19** from the Parvovirus B19 R-gene® kit.

⇨ 100% (8/8) of the samples tested agreed with the expected results for both the amplification platforms tested.

QCMD Results				Parvovirus B19 R-gene®		delta log_{10} cp/mL	
Panel Code	concentration (log_{10} cp/mL)	Sample status	Sample Type	ABI 7500 Fast	Biorad Dx RTS	QCMD -ABI	QCMD-Biorad
B19-01	2.32	detected		2.56	2.38	0.24	0.06
B19-02	4.18	frequently detected	Core	4.31	4.42	0.13	0.24
B19-03		negative	Core				
B19-04	7.01	frequently detected	Core	7.27	7.26	0.26	0.25
B19-05	5	frequently detected	Core	5.28	5.31	0.28	0.31
B19-06	3.27	frequently detected	Core	3.34	3.41	0.08	0.15
B19-07	6.08	frequently detected	Core	6.28	6.26	0.2	0.18
B19-08	3.16	detected	Core	3.49	3.36	0.33	0.2

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17. References

Programming and analysis assistance sheets, per device type, downloadable at www.biomerieux.com/techlib

Publications:

(1) Servant A. et al, **Genetic Diversity within Human Erythroviruses: identification of three genotypes.**
Journal of Virology, vol.78, No.18, p.9124-9134 (2002)

(2) Knöll A. et al., **Parvovirus B19 infection in pregnancy: quantitative viral DNA Analysis Using a Kinetic fluorescence Detection system (TaqMan PCR)**
Journal of Medical Virology, 67, p.259-266 (2002)

Posters:

(1) MARECHAL P., FOUCAUD G., MAGRO S. and BARRANGER C.
"Development of a new diagnostic tool for the detection and quantification of Parvovirus B19 by Real Time PCR ".
Poster, ECV 2013, Lyon, France

(2) SCHNURIGER A., SALOUM K., MICHEL Y., MARINHO V., ANSART I. and GARBARG-CHENON A.,
Evaluation of a novel real time PCR assay for Parvovirus B19 (B19V) genome detection and quantification.
Poster, ECV 2013, Lyon, France

18. Related products

- DICO Ampli r-gene® ref.: 71-100
- DICO Extra r-gene® ref.: 71-101
- Colour compensation r-gene® ref.: 71-103
- CELL Control r-gene® ref.: 71-106

- Instrument:
  - NucliSENS® easyMAG® ref.: 200110

- Reagents:
  - Lysis buffer NucliSENS® Lysis Buffer (4 x 1000 ml/bottle) ref.: 280134
  - Extraction buffer NucliSENS® easyMAG® Extraction Buffer 1 (4 x 1000 ml/bottle) ref.: 280130
  - Extraction buffer NucliSENS® easyMAG® Extraction Buffer 2 (4 x 1000 ml/bottle) ref.: 280131
  - Extraction buffer NucliSENS® easyMAG® Extraction Buffer 3 (4 x 1000 ml/bottle) ref.: 280132
  - Magnetic silica NucliSENS® easyMAG® Magnetic Silica (48 x 0.6 ml) ref.: 280133

- Consumables:
  - NucliSENS® easyMAG® shuttles ref.: 280135

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19. Index of symbols

Symbol	Meaning
	Catalogue number
	In Vitro Diagnostic Medical Device
	Manufacturer
	Temperature limite
	Use by
	Batch code
	Consult Instructions for Use
	Contains sufficient for <n> tests
	Protect from light
	Keep dry
	Identification of notified body

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