LightMix® Kit Parvovirus B19 EC

Cat.-No. 40-0585-32 Universal Extraction Control Target (^{n}ECT)

Kit with reagents for the detection of *Parvovirus B19* DNA using the Roche Diagnostics LightCycler® 1.x / 2.0 / 480 II Instruments or cobas z 480 Analyzer (UDF open channel).

Lyophilized mix of primers and probes for a total of 96 reactions with a final volume of 20 µl each. **Store protected from light at room temperature (18-25°C), do NOT freeze!**

Instructions for use with the LightCycler® 1.x / 2.0 Instruments see pages 4-5
Instructions for use with the LightCycler® 480 II Instrument / cobas z 480 Analyzer see pages 6-7

1. Introduction

Parvovirus B19 is a human, non-enveloped, singlestranded DNA virus. From the three genotypes GT1 is most frequent. Infection provides normally a lifelong immunity. The most common manifestation of a infection is *erythema infectiosum* (Ringröteln), also known as fifth disease, is a mild rash experienced during childhood.

In immunosupressed and anemic individuals *Parvovirus B19* may cause an acute, severe anemia. Individuals with sickle cell anemia may experience an aplastic crisis that has to be treated with blood transfusion. An undetected virus infection of immunocompromised individuals, such as AIDS patients, may develop a chronic anemia. Infection of pregnant women with Parvovirus can lead to miscarriage or stillbirth. In all these cases detection and monitoring of *Parvovirus B19* is crucial.

Typical clinical specimen are EDTA blood or serum, but can be also amniotic fluid.

Common targets for PCR based detection of the virus are the open reading frames orf1[1] and orf2[2].

2. Description

This kit provides a fast and accurate system to detect *Parvovirus B19* genotypes GT1-3 in a nucleic acid extract; a control reaction allows to monitor extraction and eventual PCR inhibtion.

A 184 bp fragment of the orf2 region of the *Parvovirus B19* genome is amplified with specific primers. The resulting PCR fragment is analyzed with hybridization probes labeled with LightCycler® Red 640.

The assay is monitored by an additional PCR product of 135 bp, formed from the control target. This control does not interfere with the virus-specific reaction. The amplification will usually fail in the presence of higher concentrated samples (1,000 copies or higher) while displaying an amplification signal in negative and low-concentrated samples. The hybridization probes are labeled with LightCycler® Red 690 (recorded in channel 705).

The PhHV DNA Internal Control (IC) has been changed to a spiked Extraction Control (sEC) in order to monitor successful extraction and to demonstrate the ability to run a PCR reaction (no inhibition).
We recommend to use the 'Extraction Control' procedure; in case that the former procedure shall be maintained the usage as IC is described. Target and control primer/probe sequences remained unchanged. The novel extraction control target ^{n}ECT contains Lamdba and PhHV DNA and may be used also for other LightMix kits, without adding the ECT provided with the other kit(s).
Note: With a MagNA Pure Compact extraction the recovery rate for the EC target can be very low or the DNA target even gets lost; the amount of ECT might has to be adapted to the extraction method.

The use of a color compensation file generated with the LightMix® Color Compensation HybProbe 530/640/690 kit (order no. 40-0318-00) is a prerequisite to detect the control reaction.

The supplied standard row allows to determine the linear range of the reaction and to estimate the quantity of the target sequence in unknown samples.

The kit is tested with 'LightCycler® FastStart DNA Master HybProbe' only.

3. Set Contents

3 Vials with **blue** cap containing premixed primers and probes for 32 reactions of *Parvovirus B19*
3 Vials with **white** cap containing premixed primers and probes for 32 control reactions

1 Standard row with 6 lyophilized standards of *B19* from 10^1 to 10^6 target equivalents per rxn
1 Sealing foil for the standard row

1 Vial with **white** cap containing Extraction Control Target (^{n}ECT) with 4.8 x 10^6 copies (total)
1 Vial with **black** cap containing the stabilizer for preparation of the 'No Template Control' (NTC)

1 Certicate of Analysis (CoA) with lot-specific data

4. Additional Reagents and Items Required

	Roche Diagnostics
Color Compensation HybProbe order n°40-0318-00	Cat .-No. 05 997 704 001
LightCycler® FastStart DNA Master HybProbe	Cat.-No. 03 003 248 001
LightCycler® Capillaries (20 µl) (LightCycler® 1.x / 2.0 Instruments)	Cat.-No. 04 929 292 001
LightCycler® 480 Multiwell Plate 384, white (LightCycler® 480 Instrument)	Cat.-No. 04 729 749 001
or LightCycler® 480 Multiwell Plate 96, white (LightCycler® 480 Instrument)	Cat.-No. 04 729 692 001

For use in LightCycler® 1.x / 2.0 Instruments select channel F2 instead of channel 640, channel F3 instead of channel 705 for detection. We recommend to upgrade to software 4.10 or later.

4.1. Optional Additional Reagents

High Pure Viral Nucleic Acid Kit	Cat.-No. 11 858 874 001
or High Pure PCR Template Preparation Kit	Cat.-No. 11 796 828 001

5. Product Characteristics

PCR results are obtained within 50 minutes (50 cycles and melting curve) with the LightCycler® 1.x / 2.0 Instruments and within 80 minutes (50 cycles and melting curve) with the LightCycler® 480 II Instrument.

Sensitivity
These reagents detect 10 copies of *Parvovirus B19* DNA using the Roche 'LightCycler® FastStart DNA Master HybProbe' (in an exemplary system, using cloned targets as reference).

Measuring range
The linear measuring range of the assay is 10^2 to 10^6 copies of *Parvovirus B19* DNA using the Roche 'LightCycler® FastStart DNA Master HybProbe'.

Storage and Stability
- Lyophilized reagents are stable for at least 6 months after shipment when stored protected from light at room temperature (18-25°C). See expiry date o n the product label.
- **Do not freeze** lyophilized reagents.
- Dissolved reagents are stable for at least 10 days when stored protected from light and refrigerated (4°C).

6. Experimental Protocol

Start programming before preparing the solutions. See the Instrument operator's manual for details.

6.1. Preparation of Parameter-Specific Reagents (PSR) and Control Reaction :

One reagent vial with a **blue** cap contains primers and probes to run 32 reactions of *Parvovirus B19.*
One reagent vial with a **white** cap contains primers and probes to run 32 Control Reactions.

Check for the colored pellet, then **add 66 µl** PCR-grade water, mix (vortex) and spin down.

►**Use 2 µl** reagent for a 20 µl PCR reaction.

This solution is stable at least ten days when stored refrigerated at 4°C. Avoid prolonged exposure to light.

6.2. Preparation of Extraction Control Target (ECT)

Add **1,200 µl** PCR-grade water to the vial with **white** cap containing the Extraction Control Target

6.3. Sample Preparation:

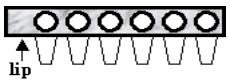
Before extraction add **10 µl** of ECT (vial **white** cap) to the sample. Skip if IC procedure is used.
Perform nucleic acid preparation as described in the protocol of the extraction kit used (**see 4.1**).

6.4. Preparation of No Template Control (NTC) including the Extraction Control Target (ECT)

Add **900 µl** PCR-grade water to the **NTC** (vial **black** cap) and
add **100 µl** of ECT (vial **white** cap).

►Use **5 µl** No Template Control DNA for a 20 µl PCR reaction.

6.5. Preparation of the Standard Row



The target DNA is provided in 6 different quantities to yield from 10^1 to 10^6 target molecules in 5 µl once resolved. Start with the lowest concentrated standard (first tube from the extended lip). Use the pipette tip to punch a hole through the sealing foil. Add **40 µl** of **NTC** (vial **black** cap) to each well. Use water if the IC procedure is selected. Mix the target DNA by pipetting the solution up and down 10 times.

►**Use 5 µl** standard DNA for a 20 µl PCR reaction.

This standard solution is not long-term stable and will lose sensitivity under prolonged storage. After adding the target DNA to reaction mix, use the provided sealing foil to close the vials in order to avoid contaminations.

6.6. Preparation of the Reaction Mix

Include Positive Control(s) and at least one 'No Template Control' (NTC). In a cooled tube, prepare the reaction mix by multiplying the single reaction volumes by the number of reactions plus one reserve :

sEC Procedure	For use with the Roche FastStart Master	IC Procedure
Single reaction	**Component**	**Single reaction**
6.6 µl	Water, PCR-grade (colorless cap, provided with the Roche Master kit)	6.35 µl
2.4 µl	Mg^{2+} solution 25 mM (blue cap, provided with the Roche FastStart kit)	2.4 µl
2.0 µl	**PSR** mix (parameter specific reagents, primers and probes, see **6.1.**)	2.0 µl
2.0 µl	**Control Reaction** (see **6.1.**)	2.0 µl
---- µl	ECT Control Target (vials **white** cap)	0.25 µl
2.0 µl	Roche Master (red cap, for preparation see Roche manual)	2.0 µl
15.0 µl	**Volume of reaction mix**	**15.0 µl**

Table 1

To run the assay without the control reaction substitute ECT with 0.25 µl PCR-grade water.

Mix gently, spin down and **transfer 15 µl** of the reaction mix to a capillary or well.

Add 5 µl of sample or standard to each capillary or well for a final reaction volume of 20 µl.
Close the capillaries / attach a foil to the multiwell plate and seal, and spin down.

Start run.

7. LightCycler® 1.x / 2.0 Instruments

7.1. Programming

The protocol consists of four program steps

- 1: Denaturation: sample denaturation and enzyme activation
- 2: Cycling: PCR-amplification of the target DNA
- 3: Melting: melting curve analysis for identification of the PCR product derived from the target DNA
- 4: Cooling: cooling the instrument

Program Step:	Denaturation	Cycling			Melting			Cooling
Parameter								
Analysis Mode	None	Quantification mode			Melting Curves mode			None
Cycles	1	50			1			1
Target [°C]	95	95	62	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:05	00:00:05	00:00:15	00:00:20	00:00:20	00:00:00	00:00:30
Ramp Rate [°C/s]	20	20	20	20	20	20	0.2	20
Sec Target [°C]	-	-	55	-	-	-	-	-
Step Size [°C]	-	-	0.5	-	-	-	-	-
Step Delay (Cycles)	-	-	1	-	-	-	-	-
Acquisition Mode	None	None	Single	None	None	None	Cont	None

(Melting not relevant for detection) Table 2

7.2. Data Analysis

Switch the color compensation mode on. If this mode is not enabled run the color compensation program. Follow the instructions in the manual of the LightMix® Kit - Color Compensation HybProbe.

Perform data analysis, as described in the Instrument operator's manual.

We recommend using the Second Derivative Maximum method (Automated (F'' max)). The cycle number of the Crossing Point (Cp) of each sample is calculated automatically. The Fit Points method is more-error prone due to the user's influence.

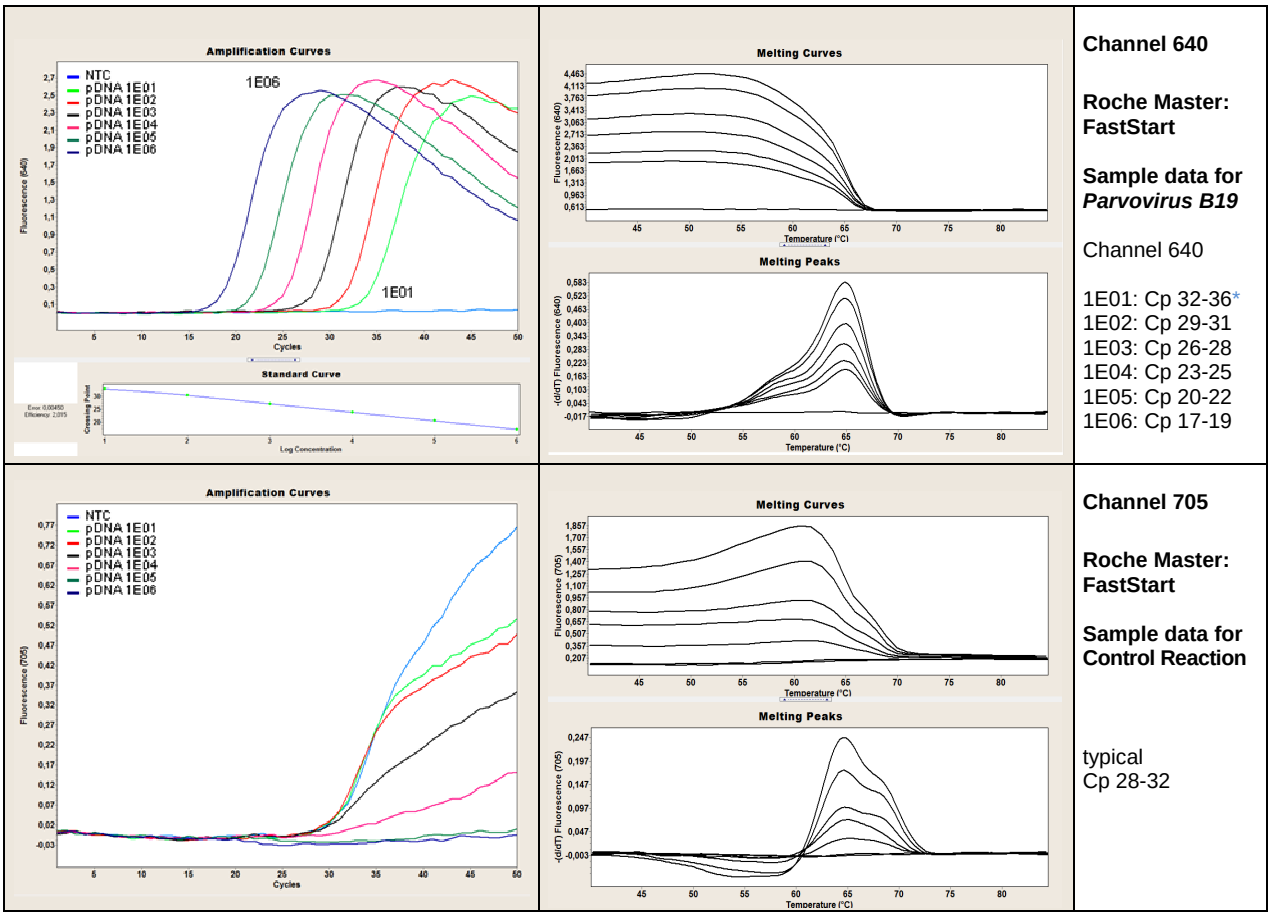
View *Parvovirus B19* data in channel 640, Quantification mode. The negative control must show no signal. For the identification of the PCR product view *Parvovirus B19* data in channel 640 Melting Curves mode (not relevant for detection).

For the Control Reaction view channel 705 data. The negative control and the low-concentrated *Parvovirus B19* DNA samples (10 to 1,000 copies) will show an amplification curve with a Cp at approximately cycle 28-32.

The provided standard row with concentrations in the range from 10^6 to 10^1 copies/rxn of target should have Cp values between cycles 17 and 36 (see figure 1).

For use in LightCycler® 1.x Instruments select channel F2 instead of channel 640, channel F3 instead of channel 705 for detection. We recommend to upgrade to software version 4.10 or higher.

7.3. Sample Data – Typical Results



Channel 640

Roche Master: FastStart

Sample data for *Parvovirus B19*

Channel 640

1E01: Cp 32-36*
1E02: Cp 29-31
1E03: Cp 26-28
1E04: Cp 23-25
1E05: Cp 20-22
1E06: Cp 17-19

Channel 705

Roche Master: FastStart

Sample data for Control Reaction

typical
Cp 28-32

Fig.1. LightCycler® 2.0 sample data for the *Parvovirus B19* detection system.

Upper panels: Left panel channel 640 quantification mode (Sec. Derivative Maximum) with amplification curves for *Parvovirus.* Right panel channel 640 melting analysis for *Parvovirus B19* (not relevant for detection).
Lower panels: Left: channel 705 quantification mode (Sec. Der. Maximum).Right: channel 705 melting peaks control reaction.

* **Note:** Fluorescence levels depend on instrument settings and may vary. The characteristics of the curve (shape and trend of fluorescence levels) should be similar to the curve in the manual. The fluorescent curves over cycles (quantification mode) must be smooth and not zigzag shaped. The Cp values will vary from instrument to instrument by up to 2 cycles, while the distance between two dilution steps should be relative constant (delta Cp). The Cp values described in this manual (chart text) have been obtained using the supplied standard row; the entire set of curves can be horizontal shifted.

7.4. Interpretation of Data

Sample 640 *Parvovirus B19*	**Sample 705** Control Reaction	**Channel 640** PositiveControl	Negative Control (NTC)	**Result (warnings)**
no amplification	detectable	amplification	negative	**Negative (not detectable)**
Cp < 38+	not relevant	amplification	negative	**Positive for *Parvovirus B19***
no amplification	not detectable	amplification	not relevant	PCR failure, repeat experiment
not relevant	not relevant	no amplification	not relevant	PCR failure, repeat experiment
not relevant	not relevant	not relevant	positive	Contamination, repeat experiment

Table 3. Typical analysis results (LightCycler® 2.0 Instrument, Roche Master: Fast Start)

+ The cut off value is a recommendation only - this value has to be defined by the user. Compare with the lot specific values for 10 copies as reported in the Certificate of Analysis (CoA). 1-2 cycles later than observed for 10 copies coresponds to ~5 copies.

8. LightCycler® 480 II Instrument and cobas z 480 Analyzer

8.1. Programming

The protocol consists of four program steps

- 1: Denaturation: sample denaturation and enzyme activation
- 2: Cycling: PCR-amplification of the target DNA
- 3: Melting: melting curve analysis for identification of the PCR product derived from the target DNA
- 4: Cooling: cooling the instrument

Detection Format (Filter Setting):
LightCycler® 480 II Instrument: 465-510, 498-640, 498-660
cobas z 480 Analyzer: 465-510, 498-645, 498-700

Program Step:	Denaturation	Cycling			Melting			Cooling
Parameter								
Analysis Mode	None	Quantification mode			Melting Curves mode			None
Cycles	1	50			1			1
Target [°C]	95	95	62	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:05	00:00:05	00:00:15	00:00:30	00:02:00	00:00:00	00:00:30
Ramp Rate [°C/s] **96**	4.4	4.4	2.2	4.4	4.4	1.5	-	1.5
Ramp Rate [°C/s] **384**	4.6	4.6	2.4	4.6	4.6	2.0	-	2.0
Sec Target [°C]	-	-	55	-	-	-	-	-
Step Size [°C]	-	-	0.5	-	-	-	-	-
Step Delay (Cycles)	-	-	1	-	-	-	-	-
Acquisition Mode	None	None	Single	None	None	None	Continuous	None
Acquisitions [per °C]	-	-	-	-	-	-	1	-

(Melting not relevant for detection) Table 4

8.2. Data Analysis

Note: cobas z 480 Analyzer signal levels are about 50% compared to LightCycler® 480 II results.

Switch the color compensation mode on. If this mode is not enabled run the color compensation program. Follow the instructions in the manual of the LightMix® Kit Color Compensation 530/640/690.

Perform data analysis, as described in the Instrument operator's manual.

We recommend using the Second Derivative Maximum method (Automated (F" max)). The cycle number of the Crossing Point (Cp) of each sample is calculated automatically. The Fit Points method is more-error prone due to the user's influence.

View *Parvovirus B19* data with Filter Combination 498-640 Quantification mode. The negative control must show no signal.

For the Control Reaction view Filter Combination 498-660 data, Quantification mode. The negative control and the low-concentrated DNA samples (10 to 1,000 copies) will show an amplification curve with a Cp at approximately cycle 27-30.

The provided standard row of target with concentrations in the range from 10^6 copies/rxn to 10^1 copies/rxn of *Parvovirus B19* should have Cp values between cycles 17 and 36 (see figure 2).

8.3. Sample Data – Typical Results

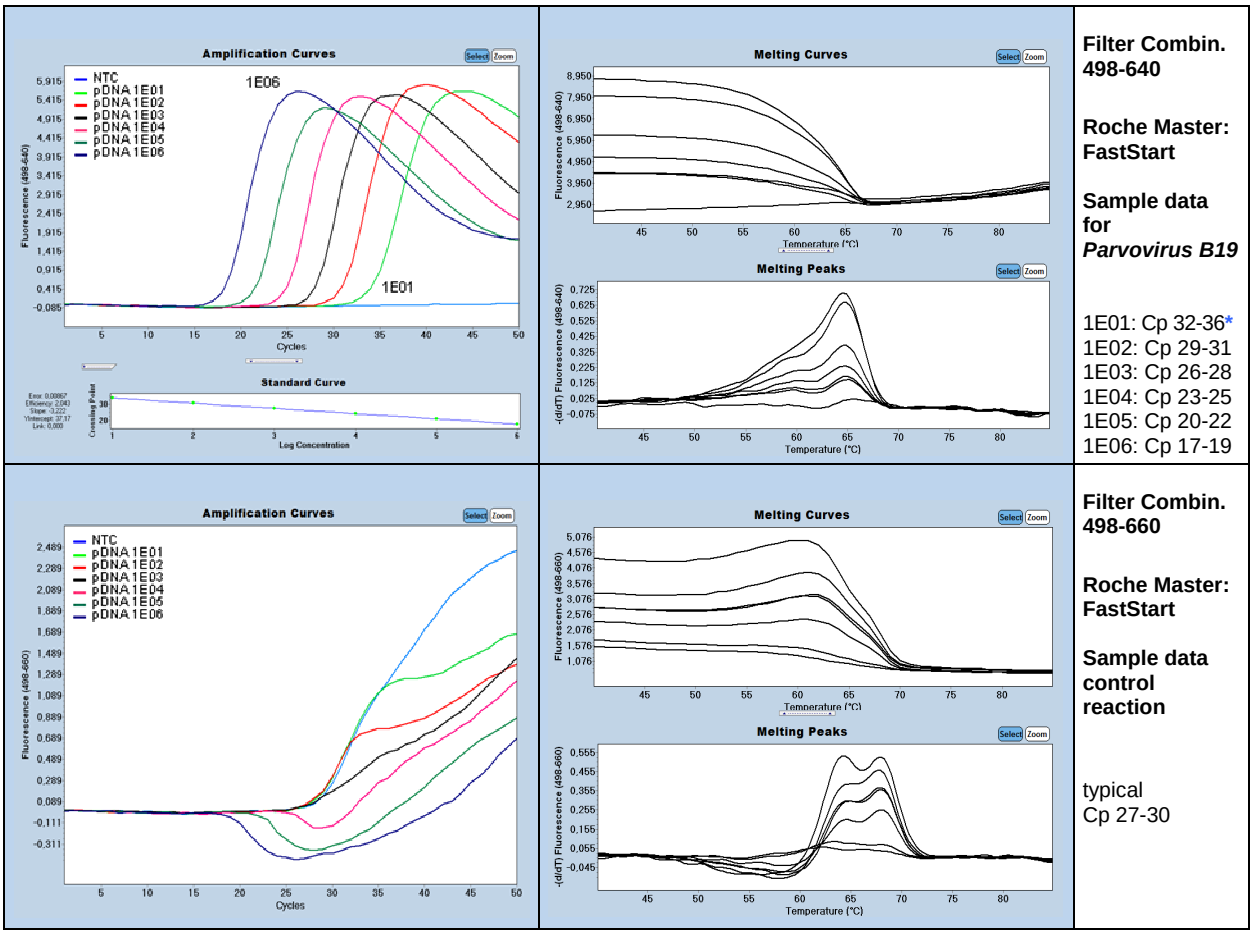


Fig.2. LightCycler® 480 II sample data for the *Parvovirus B19* detection system.

Upper panels: Left: Filter Comb. 498-640 quantification mode (Sec Der. Maximum) with amplification curves for *Parvovirus.* Right panel Filter Combination 498-640 melting analysis for *Parvovirus B19* (not relevant for detection).
Lower panels: Control Reaction. Left: Filter 498-660 quantification mode (Sec. Derivative Maximum). The negative curves in the amplification plot are a result of the 640 curves due to over-compensation (color compensation).
Right: Melting analysis (not relevant for detection).

* **Note:** Fluorescence levels depend on instrument settings and may vary. The characteristics of the curve (shape and trend of fluorescence levels) should be similar to the curve in the manual. The fluorescent curves over cycles (quantification mode) must be smooth and not zigzag shaped. The Cp values will vary from instrument to instrument by up to 2 cycles, while the distance between two dilution steps should be relative constant (delta Cp). The Cp values described in this manual (chart text) have been obtained using the supplied standard row; the entire set of curves can be horizontal shifted.

8.4. Interpretation of Data

Sample 640 *Parvovirus B19*	**Sample 660** Control Reaction	**Channel 640** PositiveControl	**Negative Control (NTC)**	**Result (warnings)**
no amplification	detectable	amplification	negative	**Negative (not detectable)**
Cp < 37+	not relevant	amplification	negative	**Positive for *Parvovirus B19***
no amplification	not detectable	amplification	not relevant	PCR failure, repeat experiment
not relevant	not relevant	no amplification	not relevant	PCR failure, repeat experiment
not relevant	not relevant	not relevant	positive	Contamination, repeat experiment

Table 5. Typical analysis results (LightCycler® 480 Instrument II, Roche Master: Fast Start)

+ The cut off value is a recommendation only - this value has to be defined by the user. Compare with the lot specific values for 10 copies as reported in the Certificate of Analysis (CoA). 1-2 cycles later than observed for 10 copies coresponds to ~5 copies.

9. Conversion Factor

The amount of virus per sample (Viral Load) is usually reported in copies / ml while PCR reports copies per reaction. The conversion factor between both depends on sample and extraction volumes. Extraction starts usually from less than one milliliter and PCR does not use the total extracted volume.

The viral load (VL) can be calculated using the following general formula:

VL [copies/ml] = MV (Measured Value) x EVF x SF

where:

VL = **Viral Load**
MV = **M**easured **V**alue [copy number per reaction]
EVF = **E**xtraction **V**olume **F**actor [Final extraction volume / PCR sample volume]
SF = **S**ample **F**actor [1,000 µl / extracted volume of clinical sample]

Example: Extracting 200 µl clinical sample results in a correction factor of 5. Using 5 µl from a total eluation volume of 100 µl results in a correction factor of x 20, resulting in a conversion factor of x100:

VL [copies/ml] = Measured **V**alue [copy number per reaction] **x 100**

10. References

[1] New LightCycler PCR for rapid and sensitive quantification of parvovirus B19 DNA guides therapeutic decision-making in relapsing infections. Harder et al.. JCM 39 (2001) 4413-4419

[2] Infection of the erythroid cell line, KU812Ep6 with human parvovirus B19 and its application to titration of B19 infectivity. Miyagawa et al., J.Virol.Methods 83 (1999)

11. Material Safety Data

According to OSHA 29CFR1910.1200, Australia [NOHSC:1005, 1008 (1999)] and the European Union Directives 67/548/EC and 1999/45/EC any products which not contain more than 1% of a component classified as hazardous or classified as carcinogenic do not require a MSDS.

Product is not hazardous, not toxic, not IATA-restricted. Product is not from human, animal or plant origin. Product contains synthetic oligonucleotide primers and probes.

11. Version History

Events require changes in procedures marked red, mod. sequences blue

V110210	Release version
V130813	Left primer changed to ensure to detect GT1-3 (fragment size 270 bp). MSDS and Conversion factor included.
V141010	IC changed to Extraction Control Extraction target changed to PhHV Improved performance for the capillary based instruments : Forward primer moved downstream; fragment shortened by 85 bp
V150707	Change to universal ECT target containing Lambda and PhHV DNA

Roche SAP order n° 06296866001