Parvovirus B19 Quantification Kit v1

USER MANUAL

For *in vitro* Diagnostic Use

Document Code: MB06v5
Approval Date: August 2016

IVD CE

Contents

1. PRODUCT DESCRIPTION

Bosphore® Parvovirus B19 Quantification Kit v1 detects and quantitates Parvovirus B19 DNA in human serum or plasma, encompassing all the major Parvovirus B19 genotypes. The linear range of quantitation is $2x10^2$-$1x10^6$ IU/ml and the analytic sensitivity is 75 IU/ml. A region within the NS gene is amplified and fluorescence detection is accomplished using the FAM filter.

An internal control has been integrated into the kit in order to check PCR inhibition. The amplification data of the internal control is detected with the Cy5/HEX/JOE filter. The internal control can be added either during DNA extraction or PCR step.

2. CONTENT

Bosphore® Parvovirus B19 DNA Quantification Kit v1 is composed of Real-Time PCR reagents and quantitation standards which have been calibrated against WHO International Standard (NIBSC Code 99/802):

Component	REAGENT	100 Reactions	50 Reactions	25 Reactions
1	dH_2O	(1000 µl)	(1000 µl)	(500 µl)
2	PCR Mix	(1400 µl)	(700 µl)	(350 µl)
3	Detection Mix1	(140µl)	(70 µl)	(35 µl)
4	Detection Mix2	(80 µl)	(40 µl)	(20 µl)
5	Internal Control	(560 µl)	(280 µl)	(140 µl)
6	Positive Control 1	(25 µl)	(25 µl)	(12 µl)
7	Standard 1 (1 x 10^6) IU/ml	(88 µl)	(44 µl)	(22 µl)
8	Standard 2 (1 x 10^5) IU/ml	(88 µl)	(44 µl)	(22 µl)
9	Standard 3 (1 x 10^4) IU/ml	(88 µl)	(44 µl)	(22 µl)
10	Standard 4 (1 x 10^3) IU/ml	(88 µl)	(44 µl)	(22 µl)

3. STORAGE

Bosphore® Parvovirus B19 Quantification Kit v1 PCR reagents should be stored at -20°C. Repeated thawing and freezing (>3x) should be avoided since it may reduce sensitivity. If the components are to be used in small amounts, they should be frozen in aliquots.

While preparing the PCR; the components should not be exposed to room temperature for more than 10 min. and the components and the PCR master mixes should not be exposed to light and air more than necessary, vials must be kept closed except during pipetting. We recommend preparing the PCR on a cooling block and keeping the detection mixes within a closed container.

The components maintain their stability until the expiry dates on the labels, if they are stored at advised conditions.

4. REQUIRED MATERIALS AND DEVICES

- Montania® 483, Montania®484 and Montania® 4896 Real-Time PCR Instruments (Anatolia Geneworks), or another Real-Time PCR system with FAM and Cy5/HEX/JOE filters (iCycler, iQ5, CFX–BioRad, LightCycler 480-Roche, 7300, 7500 Real-Time PCR System, ABI StepOne or StepOne Plus systems -ABI, Stratagene Mx3005P, Mx3000P-Agilent, LineGeneK, LineGene 9600-Bioer, Rotorgene 2000, 3000, 6000, Q-Qiagen)
- 0.2 ml Thin-Wall PCR tubes, plates or strips
- Magnesia® 16 Nucleic Acid Extraction System/Magnesia® Viral Nucleic Acid Extraction Kit or Bosphore® Viral DNA Extraction Spin Kit or Magnesia® 2448 Nucleic Acid Extraction & PCR

Setup Robot/Magnesia® 2448 Viral DNA/RNA Extraction Kit or Magrev24/Magrev Viral DNA/RNA Extraction Kit (Anatolia Geneworks) or other high quality viral DNA extraction kits and systems

- Deep freezer (-20°C)
- Desktop centrifuge with rotor for 2 ml. microcentrifuge tubes
- Calibrated adjustable micropipettes
- DNAse, RNAse, pyrogen free micropipette tips with filters
- DNAse, RNAse, pyrogen free 1.5 or 2 ml. microcentrifuge tubes
- Disposable laboratory gloves

5. IMPORTANT NOTES AND SAFETY INSTRUCTIONS

Important!:

- The product should be delivered on dry ice. Check for presence of dry ice upon arrival.
- Check for the expiry dates on the box and tube labels, upon arrival. Do not use expired products or components.
- Calibrated or verified micropipettes, DNAse, RNAse, pyrogen free micropipette tips with filters, and DNAse, RNAse, pyrogen free microcentrifuge tubes should be used.
- Before starting a test procedure, all components should be thoroughly thawed. After thawing, all components should be centrifuged briefly (spin-down for 3-5 seconds), and mixed well to ensure homogeneity prior to use.
- The kit components should be kept on ice or a cooling block until the reaction is prepared, and they should be quickly returned to -20ºC.
- PCR and nucleic acid isolation must be performed in different compartments. Samples should be stored separately to avoid contact with the kit components.
- Pathogen information should be reviewed to be aware of the health related risks.
- Serum or plasma samples (including the standards) should be handled with extreme caution: Physical contact with pathogens should be avoided by; wearing lab coats and gloves, no allowance for eating or drinking within the workspace, prevention of unauthorized individuals' access to the working area.
- All the pathogenic wastes produced during the nucleic acid isolation step; including the serum or plasma samples and material contacted with them, should be discarded into medical waste and disposed safely.

6. PRODUCT USE LIMITATIONS

- All the components may exclusively be used for in vitro diagnostics.
- This product should be used in accordance with this user manual.
- This product is to be used by personnel specially trained to perform in vitro diagnostic procedures.

7. PATHOGEN

Causative Agents

Parvovirus B19 belongs to family of Parvoviridae, genus Erythrovirus. Parvovirus B19 is the only member of Parvoviridae family that is pathogenic to humans. The virus mainly causes erythema infectiosum, arthropathy, and erythrocyte aplasia. Genome of Parvovirus B19 has 5596 nucleotides. Internal coding sequence (4830 nt) is flanked by terminal palindromic repeat sequences (383 nt each). Low DNA content and lack of lipid envelope make B19 highly resistant to heat, radiation, and detergents. [1], [2]

Epidemiology

B19 is a common infection in humans that is present throughout the year. Outbreaks of infection,more commonly seen in the spring and summer, are centered by age 15 approximately 50% of cildren with detectable IgG. It occurs throughout adult life, thus 80% of the elderly are seropositive. In women of child-bearing age, annual seroconversion rate is estimated of 1.5%. While studies in different countries like USA, France, Germany, Japan show smilar patterns, higher prevalence is shown in Brazil and African continent. [3]

Modes of Transmission

Parvovirus B19 is mainly spread by infected respiratory droplets and blood. It can be spread transplacentally to fetus from mother.

8. METHOD

Bosphore® Parvovirus B19 Quantification Kit v1 is based on the Real-Time PCR method. Polymerase chain reaction is a technique that is used for amplification of a DNA region. The reaction occurs by the repeating cycles of heating and cooling. The main components of PCR are primers, dNTPs, Taq polymerase enzyme, buffer solution and template. As a brief explanation, primers are small synthetic DNA those anneal to the specific regions of the template in order to start the synthesis. dNTPs are the building blocks of the amplified products. Taq polymerase amplifies the DNA template. Buffer solution provides the pH adjustment required for the reaction and template, as referred, is the target region for synthesis.

In Real Time PCR technique, in contrast to conventional PCR, PCR product can be monitored during the reaction. Therefore Real-Time PCR obviates the need for further analysis methods like gel electrophoresis, whereby minimizing the risk of contamination. Dual labeled probes employed in the reaction in addition to the conventional PCR reagents, enable detection of the amplified target with increased sensitivity.

The assay utilizes the 5′ exonuclease activity of Taq Polymerase to cleave a dual-labeled fluorescent probe during the extension phase of PCR. The probe is labeled at the 5′ end with a fluorescent 'reporter' molecule, and at the 3′end with another fluorescent molecule that acts as a 'quencher' for the 'reporter'. When the two fluorophores are in close proximity, and the reporter is excited by light, no reporter fluorescence can be detected. During the elongation step of PCR, Taq Polymerase encounters and cleaves the probe bound to the template. As the reporter is freed from the suppressing effect of the quencher, fluorescence signal can be detected.

The fluorescence generated by the reporter increases as the PCR product is accumulated; the point at which the signal rises above background level and becomes distinguishable, is called the threshold cycle (C_T). There is a linear relationship between the log of the starting amount of a template and its threshold cycle, thus starting amount of unknown templates can be determined using standard curves constructed using C_T values of the known starting amounts of target templates.

Bosphore Parvovirus B19 Quantification Kit v1 employs multiplex PCR, and an internal control is incorporated into the system in order to control the isolation procedure and to check for possible PCR inhibition. Parvovirus B19 DNA and an internal control are co-amplified in a single reaction, using sequence-specific primers. The fluorescent signal generated by the Parvovirus B19 amplification is detected by a probe labeled at the 3′ end with FAM, through the FAM channel. The fluorescent signal generated by the internal control amplification, is detected by a second probe (labeled at the 5′ end with a different reporter molecule, Cy5/HEX/JOE) through the Cy5/HEX/JOE channel.

9. PROCEDURE

9.1. DNA Isolation

We recommend that the Magnesia® 16 Nucleic Acid Extraction System/Magnesia® Viral Nucleic Acid Extraction Kit or Bosphore® Viral DNA Extraction Spin Kit or Magnesia® 2448 Nucleic Acid Extraction & PCR Setup Robot/Magnesia® 2448 Viral DNA/RNA Extraction Kit or Magrev24/Magrev Viral DNA/RNA Extraction Kit (Anatolia Geneworks) or other high quality viral DNA extraction kits and systems are used with Bosphore® Parvovirus B19 Quantification Kit v1. The DNA isolation should be performed according to the manufacturers' instructions. The amount of internal control that should be used during isolation for each sample is 5 µl. If another DNA extraction kit is used a correction factor must be introduced, depending on the extraction starting and elution volumes.

9.2. Kit Components

9.2.1. PCR Mix

HotStarTaq DNA Polymerase: HotStarTaq DNA Polymerase is a modified form of a recombinant 94 kDa DNA polymerase, originally isolated from *Thermus aquaticus,* cloned into E.Coli. The enzyme is provided in an inactive form. It is activated by a 15-minute 95 ºC incubation step. This prevents the formation of misprimed products and primer-dimers during reaction setup and the first denaturation step, leading to high PCR specificity and accurate quantification. PCR Buffer: contains Tris-Cl, KCl, $(NH_4)_2SO_4$, 8 mM $MgCl_2$, pH 8.7 (20ºC). dNTP Mix: Contains ultrapure quality dATP, dGTP, dCTP ve dTTP/dUTP.

9.2.2. Detection Mix 1

Detection Mix 1 contains Parvovirus B19-specific forward and reverse primers and a dual-labeled probe.

9.2.3. Detection Mix 2

Detection Mix 2 contains internal control-specific forward and reverse primers and a dual-labeled probe.

9.2.4. Internal Control

An internal control is included in the kit to control DNA isolation and PCR inhibition. The internal control is a synthetic DNA molecule. It is added into the serum or plasma, proteinase K and carrier RNA mixture during DNA isolation, to control the isolation efficiency and PCR inhibition. The amount of IC that should be added during isolation is 5 µl per serum or plasma sample. Alternatively, the internal control can be added directly into the PCR master mix to control the PCR inhibition exclusively. For this purpose, 0.1µl of internal control should be added for each reaction into the master mix. Lack of internal control amplification in the FAM negative samples, may indicate a problem in isolation or PCR inhibition. In this case, isolation and PCR should be repeated. In samples that contain a high viral load, internal control can be suppressed and no increase of the signal is detected. Please use the table below for the interpretation of internal control data:

Parvovirus B19 (FAM)	Internal Control (Cy5/HEX/JOE)	Interpretation
+	+	Sample positive
-	+	Sample negative
+	-	Sample positive
-	-	Repeat the test!

9.2.5. Positive Control

The positive control contains Parvovirus B19 DNA. It can be included in the PCR to test the efficiency of the PCR exclusively. The threshold cycle for the positive control is given in the acceptance criteria table (Section 10. Analysis). Threshold cycles higher than the acceptance criteria may indicate an efficiency loss in the reaction.

9.2.6. Quantitation Standards

The quantitation standards are calibrated by WHO International Standard (NIBSC Code 99/802).

9.3. Preparing the PCR

All four external quantitation standards should be added into the PCR reaction together with the samples and the negative control (PCR-grade water). Make sure that all the kit components are thawed before use. Refer to the table below for preparing the PCR. It is for only one reaction, multiply these values with the sample number to find the values required for the master mix. While preparing master mixes for more than 5 samples, an extra 10% should be added to the total sample number.

PCR Mix	12.5 µl
Detection Mix 1	1.25 µl
Detection Mix 2	0.7 µl
Internal Control*	0.1 µl
dH2O*	0.45 µl
Sample DNA Standard Negative/Positive Control	10 µl
Total Volume	25 µl

*No need to add internal control if it's already added in the extraction step.

Pipette 15 µl of the master mix into the PCR tubes or strips, and add 10 µl of DNA (sample/ standard/positive or negative control). Close the tube cap. Make sure that the solution in each tube is at the bottom of the tube. Centrifuge if necessary.

9.4. Programming the Real-Time PCR Instrument

The thermal protocol for Bosphore® Parvovirus B19 Quantification Kit v1 is composed of an initial denaturation for activation the HotStarTaq DNA Polymerase, a two-step amplification cycle and a terminal hold. The real-time data is collected at the second step of the amplification cycle.

Initial denaturation	95°C	14:30 min.	
Denaturation	97°C	00:30 min.	} 50 cycles
Annealing and Synthesis (Data Collection)	54°C	01:30 min.	
Hold	22°C	05:00	

Before starting a Real-Time PCR reaction using the Bosphore® Kits, the following steps should be completed:

- Choose the filter pairs to be used (FAM and Cy5/HEX/JOE),
- Identify unknown samples, standards, positive and negative controls, assign quantitative values to the standards,
- Select the correct thermal protocol.
- Start the experiment

10. ANALYSIS

By the end of the thermal protocol, the instrument software automatically calculates the baseline cycles and the threshold.

Please also see section 9.1. Isolation, before analyzing the results.

Example of an amplification curve is given in Fig. 1.

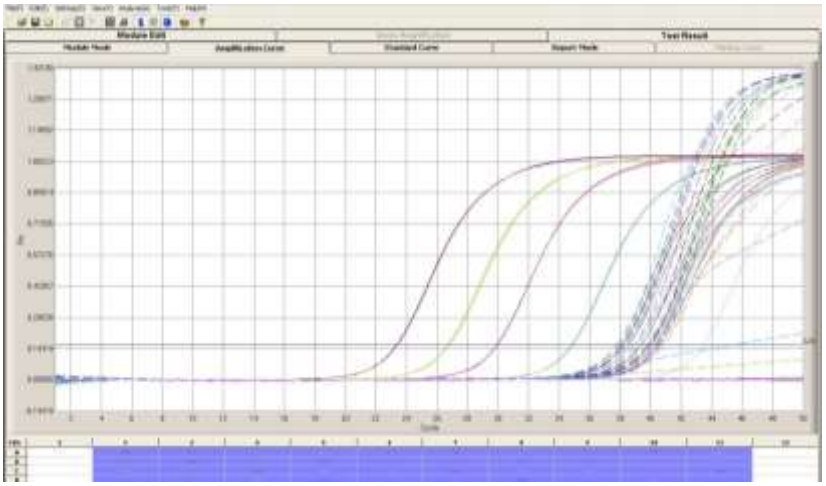

Fig. 1: Amplification Curve of a Bosphore® Parvovirus B19 v1 test

The standard curve is plotted using the data obtained from the defined standards, with the axes Ct-Threshold Cycle and Log Starting Quantity. Example of a standard curve is given in Fig. 2

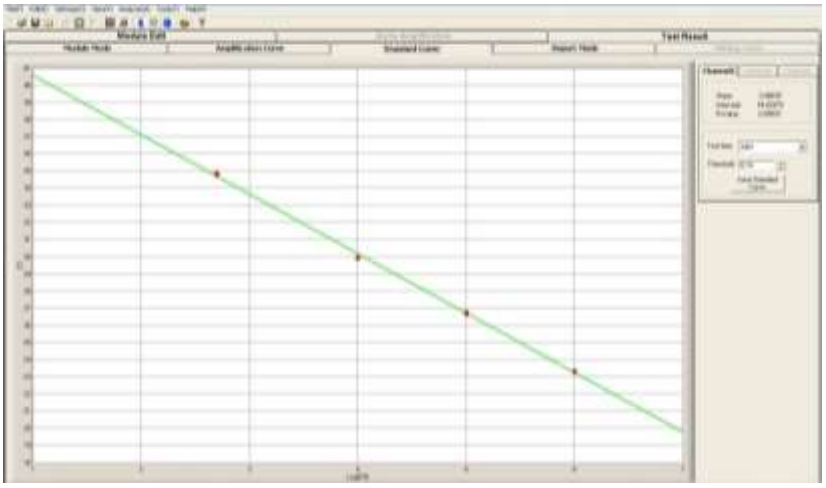

Fig.2: Standard Curve of a Bosphore® Parvovirus B19 v1 test

Analysis of the results should be performed by trained personnel who have received the required training for analysing Real-Time PCR data. We recommend that the test results must be evaluated by an expert clinician, taking the patient's clinical findings and the results of other tests into consideration.

All analysis is done automatically in routine use. However, when the trained personnel, who have received the required training from manufacturer, consider it as necessary, and if the system allows pulling down the threshold as much as possible in order to detect low positive samples, attention should be paid to keep the threshold line above the background and to keep the correlation coefficient at the maximum possible value (and within its acceptance criteria.)

The table below displays the acceptance criteria for Bosphore® Parvovirus B19 v.1.

Component/Parameter	Cycle Threshold (C_T)
Standard 1	25±3
Standard 2	28±3
Standard 3	31±3
Standard 4	34±3
Positive Control	31±4
Correlation Coefficient	>0.970
PCR Efficiency	>%80

Test results should not be reported unless the assay results meet the criteria stated above. Please contact the manufacturer if an impairment in the product's performance is observed (See the last page for contact information).

The samples that cross the threshold in FAM are displayed with a calculated starting quantity, samples that do not cut the threshold are displayed as "No Ct". These samples are regarded as negative or having a viral load below the detection limit of the assay. For these undetectable samples, the Cy5/HEX/JOE data of the internal control should also be checked to avoid false negative results.

The following table shows the possible results and their interpretation:

Signal detected in FAM filter pair	The sample contains Parvovirus B19 DNA, the result is positive	No need to check the internal control since the sample is positive (high positive samples may suppress the signal from the internal control)
No signal in FAM, signal in Cy5/HEX/JOE	The Parvovirus B19 DNA in the sample is not detectable	Signal from Cy5/HEX/JOE filter pair rules out the possibility of PCR inhibition
No signal in FAM and Cy5/HEX/JOE	The diagnosis is inconclusive	No signal in Cy5/HEX/JOE points out to PCR inhibition or to a problem in DNA isolation

In case of PCR inhibition due to medication or other PCR inhibitors in the sample, we recommend to repeat the test of inhibited samples, by freezing and thawing the DNA samples and using them in the PCR after diluting them 1:4 with dH_2O.

11. SPECIFICATIONS

11.1. Sensitivity and Genotype Detection

Analytical sensitivity may be expressed as the limit of detection: i.e. the smallest amount of the target marker that can be precisely detected. The detection limit of an individual analytical procedure is the lowest amount of nucleic acid in a sample which can be detected but not necessarily quantitated as an exact value. The analytical sensitivity or detection limit for NAT assays is expressed by the 95% positive cut-off value.

The analytical detection limit for Bosphore® Parvovirus B19 v1 was found to be $7.5x10^1$ IU/ml (p=0.05). The sensitivity was determined using serial dilutions of DNA calibrated with the WHO International Standard for Parvovirus B19 DNA NAT assays, (NIBSC Code 99/802). The dilutions were tested in different runs in replicates. The results were analyzed by probit method.

11.2. Linear Range

The linear range of Bosphore® Parvovirus B19 Quantification Kit v1 was determined to be $2x10^2$-$1x10^6$ IU/ml.

In order to assess the linear range, a dilution series which has been calibrated against the WHO International Standard for PARVOVIRUS B19 DNA NAT assays, (NIBSC Code 99/802) was analyzed by testing each dilution in 4 replicates (Fig. 3a and 3b). The standard curve correlation coefficient was found to be 0.99961.

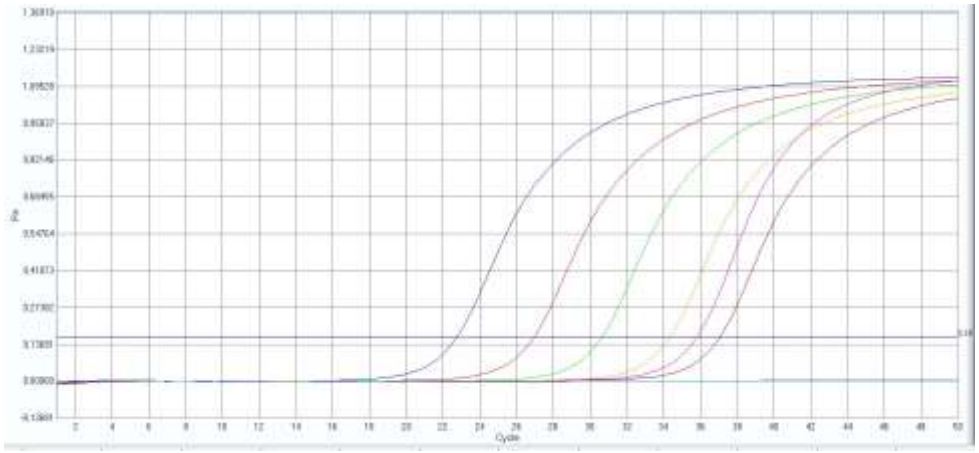

Fig. 3a: Linear Range Amplification Curve

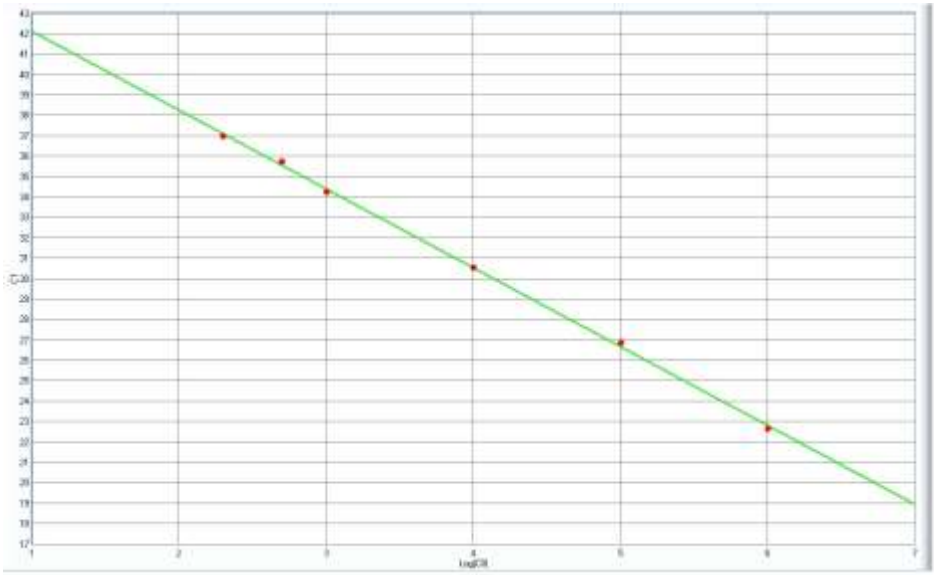

Fig. 3b: Linear Range Standard Curve

11.3. Cross-Reactivity

To eliminate potential cross-reactivity, both assay design evidence and experimental studies were employed. Primer and probe sequences were checked for possible homology to other known pathogen sequences by sequence comparison analysis using database alignment. Samples of CMV, EBV, MTBC, JCV, BKV, HSV and HBV with known high positivity were tested, and found negative.

11.4. Reproducibility

Reproducibility data (on C_T value basis) were obtained by the analysis of one of the quantitation standards of the Bosphore® Parvovirus B19 Quantification Kit v1. Test was performed in at least 4 replicates by 3 different operators, on multiple days, using 3 different lots. The resulting data is given in Table 1:

Table 1: Reproducibility Data.

PARVOVIRUS B19 (10^4 IU/ml)	Standard deviation	Variance	Coefficient of variation [%]
Intra-assay Variability N=4	0.06	0.004	0.22
Inter-lot Variability N=3	0.07	0.004	0.21
Inter-operator Variability N=3	0.15	0.023	0.49
Total Inter-assay Variability N=5	0.11	0.012	0.36

12. REFERENCES

1. Heegaard E D, Brown K E (2002) Human Parvovirus B19. Clinical Microbiology Reviews 15(3): 485-505.
2. Sabella C, Goldfarb J (1999) Parvovirus B19 infections. American Family Physician 60:1455-60.
3. Servey JT, Reamy BV, Hodge J (February 2007). "Clinical presentations of parvovirus B19 infection".AmFam Physician 75 (3): 373–6. PMID 17304869.Retrieved 2009-11-06. PMID 17304869

13. SYMBOLS


Use by

Lot/Batch

Catalog number


Temperature limitation


Caution, consult accompanying documents


Manufacturer

In Vitro Diagnostic Medical Device

14. CONTACT INFORMATION

Anatolia Tanı ve Biyoteknoloji Ürünleri Ar-Ge San. Ve Tic. A.S.

Address: Eğitim Mh. Kasap İsmail Sokak
No:10/23 Kadikoy 34722
ISTANBUL-TURKEY
Phone: +90 216 330 04 55
Fax: +90 216 330 00 42
E-mail: info@anatoliageneworks.com
www.anatoliageneworks.com