

ParoReal Kit

Histomonas meleagridis

Manual



For veterinary use only



DVEP00311, DVEP00313



100



DVEP00351, DVEP00353



50



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1. Product description

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

ParoReal Kit *Histomonas meleagridis* detects the 18S rRNA gene of *H. meleagridis*. A probe-specific amplification-curve at 530 nm (FAM channel) indicates the amplification of *H. meleagridis* specific DNA.

An internal positive control system for detection in VIC/HEX channel, (554 nm, order no. DVEP00311 or DVEP00351) or Cy5 channel (667 nm; order no. DVEP00313 or DVEP00353) 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.

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

2. Pathogen information

Histomonas meleagridis is a parasitic protozoan causing blackhead disease, infectious enterohepatitis, or histomoniasis. It can infect turkeys, chickens, and occasionally other galliform birds but it is most deadly in turkeys. Chickens are more resistant to the effects of the infection but may act as carriers. *Histomonas meleagridis* induces extensive and severe necrosis of the tissues of the mucosa and submucosa of cecum and parenchyma of the liver. It is transmitted by the nematode *Heterakis gallinarum* or directly from the droppings of infected birds. *Histomonas meleagridis* interacts with other gut organisms, such as bacteria and coccidia, and depends on these for full virulence.

References:

McDougald LR. 2005. Blackhead disease (histomoniasis) in poultry: a critical review. Avian Dis. 49:462-76.

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 amplicates.

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 *H. meleagridis* 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 *Histomonas meleagridis* order no. DVEP00311 or DVEP00351

Labelling	Content	Amount		Storage
		DVEP00311	DVEP00351	
<i>Histomonas meleagridis</i> Assay Mix (green cap)	Primer and probe (FAM) for detection of <i>H. meleagridis</i>	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
<i>Histomonas meleagridis</i> 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 *Histomonas meleagridis* order no. DVEP00313 or DVEP00353

Labelling	Content	Amount		Storage
		DVEP00313	DVEP00353	
<i>Histomonas meleagridis</i> Assay Mix (green cap)	Primer and probe (FAM) for detection of <i>H. meleagridis</i>	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
<i>Histomonas meleagridis</i> 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 ParoReal Kit *Histomonas meleagridis* 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
	<i>Histomonas meleagridis</i> Assay Mix	1.0 µl
	CR Assay Mix	1.0 µl
	Total volume Master Mix	15.0 µl
Preparation of PCR	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 an amount other than 5 µl of the sample, the amount of H₂O has to be changed accordingly.

Positive Control: As positive control use 1 µl of the *Histomonas meleagridis* Positive Control + 4 µl H₂O. 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 *Histomonas meleagridis*
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

For ABI PRISM® 7500:
Ramp speed: Without "fast cycling" parameter

For LightCycler® 480 instrument:
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 BactoReal®, MycoReal®, ParoReal®, and ViroReal® 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) <i>H. meleagridis</i> 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
Or: positive control (1:10 diluted, 1 µl/PCR)	31.0-34.0	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 *Histomonas meleagridis* please select fluorescence display options FAM channel for the *Histomonas meleagridis* target and VIC/HEX channel (order no. DVEP00311, DVEP00351) or Cy5 channel (order no. DVEP00313, DVEP00353) for the internal positive control target. Samples with a positive Cp or Ct-value are considered positive. Please also check the presence of amplification-curves manually.

8.1. Signal in FAM channel:

→ DNA of *H. meleagridis* was amplified. The sample has to be interpreted as positive.

Histomonas meleagridis DNA can lead to a reduced or absent fluorescence signal of the internal positive control assay (competition of PCR).

8.2. No signal in FAM channel but signal of the internal positive control:

→ No *H. meleagridis* DNA is detectable in the sample. The sample has to be interpreted as negative.

The positive signal of the internal positive control assay excludes a putative PCR inhibition.

8.3. No signals in FAM channel and no signal with internal positive control:

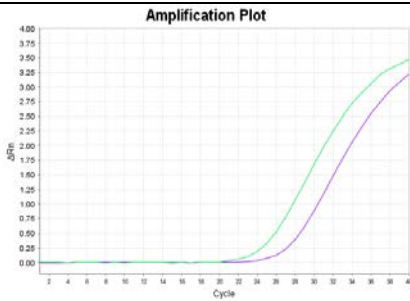
→ No interpretation statement can be made.

Information about possible sources of error and their solution can be found in 9. Troubleshooting.

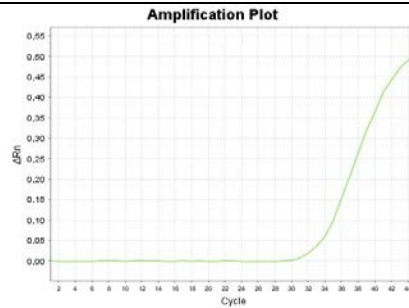
Detection of *Histomonas meleagridis*

Detection of internal positive control CR-3

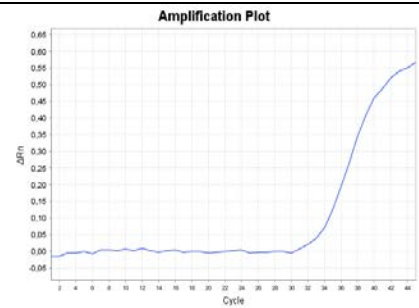
Detection of internal positive control CR-1



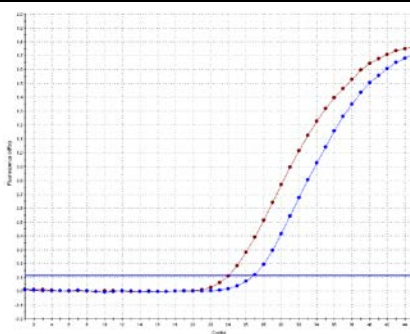
ABI Prism® 7500: FAM channel, 530 nm
1:10 serial dilution of *H. meleagridis* DNA



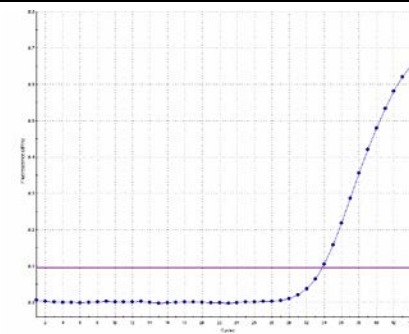
ABI Prism® 7500: Cy5 channel, 667 nm
Internal positive control



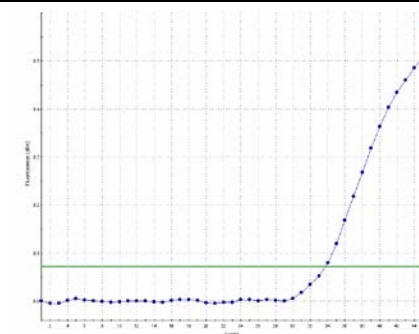
ABI Prism® 7500: VIC channel, 554 nm
Internal positive control



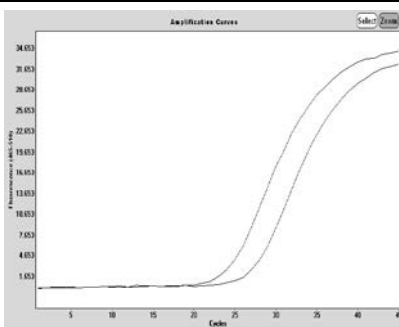
Mx3005P®: FAM channel
1:10 serial dilution of *H. meleagridis* DNA



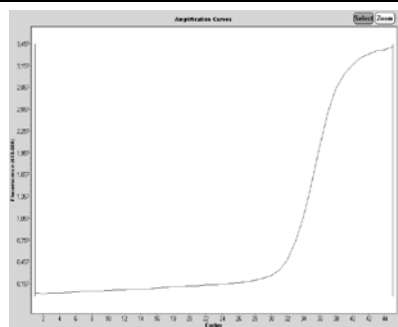
Mx3005P®: CY5 channel
Internal positive control



Mx3005P®: HEX channel
Internal positive control



LightCycler® 480: FAM channel
1:10 serial dilution of *H. meleagridis* DNA



LightCycler® 480: Cy5 channel
Internal positive control

9. Troubleshooting

9.1. No *H. meleagridis* 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 *H. meleagridis* 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 amount of H₂O).
- Incorrect PCR conditions.
→ Check the PCR conditions and repeat the PCR, if necessary.

9.3. *Histomonas meleagridis* 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. *Histomonas meleagridis* specific signal with negative control of DNA-extraction:

- 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

ParoReal Kit *Histomonas meleagridis* was evaluated with the ABI PRISM® 7500 (Fast) instrument (Thermo Fisher Scientific), with the LightCycler® 480 (Roche) and the Mx3005P® (Agilent). For further validation data please contact ingenetix.

10.1. Analytical sensitivity

The analytical sensitivity is 10 target copies/PCR reaction. The limit of detection (LoD95 = smallest number of copies of target DNA which can be detected in 95% of cases) is 12 target copies/reaction and was determined by several replicates around the detection limit.

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 *H. meleagridis* strains.

Since 2017, the kit has an improved and more specific design. Specificity was tested on six samples of *H. meleagridis* genotype I and II, and on isolates of *Dientamoebidae* sp., *Simplicimonas* sp. and *Tetratrichomonas gallinarum*. The *H. meleagridis* isolates were correctly identified. The kit shows no cross reaction with the other tested isolates.

11. Annex – symbols



Batch code



Catalogue number



Contains sufficient for <n> tests



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