ParoReal Kit
Cryptosporidium parvum

Manual

For veterinary use only

REF DVEP00611, DVEP00613 Σ **100**

REF DVEP00651, DVEP00653 Σ **50**

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Explanation of symbols

LOT	Batch code		Use by
REF	Catalogue number		Manufactured by
Σ	Contains sufficient for <n> tests		Store at

1. Product description

ParoReal Kit *Cryptosporidium parvum* is a real-time PCR kit for detection of *Cryptosporidium parvum* DNA. This test was developed for the ABI PRISM® 7500 (Fast) instrument (Thermo Fisher Scientific), LightCycler® 480 (Roche) and for Mx3005P® (Agilent), but is also suitable for other real-time PCR instruments. This test allows the rapid and sensitive detection of DNA of *Cryptosporidium parvum* from samples purified from stool (e.g. with the QIAamp DNA Mini Kit).

ParoReal Kit *Cryptosporidium parvum* detects the GP60 gene of *Cryptosporidium parvum.* A probe-specific amplification-curve at 530 nm (FAM channel) indicates the amplification of *Cryptosporidium parvum* DNA.
An internal positive control system (IPC) for detection in VIC/HEX channel, (554 nm, order no. DVEP00611 or DVEP00651) or Cy5 channel (667 nm; order no. DVEP00613 or DVEP00653) excludes false-negative interpretation of results due to inhibition of real-time PCR (see 8. Interpretation of PCR-data).
When using PCR-platforms not validated by ingenetix, an evaluation of the multiplex-PCR is recommended. Please be aware that some PCR-platforms have to be calibrated with the corresponding dye before performing multiplex-PCR.

ViroReal®, BactoReal®, MycoReal and ParoReal Kits are optimized to run under the same thermal cycling conditions. RNA and DNA material can be analysed in one run.

2. Pathogen information

Cryptosporidium parvum is one of several protozoal species that cause cryptosporidiosis, a parasitic disease of the mammalian intestinal tract. It is a zoonotic and obligate intracellular parasite. Primary symptoms of *C. parvum* infection are acute, watery, and non-bloody diarrhea. *C. parvum* infection is of particular concern in immunocompromised patients. Extra-intestinal sites include the lung, liver and gall bladder where it causes respiratory cryptosporidosis, hepatitis and cholecystitis. Infection is caused by ingestion of sporulated oocysts transmitted by the faecal-oral route.

References:
Nurul Fariza Rossle and Baha Latif. Cryptosporidiosis as threatening health problem: A review. 2013. Asian Pac J Trop Biomed. 3(11): 916–924.

3. Principle of real-time PCR

A specific DNA sequence of the pathogen genome is amplified and the generated PCR-product is detected by an oligonucleotide-probe labelled with a fluorescent dye. This technology allows for a sequence-specific detection of PCR amplificates.

4. General Precautions

The user should always pay attention to the following:

- Always include a negative control per PCR-run (water instead of sample).
- Optional: for valid interpretation of results, a negative control should be included during DNA-extraction (for example extraction of water instead of sample material), in order to exclude false-positive results due to contamination with *Cryptosporidium parvum* DNA during extraction.
- Be careful when handling the positive control.
- Store and extract positive material (specimens, controls and amplicons) separately from all other reagents and add it to the reaction mix in a spatially separated workspace.
- Periodically decontaminate benches and devices.
- Use sterile pipette tips with filters.
- Thaw all components thoroughly at room temperature before starting an assay. When thawed, mix the components and centrifuge briefly.
- For MSDS, see www.ingenetix.com.

5. Contents of the Kit

5.1. ParoReal Kit *Cryptosporidium parvum* order no. DVEP00611 or DVEP00651

Labelling	Content	Amount		Storage
		DVEP00611	DVEP00651	
Cryptosporidium parvum Assay Mix (green cap)	Primer and probe (FAM) for detection of *C. parvum*	2 x 50 µl	1 x 50 µl	-20°C
CR-1 Assay Mix (yellow cap)	Primer, probe (VIC/HEX) and target for detection of IPC	2 x 50 µl	1 x 50 µl	-20°C
Cryptosporidium parvum Positive Control (red cap)	Control-DNA (approx. 10,000 target copies/µl)	1 x 25 µl	1 x 25 µl	-20°C
DNA Reaction Mix (white cap)#	Reaction Mix	2 x 500 µl	1 x 500 µl	**-20°C until first use, then at +4°C**
Water (blue cap)	Water	1 x 1000 µl	1 x 1000 µl	-20°C to +4°C

#DNA Reaction Mix contains uracil-N glycosylase (UNG)

5.2. ParoReal Kit *Cryptosporidium parvum* order no. DVEP00613 or DVEP00653

Labelling	Content	Amount		Storage
		DVEP00613	DVEP00653	
Cryptosporidium parvum Assay Mix (green cap)	Primer and probe (FAM) for detection of *C. parvum*	2 x 50 µl	1 x 50 µl	-20°C
CR-3 Assay Mix (yellow cap)	Primer, probe (Cy5) and target for detection of IPC	2 x 50 µl	1 x 50 µl	-20°C
Cryptosporidium parvum Positive Control (red cap)	Control-DNA (approx. 10,000 target copies/µl)	1 x 25 µl	1 x 25 µl	-20°C
DNA Reaction Mix (white cap)#	Reaction Mix	2 x 500 µl	1 x 500 µl	**-20°C until first use, then at +4°C**
Water (blue cap)	Water	1 x 1000 µl	1 x 1000 µl	-20°C to +4°C

#DNA Reaction Mix contains uracil-N glycosylase (UNG)

The components of BactoReal® Kit *Cryptosporidium parvum* are stable until the expiry date stated on the label. Repeated thawing and freezing should be avoided. Please protect kit components from light.

6. Additionally required materials and devices

- Reagents and devices for DNA-extraction
- PCR-grade water
- Disposable powder-free gloves
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Vortex mixer
- Desktop centrifuge with rotor for 2 ml reaction tubes
- Real-time PCR instrument which is able to detect and differentiate fluorescence in FAM and VIC/HEX or Cy5 channel
- Appropriate 96 well reaction plates or reaction tubes with corresponding (optical) closing material

7. Preparation of real-time PCR

Please make sure that at least one negative control (water, blue cap), as well as one positive control (red cap) and one extraction negative control (optional, recommended) are included per PCR run.
Ingenetix highly recommends performing PCR analyses in duplicates, which increases the probability of detection of the pathogen and facilitates interpretation of results.

7.1. Pipetting scheme

		Per sample
Preparation of Master Mix (mix well)	Water*	3.0 µl
	DNA Reaction Mix (2x)	10.0 µl
	Cryptosporidium parvum Assay Mix	1.0 µl
	CR Assay Mix	1.0 µl
	Total volume Master Mix	**15.0 µl**
Preparation of PCR assay	Master mix	15.0 µl
	Sample*	5.0 µl
	Total volume	**20.0 µl**

*1-8 µl of the sample can be used. When using a volume other than 5 µl, the volume of H_2O has to be changed accordingly.

Positive Control: As positive control use 1 µl of the *Cryptosporidium parvum* Positive Control + 4 µl H_2O. Optional: a 1:10 dilution of the positive control can be used and defined as second standard value (approx. 1000 target copies/µl).

7.2. Programming of the temperature profile

Please find further information on programming the real-time PCR instrument in the respective operator's manual. Please be aware that some PCR-platforms have to be calibrated with the corresponding dye before performing multiplex-PCR.

Select dyes: FAM-TAMRA for detection of *Cryptosporidium parvum*
Cy5-NONE (CR-3 Assay Mix) **or** VIC-TAMRA (CR-1 Assay Mix) for detection of IPC

Select reference dye (passive reference): ROX

Sample Volume: 20 µl

Temperature Profile:

Program 1	Program 2	Program 3
Cycles: 1 Analysis: None	Cycles: 1 Analysis: None	Cycles: 45 Analysis: Quantification Acquisition at 60°
50°C 2 min*	95°C 20 sec	95°C 5 sec 60°C 1 min

<u>For ABI PRISM® 7500:</u>
Ramp speed: Without "fast cycling" parameter

<u>For LightCycler® 480 instrument:</u>
Detection format: 2 Color Hydrolysis Probe (dyes see above)

***Note:** If viral RNA should be also detected in the same PCR run, program 1 has to be prolonged to 15 min at 50°C. This temperature profile can be used for all ViroReal®, BactoReal®, MycoReal and ParoReal kits for the detection of DNA or RNA.

8. Interpretation of PCR-data

Examples for interpretation of positive reactions are shown in the amplification plots below.

For a valid interpretation, the following criteria must be fulfilled:

	Ct/Cp (FAM channel) *C. parvum* target	Ct/Cp IPC target	Interpretation
Negative control	Negative	36.0 ± 2	Valid
Positive control (undiluted, 1 µl/PCR)	28.0-31.0	36.0 ± 2	Valid
Extraction negative control (optional)	Negative	36.0 ± 2	Valid
Negative sample	Negative	36.0 ± 2	Valid
Positive sample	Positive	Positive/Negative	Valid

For analysis of PCR data please proceed as follows:
For analysis of PCR results gained with ParoReal Kit *Cryptosporidium parvum* please select fluorescence display options FAM channel for the *C. parvum* target and VIC/HEX channel (order no. DVEP00611, DVEP00651) or Cy5 channel (order no. DVEP00613, DVEP00653) for the internal positive control target (IPC). Samples with a positive Cp or Ct-value are considered positive. Please also check amplification-curves manually.

8.1. Signal in FAM channel:
→ DNA of *C. parvum* was amplified. The sample has to be interpreted as positive.
Cryptosporidium parvum DNA can lead to a reduced or absent fluorescence signal of the IPC.

8.2. No signal in FAM channel but signal of the IPC:
→ No *C. parvum* DNA is detectable in the sample. The sample has to be interpreted as negative.
The positive signal of the IPC assay excludes a putative PCR inhibition.

8.3. No signals in FAM channel and no signal with the IPC:
→ No interpretation statement can be made.
Information about possible sources of error and their solution can be found in 9. Troubleshooting.

9. Troubleshooting

9.1. No *C. parvum* specific signal with positive control:
- Incorrect programming of the temperature profile of the real-time PCR instrument.
  → Compare the temperature profile with the protocol (see 7. Preparation of real-time PCR).
- Incorrect configuration of the PCR reaction.
  → Check your work steps (see 7. Preparation of real-time PCR) and repeat the PCR, if necessary.

9.2. No signal with IPC and no *C. parvum* specific signal with sample:
- The PCR reaction was inhibited. No interpretation can be made.
  → Make sure that you use a recommended method for DNA isolation and stick closely to the manufacturer's instructions.
  → If no operating mistakes during extractions can be retraced, it is recommended to repeat the PCR with lower amounts of DNA-eluate (1/5 or 1/10 of sample volume + the adequate volume of H_2O).
- Incorrect PCR conditions.
  → Check the PCR conditions and repeat the PCR, if necessary.

9.3. *Cryptosporidium parvum* specific signal with negative control:
- A contamination occurred during preparation of the PCR.
  → Repeat the PCR with new reagents in replicates.
  → Strictly pipette the positive controls at last.
  → Make sure that work space and instruments are decontaminated at regular intervals.

9.4. *Cryptosporidium parvum* specific signal with negative control of extraction (optional):
- A contamination occurred during extraction.
  → Repeat the extraction and PCR using new reagents.
  → Make sure that work space and instruments are decontaminated at regular intervals.

10. Specifications and performance evaluation

ParoReal Kit *Cryptosporidium parvum* was evaluated with the ABI PRISM® 7500 (Fast) instrument (Thermo Fisher Scientific). For further validation data please contact ingenetix GmbH.

10.1. Analytical sensitivity and linearity

ParoReal Kit *Cryptosporidium parvum* was tested with a 10-fold dilution series of a plasmid containing a fragment of *Cryptosporidium parvum* DNA. At least 10 target copies/PCR reaction could be detected.
The assay shows **linearity** over the range of 100 to 1,000,000 target copies/reaction with a slope of -3.6 and a R_2 of > 0.997 as shown in Figure 1.

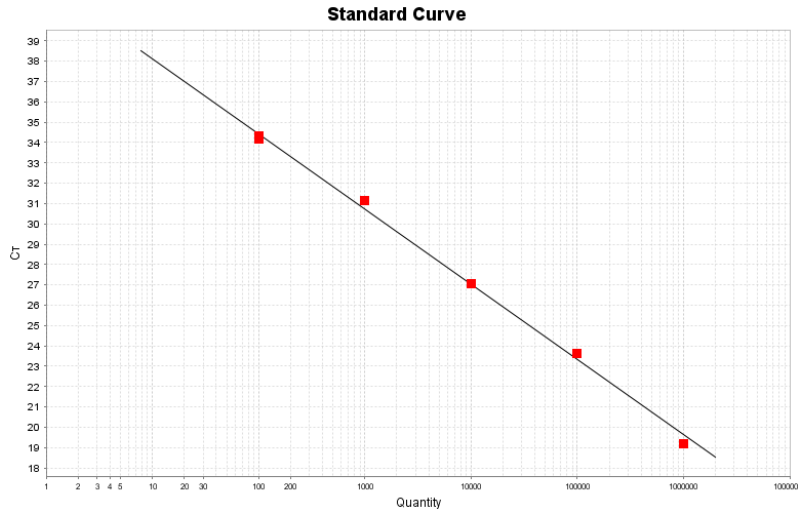


Figure 1 Ten-fold dilution series of a *Cryptosporidium parvum* DNA standard plotted against CT

10.2. Analytical specificity

The specificity is ensured by the selection of highly specific primers and probes. The primers and probes were checked for possible homologies to currently published sequences by sequence comparison analyses. This also validated the detection of so far known *Cryptosporidium parvum* strains. The kit is specific for *Cryptosporidium parvum* and *Cryptosporidium felis.*
ParoReal Kit *Cryptosporidium parvum* was tested on three *Cryptosporidium parvum* isolates.

10.3. Kit performance

Performance of ParoReal Kit *Cryptosporidium parvum* with an Applied Biosystems® 7500 Fast Real-time PCR System (Thermo Fisher Scientific) is shown in Figure 2.

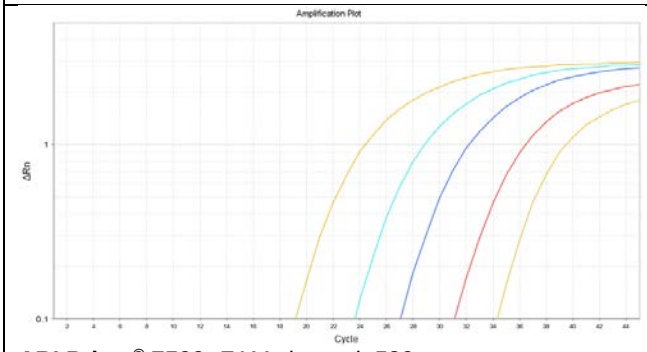
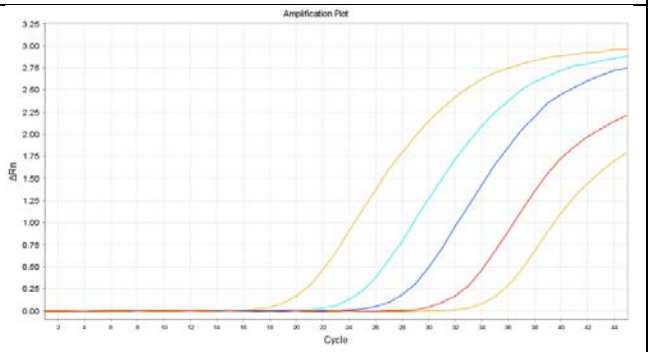
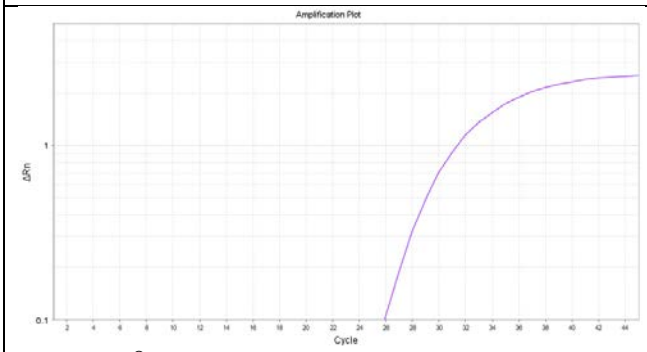
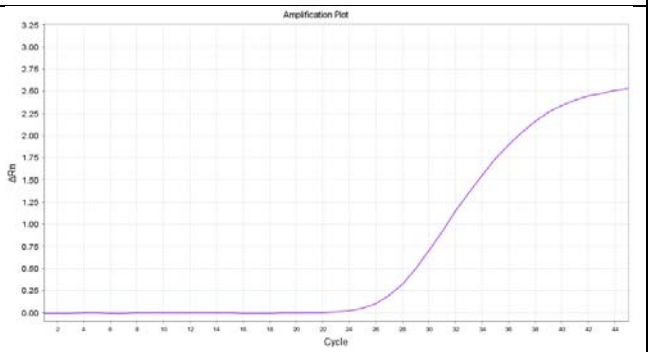
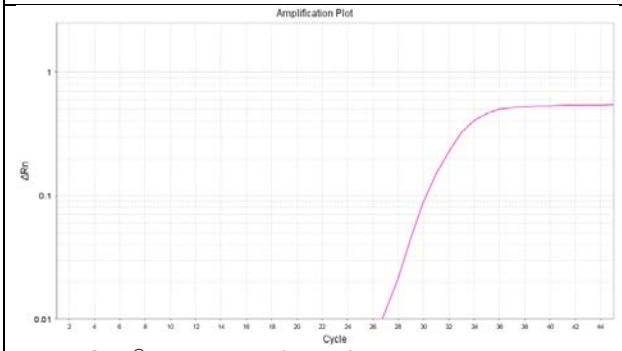
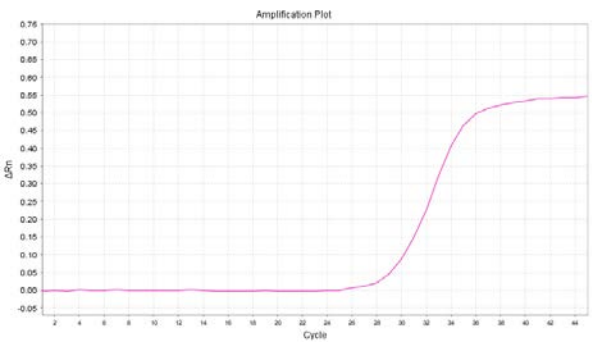
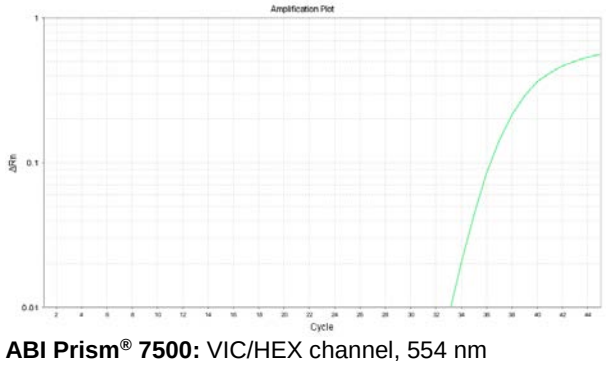
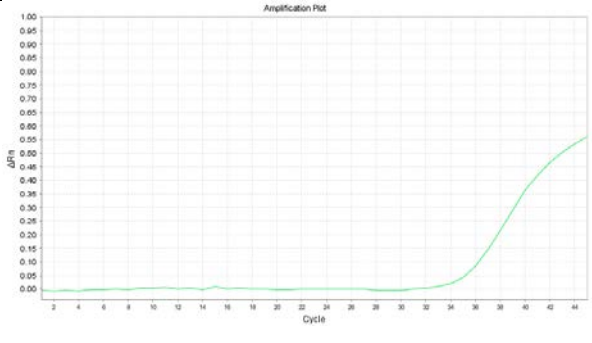
Logarithmic view	Linear view
	
ABI Prism® 7500: FAM channel, 530 nm 1:10 serial dilution of a *Cryptosporidium parvum* DNA target	
	
ABI Prism® 7500: FAM channel, 530 nm *Cryptosporidium parvum* positive control	
	
ABI Prism® 7500: Cy5 channel, 667 nm Internal DNA positive control	
	
ABI Prism® 7500: VIC/HEX channel, 554 nm Internal DNA positive control	

Figure 2 Performance of ParoReal Kit *Cryptosporidium parvum*