ParoReal Kit *Acanthamoeba* T4

Instructions for Use

CE

IVD **For *in vitro* diagnostic use**

REF **DHUP00153**

Σ **50 reactions**

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Index

Explanation of symbols

LOT	Batch code		Use by date
REF	Catalogue number		Manufacturer
Σ	Contains sufficient for <n> tests		Temperature limit (Store at)
CE	This product fulfils the requirements of the European Directive 98/79 EC for *in vitro* diagnostic medical devices	IVD	In vitro diagnostic medical device
	Consult instructions for use	UDI	Unique device identifier
	Keep away from sunlight	QTY	Contents
	Corrosion, GHS05		Exclamation mark, GHS07

1. Intended purpose

ParoReal Kit *Acanthamoeba* T4 is a non-automated IVD test, based on real-time PCR, for the qualitative detection of DNA (18S rRNA gene) of *Acanthamoeba* species of genotype T4 *(A. castellani, A. lugdunensis, A. mauritaniensis, A. polyphaga, A. rhysodes, A. royreba).* T4 genotype is the most prevalent (approx. 86%) *Acanthamoeba* genotype causing keratitis worldwide. The test does not detect other *Acanthamoeba* genotypes (T3, T15, T11, and T5) also causing keratitis.

Proper specimens are DNA extracts from human clinical specimens associated with keratitis (ocular swabs, corneal biopsies, ocular punctates, corneal scrapings) as well as contact lenses and contact lens solution.

This test is suitable for patients of all ages with suspected infection with *Acanthamoeba* genotype T4 (causative agent of *Acanthamoeba* keratitis, AK) and is intended as an aid in the diagnosis of infection with this pathogen in combination with patient history and additional clinical information.

The test is for professional use only and the use is limited to qualified personnel instructed in real-time PCR and *in vitro* diagnostic procedures.

2. Product description

ParoReal Kit *Acanthamoeba* T4 is a real-time PCR test and detects the 18S rRNA gene of *Acanthamoeba* species of genotype T4, which cause approx. 86% of *Acanthamoeba* keratitis worldwide (Diehl et al., 2021). The 18S rRNA gene of *Acanthamoeba* is a multicopy gene. The number of rRNA repeats In *Acanthamoeba* cells is 24 per haploid genome. However, because *Acanthamoeba* is polyploid, each cell contains approximately 600 rRNA genes (Yang et al., 1994).

A probe-specific amplification-curve in the FAM channel (530 nm) indicates the amplification of *Acanthamoeba* specific DNA. An internal DNA positive control (DNA IPC) is detected in Cy5 channel and is used as DNA extraction as well as real-time PCR inhibition control. The target for the DNA IPC (artificial target DNA) is extracted with the sample.

This test has been validated with the ABI® 7500 instrument (Thermo Fisher Scientific, fast cycle parameters are not supported) and was also tested with a LightCycler® 480 II (Roche Diagnostics), QuantStudio™ 7 real-time PCR system (Thermo Fisher Scientific), and Mic instrument (bio molecular systems).
The test is also compatible with other real-time PCR instruments which detect and differentiate fluorescence in FAM and Cy5 channel (e.g., QuantStudio™ 7 real-time PCR system (Thermo Fisher Scientific), qTOWER^{3}G (Analytik Jena), cobas z 480 Analyzer (Roche)).
When using PCR-platforms not tested by ingenetix, an evaluation of the multiplex-PCR shall be done. Keep in mind that some PCR-platforms first have to be calibrated with the corresponding dye before performing multiplex-PCR.

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

3. Pathogen information

The genus *Acanthamoeba* is primarily a free-living protozoan in natural habitats, but also causative agent of human and animal disease. *Acanthamoeba* serves as host for a variety of pathogenic bacteria such as *Mycobacterium avium*. Infestation with *Acanthamoeba* is associated with potentially sight-threatening contact lens-related keratitis, serious infections of other organs and fatal granulomatous amoebic encephalitis. More than 20 species of *Acanthamoeba* are known, which can be classified into three morphologic groups (Group I, II and III) and 22 genotypes (T1-T22). Some species ((*A. castellanii* (T4), *A. lugdunensis* (T4), *A. polyphaga* (T4), *A. rhysodes* (T4), *A. quina* (T4), *A. palestinensis* (T2), *A. griffinii* (T3), *A. lenticulata* (T5), *A. astronyxis* (T7), *A. culbertsoni* (T10), *A. hatchetti* (T11), *A. healyii* (T12), *A. byersi* (T18), *A. divionensis*) have been recently associated with human disease. Genotype T4 has been considered the most important genotype in both ocular and CNS infections. According to literature, T4 genotype is the most prevalent *Acanthamoeba* genotype causing keratitis worldwide (approx. 86%) (Diehl et al., 2021).

4. Principle of real-time PCR

The test is based on multiplex real-time PCR by 5´-nuclease-assay technology. Specific DNA sequences are amplified and the generated PCR-products are detected by oligonucleotide-probes labelled with fluorescent dyes (FAM und C5). This allows a sequence-specific detection of PCR amplificates.

During PCR, primers are extended by *Taq* polymerase and probes hybridized to the target are cleaved by the 5'-exonuclease activity of *Taq* polymerase. According to the accumulation of PCR product, the fluorescence of the probe increases with each PCR cycle. The change in fluorescence of the different dyes is recorded cycle by cycle in the real-time PCR instrument in the closed reaction tube at different fluorescence wavelengths.
The Ct value (Ct = Cycle threshold, Cq = Quantification cycle, Cp = Crossing point) describes the cycle at which the fluorescence first rises significantly above the background fluorescence.

5. Contents of the kit, stability and storage

Labelling	Content	Amount	Storage
Acanthamoeba T4 Assay Mix (green cap)	Primers and probes for detection of - *Acanthamoeba* (FAM) - DNA IPC (Cy5)	1 x 50 µl	-25 to -15 °C
DNA IPC Target (orange cap)	Target for DNA IPC (internal DNA positive control system)	1 x 200 µl	-25 to -15 °C
Acanthamoeba T4 Positive Control (red cap)	DNA positive control (approx. 1,000 target copies/µl)	1 x 300 µl	-25 to -15 °C
DNA Reaction Mix (white cap)	DNA amplification mix	1 x 500 µl	**-25 to -15°C, after first opening 2 to 8 °C**
Nuclease-free water (blue cap)	Nuclease-free water	1 x 1000 µl	-25 to -15 °C

DNA Reaction Mix
The Master Mix provided with the kit has been designed for reliable, high-sensitivity real-time PCR. The Master Mix contains a highly purified Taq Polymerase for rapid hot-start PCR, dNTPs with dUTP and Uracil-N glycosylase (UNG) to eliminate amplicon carryover, ROX™ dye (passive reference) and buffer components – additives optimized to handle RT-PCR inhibitors.

Delivery and Storage
Shipment is at approx. -20 °C or +4 °C.
The unopened kit is stable at -25 to 15 °C until the expiry date stated on the label. After first opening, the in-use stability is a maximum of 12 months but no longer than the expiry date indicated on the label. Store kit protected from light.

Quality Control Release Testing
In accordance with the ISO 13485-certified Quality Management System of ingenetix, each lot is tested against predetermined specifications to ensure consistent product quality.
Quality control is performed with a plasmid containing parts of the pathogen DNA. The DNA concentration of the plasmid was determined at an OD of 260 nm and the copy number was calculated.

6. Additionally required materials and devices

- Reagents and devices for DNA-extraction appropriate for the listed sample material (see 9. Preparation of the samples)
- Optional: Nuclease-free water for dilution of DNA IPC Target
- Powder-free disposable gloves
- Pipettes (adjustable)
- Filter pipette tips
- Real-time PCR instrument which is able to detect and differentiate fluorescence in FAM and Cy5 channel
- Appropriate 96 well reaction plates or reaction tubes with corresponding (optical) closing material

7. Precautions and safety information

- For *in vitro* diagnostic use. The use of this kit is limited to qualified personnel instructed in real-time PCR and *in vitro* diagnostic procedures.
- Transportation of clinical specimens must comply with local regulations for the transport of etiologic agents.
- Improper collection, transport or storage of specimens may hinder the ability of the assay to detect the target sequences.
- The real-time PCR instrument should be serviced and cleaned regularly.
- Clean benches and devices periodically.
- Use sterile filter pipette tips and powder-free disposable gloves.
- Specimens should be handled as if infectious in accordance with safe laboratory procedures. Wear protective powder-free disposable gloves when handling kit reagents and specimens.
- Use separate areas for specimen preparation, reagent preparation and amplification. Supplies and equipment must be dedicated to each of these separate areas and ensure workflow in the laboratory from pre- to post-PCR.
- Be careful when handling samples and positive control to avoid cross contamination. Change gloves after handling of samples or positive control.
- Store positive or potentially positive material separately from reagents.
- Prevent contamination of work equipment and reagents with DNA/RNA, nuclease or amplification products by good laboratory practice.
- Quality of DNA has a profound impact on the test performance. Ensure that the used DNA extraction system is compatible with real-time PCR technology.
- For a valid interpretation of results, a negative control must be included during DNA-extraction (e.g., extraction of water instead of sample material) and tested per PCR-run, in order to exclude false-positive results due to contamination with pathogen DNA during extraction.
- Optional: include a negative control of PCR per PCR-run (nuclease-free water instead of sample, NTC).
- Do not mix reagents of different kits and lots and check expiry date of the kits.
- Use established laboratory practices according to your local safety regulations for discarding specimens, reagents and waste disposal.
- **Caution:** DNA IPC Target is stored in RNA/DNA stabilizer which contains DTT/Guanidinium thiocyanate/Triton X-100 (see MSDS, www.ingenetix.com).

8. Limitations

- Reliable results with this test are only achieved by appropriate specimen collection, transport and storage, as well as an appropriate DNA extraction procedure.
- DNA extraction and *Acanthamoeba* detection have been validated for clinical ocular specimen material and contact lens material with this kit.
- A negative test result does not exclude the possibility of *Acanthamoeba* infection, because test results may be affected by improper specimen collection, technical error, specimen mix-up or pathogen quantities below the assay sensitivity. The presence of PCR inhibitors may lead to invalid results.
- For this kit highly specific primers and probes have been selected. However, false-negative or less sensitive results might be obtained due to sequence heterogeneity within the target region of yet unknown clinical subtypes of genotype T4. The test detects *Acanthamoeba* species of genotype T4. Other *Acanthamoeba* genotypes (T3, T15, T11, and T5), which also cause keratitis, are not detected.
- Results should be interpreted in context of other clinical and laboratory findings.

9. Preparation of samples

ParoReal Kit *Acanthamoeba* T4 is suitable for analysis of DNA extracts of samples from the human eye (ocular swabs, corneal biopsies, ocular punctates, corneal scrapings) as well as contact lenses and contact lens solution.

Sample collection and storage:

- Samples from the respiratory tract can be stored in microcentrifuge tubes.
  Swabs can be collected with swab material proper for PCR (e.g. sterile polyester or rayon swabs with aluminium or plastic shaft) and put into 1 ml isotonic saline solution (NaCl 0.9%, not provided).
  It is recommended to process samples immediately after collection. Store samples at 2-8 °C for no longer than 48 hours or freeze at -20/-80 °C.

Purified DNA should be stored at -25 to -15 °C.

Extract samples with a DNA extraction system compatible with real-time PCR technology and appropriate for the sample material.

- For manual extraction recommended: - QIAamp DNA Mini Kit (Qiagen)
  - High Pure PCR Template Preparation Kit (Roche)

When using extraction methods not recommended by ingenetix, an evaluation of the extraction method must be performed.

Always include an extraction negative control during DNA-extraction (e.g., extraction of water instead of sample material).

Quality control for DNA extraction and PCR inhibition

The DNA IPC system (internal DNA positive control) is used as a control for DNA extraction, identifies possible PCR inhibition and confirms the integrity of kit reagents. An artificial target DNA (IPC Target, approx. $6x10^5$ copies/µl) is added during extraction and detected in Cy5 channel.
For control of DNA extraction, the undiluted IPC Target has to be added directly to the lysis buffer (or added to the sample after the lysis buffer has been added to the sample):

→ Per sample, add 1 µl DNA IPC Target (orange cap)

Note: The undiluted DNA IPC Target shall not be added to sample material in the absence of lysis buffer, as degradation may occur. It must be added to the lysis buffer.
If the DNA IPC Target has not been added during extraction, it can be added at a later stage to the PCR master mix as quality control for the PCR reaction. In this case, dilute the DNA IPC Target 1:100 with nuclease-free water and add 1 µl of the dilution/PCR reaction.
Caution: The IPC Target shall not be added to the master mix undiluted.

10. Preparation of real-time PCR

- Include one positive control (red cap), one extraction negative control and optional one negative control (nuclease-free water) per PCR run.
- It is generally recommended to analyse samples in duplicates, which increases the probability of pathogen detection and facilitates interpretation of results.
- Thaw DNA samples on ice.
- Thaw kit components completely at room temperature. When thawed, mix components carefully, centrifuge briefly with low speed. Mix the DNA Reaction Mix gently to ensure homogeneity of solution.
- **Positive Control**
  → Use 9 µl of Positive Control (red cap). Always, pipette positive control at last.

10.1. Pipetting scheme

		Per sample
Preparation of Master Mix (mix well)	DNA Reaction Mix	10.0 µl
	Acanthamoeba T4 Assay Mix	1.0 µl
	Total volume Master Mix	**11.0 µl**
Preparation of PCR	Master Mix	11.0 µl
	DNA-Sample*	9.0 µl
	Total volume	**20.0 µl**

*1-9 µl of the sample can be used. For ≠ 9 µl sample, the volume must be adjusted with nuclease-free water.

→ **If DNA IPC Target was not added during extraction:** Freshly dilute the DNA IPC Target (orange cap) 1:100 with nuclease-free water and add 1 µl per sample directly to the master mix. In this case, the IPC is used for quality control of the PCR reaction. Only 8 µl of DNA sample can be analyzed.
Caution: The use of more than 1 µl diluted DNA IPC Target per reaction causes inhibition of the real-time PCR reaction.

- Prepare the Master Mix according to the number of samples, calculating an additional volume of approx. 10% to account for pipetting loss.
- Pipette 11 µl of the prepared Master Mix per sample into the well of the optical reaction plate.
- Then add 9 µl of the extracted sample or controls. Pipette the positive control at last.
- Seal the plate with a suitable optical sealing material.
- Vortex the sealed plate for 1-2 seconds and briefly centrifuge the plate.

10.2. Programming of temperature profile

Further information on programming the real-time PCR instrument can be found in the respective operator's manual. Keep in mind that some PCR-platforms have to be calibrated with the corresponding dye before performing a multiplex-PCR.

Temperature Profile:

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

For ABI® 7500, QuantStudio™ 5/6/7:
Ramp speed: "Standard", without "fast cycling"

[1]The previous temperature profile with 20 sec in program 2 can still be used.

[2]**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 ingenetix BactoReal®, ViroReal®, MycoReal®, ParoReal and PanReal kits for the detection of DNA or RNA.

Select detection channels:

FAM-TAMRA: Detection of *Acanthamoeba*
Cy5-NONE: Detection of IPC
Passive reference dye, if needed (depends on device): ROX (e.g., ABI® 7500, QuantStudio™ 5/6/7, Mx3005P®)

For MIC Instrument (bio molecular systems):
FAM: Green
Cy5: Red
No ROX as passive refence dye needed

For cobas z 480 Analyzer (Roche):
FAM: Excitation at 465 nm, Emission at 510 nm
Cy5: Excitation at 610 nm, Emission at 670 nm
Detection format: 2 Color Hydrolysis Probe, no ROX as passive reference dye needed

For LightCycler® 480 II (Roche):
FAM: Excitation at 465 nm, Emission at 510 nm
Cy5: Excitation at 618 nm, Emission at 660 nm
Detection format: 2 Color Hydrolysis Probe, no ROX as passive reference dye needed

11. Interpretation of PCR-data

For the analysis of the PCR results, select the fluorescence display options FAM channel for the pathogen target and Cy5 channel for the DNA IPC Target. Please note that some PCR platforms require a color compensation for a multiplex PCR with FAM and Cy5.
Samples with Cq-values < 45 in the fluorescence channel for the pathogen are considered positive (quantification cycle (Cq) = cycle threshold (Ct) = crossing point (Cp)).
Samples without amplification curves (no Cq values, undetermined) are regarded as negative. No DNA was detected in these samples because there is no infection with *Acanthamoeba* of genotype T4 or the pathogen DNA concentration is below the detection limit of the test.
IMPORTANT: Please, also check amplification curves, not only Cq-values. Samples should be inspected both in logarithmic (Roche instrument: Abs Quant/Fit Points) and linear scale view and compared with the negative control. Adjust the Threshold (noise band), if necessary. After you have saved the new settings, export the data. For the cobas z 480 Analyzer, export tables per dye.

Table 1 shows the criteria for valid positive and negative controls. Table 2 shows interpretation of data with clinical samples.

11.1. Controls

Table 1 Criteria for valid positive and negative controls, IPC Target was added during extraction

	Cq FAM channel *Acanthamoeba* target	Cq Cy5 channel DNA IPC target[1]	Interpretation	Action
Positive control	27-30	Negative	Valid	-
Positive control	Negative	Negative	Invalid	See 12.1
Positive control	27-30	Positive	Invalid	See 12.4
Extraction negative control	Negative	27-30	Valid	-
Extraction negative control	Negative	Negative	Invalid	See 12.1
Extraction negative control	Positive	27-30	Invalid	See 12.3
Negative control[2]	Negative	Negative	Valid	-
Negative control[2]	Positive	Negative	Invalid	See 12.2
Negative control[2]	Negative	Positive	Invalid	See 12.4

[1] If the DNA IPC target was added directly to the master mix, all samples in the Cy5 channel must be positive.
[2] Optional

Assessment of clinical specimen test results should be performed after the positive and negative controls have been examined and determined to be valid. If results of controls are not valid, no interpretation of results with clinical samples is possible.

11.2. Clinical samples

Samples with positive Cq-values are considered positive, see Table 2.

Table 2 Interpretation of data with clinical samples

	Cq FAM channel *Acanthamoeba* target	Cq Cy5 channel DNA IPC target	Interpretation	Action
Clinical sample	Negative	27-30[1]	Negative	-
Clinical sample	Positive	Positive/Negative[2]	Positive	-
Clinical sample	Negative	Negative	Invalid	See 12.5

[1)] A positive signal of the DNA IPC excludes potential PCR inhibition. However, IPC Cq-values should show comparable results among samples. A shift of Cq- excludes PCR inhibition. However, IPC Cq-values should show comparable results among samples. A shift of Cq- values can indicate a partial inhibition of PCR.
[2)] High pathogen load in the sample can lead to a reduced or absent fluorescence signal of the DNA IPC.

In case of invalid data, analysis has to be repeated with the remaining or newly extracted DNA sample (see 12. Troubleshooting).

12. Troubleshooting

12.1. No pathogen specific signal with positive control and with IPC (FAM and Cy5 channel)

- Incorrect programming of the temperature profile or incorrect setting of detection channels on the real-time PCR instrument.
  → Compare temperature profile and setting of detection channels with details specified in the protocol
- Incorrect configuration of PCR reaction.
  → Check your pipetting steps with the pipetting scheme and repeat PCR, if necessary.
  → The DNA may have been degraded.
- The DNA IPC Target was added undiluted directly to the master mix and had not been freshly diluted 1:100. The PCR reaction is therefore inhibited.
  → Freshly dilute DNA IPC Target 1:100 and repeat PCR.
- No Positive Control was added.
  → Repeat PCR in case all clinical samples are negative.
- For control of real-time PCR only: 1 µl of freshly 1:100 diluted DNA IPC Target has to be added to the master mix. If the DNA IPC Target has been forgotten to be added:
  → Freshly dilute DNA IPC Target and repeat PCR.
- For control of DNA extraction and PCR inhibition, the undiluted DNA IPC Target must be added during extraction to the lysis buffer. If the DNA IPC Target was missed to be added:
  → Repeat DNA extraction.

12.2. Pathogen signal in FAM channel with negative control

- A contamination occurred during preparation of PCR.
  → Repeat PCR with new reagents in replicates.
  → Strictly pipette positive control at last.
  → Make sure that workspace and instruments are cleaned at regular intervals.

12.3. Pathogen signal (FAM channel) with extraction negative control

- A contamination occurred during extraction.
  → Repeat DNA extraction and PCR using new reagents.
  → Make sure that workspace and instruments are cleaned at regular intervals.

12.4. IPC specific signal (Cy5 channel) with negative control and positive control

- The DNA IPC Target has been added to lysis buffer during extraction, but there is IPC specific signal with negative control and positive control: Contamination with the DNA IPC Target.
  → Make sure that workspace and instruments are cleaned at regular intervals.

12.5. No signal with IPC and no pathogen specific signal with sample (Cy5 and FAM channel)

- Incorrect assignment of detection channels in sample.
  → Please verify the correct assignment of detection channels.
- The DNA might be degraded.
- If the DNA IPC Target was added during extraction:
  - Inhibition of PCR may have occurred.
  - DNA extraction was unsuccessful.
  - The DNA IPC target was not added to the lysis buffer of the sample.
  - The extracted sample was not added to the PCR-reaction.

  → No statement is possible. Verify you use a recommended method for DNA isolation and re-examine the single steps of the DNA extraction.
  → If no operating mistakes during DNA 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 nuclease-free water).

13. Specifications and performance evaluation

13.1. Test performance on different real-time PCR instruments

Performance of ParoReal Kit *Acanthamoeba* T4 with an ABI® 7500 Fast Real-time PCR System (Thermo Fisher Scientific) is shown in Figure 1.

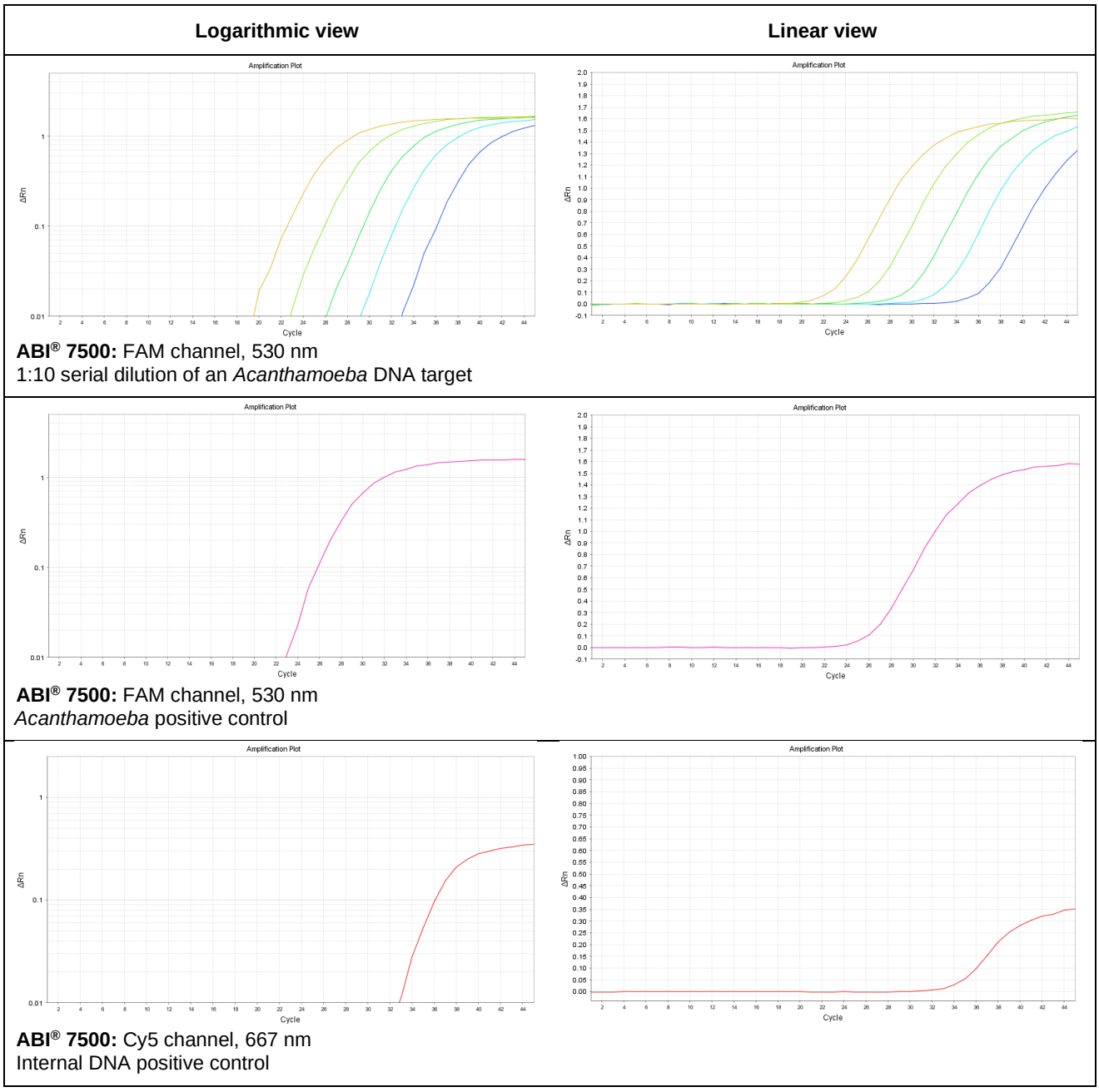

Figure 1 Performance of ParoReal Kit *Acanthamoeba* T4

This test has been validated with the ABI® 7500 instrument (Thermo Fisher Scientific) and was also tested with a LightCycler® 480 Instrument II (Roche), QuantStudio™ 7 real-time PCR system (Thermo Fisher Scientific) und Mic instrument (bio molecular systems).

13.2. Limit of detection, LoD

Method: The limit of detection (LoD95 = smallest number of copies of target DNA which can be detected in 95% of cases) was determined by testing a commercially available CE certified reference material with known concentrations of *A. castellanii* (AMPLIRUN® ACANTHAMOEBA CASTELLANII DNA CONTROL (Vircell, order No MBC054). Twenty replicates of at four different concentrations around the detection limit were tested (0.1, 0.5, 1, 3 copies). Calculation was performed with a non-linear (logistic) curve fit using GraphPad Prism Software.

Result: The LoD95 is 0.5 genome copies/reaction. The 18S rRNA gene is a multicopy gene and is present up to 22 times in the haploid genome of *Acanthamoeba*.

13.3. Linearity and dynamic measuring range

Method: ParoReal Kit *Acanthamoeba* T4 was tested with a 10-fold dilution series of a plasmid containing a fragment of *Acanthamoeba* DNA. Linearity was determined using a 10-fold dilution series (10^6 – 10^1 target copies/reaction) of the plasmid. The number of determinations (n) per dilution was nine.

Result: The assay shows linearity over the range of 100 to 1,000,000 target copies/reaction with a slope of 3.561 ± 0.04463 and an R^2 of > 0.99 as shown in Figure 2.

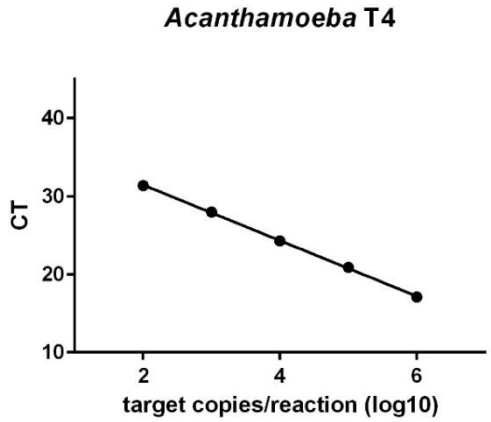

Figure 2 Ten-fold dilution series of an *Acanthamoeba* DNA standard

13.4. Precision

Method: Precision within a run (intra-assay), between multiple runs (inter-assay) and between two lots (inter-lot) was determined.

Result: The mean values of the coefficients of variation (CV%) are 1.2% for intra-assay precision, 1.2% for inter-assay precision and 0.5% for inter-lot precision.

13.5. Analytical specificity

Method BLAST analysis: The selection of highly specific primers and probes ensures analytical specificity. The specificity of primer and probes was validated *in silico* using the Basic Local Alignment Tool (BLAST) against the NCBI database. Primers and probes have been checked for possible homologies to currently published sequences. This analysis validates the detection of so far known strains of *Acanthamoeba* species of genotype T4.

Result: This test detects the 18S rDNA gene of *Acanthamoeba* species of genotype T4 *(A. castellani, A. lugdunensis, A. mauritaniensis, A. polyphaga, A. rhysodes, A. royreba).* No relevant sequence variabilities were observed in the primer and probe region thereof. The test does not detect other *Acanthamoeba* genotypes (T3, T15, T11, and T5) also causing keratitis, due to mismatches in primer and/or the probe. There is no cross-reactivity to other organisms.

13.6. Diagnostic evaluation

Method:

Diagnostic evaluation of ParoReal Kit *Acanthamoeba* T4 was performed by an external service with 168 DNA isolates from clinical specimens. Samples were DNA isolates from ocular swabs (n=63), corneal biopsies (n=20), ocular punctates (n=7), corneal scrapings (n=43) as well as contact lenses (n=7) and contact lens solution (n=26) and other material (n=2). They represented 67 samples positively tested for *Acanthamoeba* and 101 *Acanthamoeba*-negative samples. DNA was isolated from clinical specimens using the Qiagen QiaAmp Mini Kit (n=29) or Roche High Pure PCR Template Preparation Kit (n=139).
Results were compared with those obtained with a DIN EN ISO 15189 accredited pathogen-specific quantitative real-time PCR reference method detecting the 18S RRNA gene of the genus *Acanthamoeba* using hybridization probes. Real-time PCR was performed using a LightCycler® 480 II (Roche Diagnostics).

Result:

Out of the 67 samples which were positive for *Acanthamoeba* with the reference method, 59 samples were also positive with ParoReal Kit *Acanthamoeba* T4 (true positive). Cq values were between 19-37. Six out of the remaining 8 samples which were positive with the reference method but negative with ParoReal Kit *Acanthamoeba* T4 were sequenced and phylogenetically identified by BLAST analyses in the NCBI database. These samples were identified as *Acanthamoeba* T3 genotype. The 101 DNA samples which were negative with the reference method were also negative with ParoReal Kit *Acanthamoeba T4* (true negative).

The diagnostic evaluation of ParoReal Kit *Acanthamoeba* T4 is shown in Table 3 and Table 4.

Table 3 Overall results obtained with 168 tested clinical samples, 2x2 contingency table

		Reference		
		pos	**neg**	**Total**
ParoReal Kit *Acanthamoeba* T4	**pos**	**59**	**0**	**59**
	neg	**8***	**101**	**109**
	Total	**67**	**101**	**168**

* Six samples were identified as *Acanthamoeba* T3 genotype.

Table 4 Statistical evaluation of the diagnostic validation

	Value	**95% CI**
Sensitivity	88.06%	77.82% to 94.70%
Specificity	100.00%	96.41% to 100.00%
NPV	92.66%	86.82% to 96.03%
PPV	100.00%	
Prevalence	39.88%	32.42% to 47.71%

14. References

- Diehl, Maria Luisa Nunes, Paes, Júlia, Rott, Marilise Brittes. Genotype distribution of *Acanthamoeba* in keratitis: a systematic review. Parasitology Research (2021) 120:3051–3063.
- Lorenzo-Morales, Jacob; Khan, Naveed A.; Walochnik, Julia. 2015. An update on *Acanthamoeba* keratitis: diagnosis, pathogenesis and treatment. Parasite 22: 10.
- Yang, Q., Zwick, M. G., & Paule, M. R. (1994). Sequence organization of the Acanthamoeba rRNA intergenic spacer: identification of transcriptional enhancers. Nucleic acids research, 22(22), 4798–4805.

15. Revision history

Revision	Date	Description

Note:
Any serious incident that has occurred in relation to the product shall be reported to the manufacturer and the competent authority of the Member State in which the user and/or the patient is established.

Technical support
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