BactoReal® Kit
Mycoplasma gallisepticum & synoviae

Manual

For use with the

- **ABI PRISM® 7500 (Fast)**
- **Mx3005P®**
- **LightCycler® 480**

For veterinary use only

REF **DVEB05713** Σ **100**

REF **DVEB05753** Σ **50**

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1. Product description

BactoReal® Kit *Mycoplasma gallisepticum & synoviae* is a multiplex real-time PCR assay for detection and differentiation of DNA of *Mycoplasma gallisepticum* and *Mycoplasma synoviae*. This test was developed and validated for the ABI PRISM® 7500 (Fast) instrument (Life Technologies), LightCycler® 480 (Roche) and Mx3005P® (Agilent), but is also suitable for other real-time PCR instruments. The test facilitates the rapid and sensitive detection of DNA of *M. gallisepticum* and *M. synoviae* from samples purified from faecal samples, or swabs of tissues, exudates, diluted tissue homogenates, aspirates from the infraorbital sinuses or joint cavities, as well as from material from egg yolk or embryos. Sample pools with up to 20 samples per pool can be analyzed. *Mycoplasma* DNA can be recovered efficiently from faecal samples using the QIAamp DNA Stool Mini Kit, and from swabs using the QIAamp DNA Mini Kit extraction methods, for example.

BactoReal® Kit *Mycoplasma gallisepticum & synoviae* detects the mgc2 gene (cytadhesin-like protein encoding gene) of *M. gallisepticum* and the vlhA gene (haemagglutinin encoding gene) of *M. synoviae.*
A probe-specific amplification-curve at 530 nm (FAM channel) indicates amplification of *M. gallisepticum*-specific DNA. A probe-specific amplification-curve at 554 nm (VIC/HEX channel) indicates the amplification of *M. synoviae*-specific DNA.
An internal positive control system for detection in Cy5 channel (667 nm) 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

Avian mycoplasmosis is caused by several pathogenic mycoplasmas. *Mycoplasma gallisepticum* and *Mycoplasma synoviae* are the most important avian pathogens. In addition, *Mycoplasma meleagridis* and *Mycoplasma iowae* can also cause disease in poultry, but *Mycoplasma gallisepticum* and *Mycoplasma synoviae* are considered to be the main species of pathogenic mycoplasmas.
Mycoplasma gallisepticum and *Mycoplasma synoviae* cause chronic respiratory disease (CRD) and infectious synovitis of chickens and turkeys especially in the presence of stresses or other respiratory pathogens, causing economic losses due to decrease in growth rate, loss of weight and reduced egg production.

References:
Raviv, Z. and S.H. Kleven, 2009. The development of diagnostic Real-Time TaqMan PCRs for the four pathogenic avian mycoplasmas. Avian Dis., 53: 103-107.

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. Contents of the Kit

Labelling	Content	Amount		Storage
		DVEB05713	DVEB05753	
M. gallisepticum & *M. synoviae* Assay Mix (green cap)	Primer and probes (FAM and VIC/HEX) for detection of *M. gallisepticum* and *M. synoviae*	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
M. gallisepticum & *M. synoviae* 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 *Mycoplasma gallisepticum & synoviae* are stable until the expiry date stated on the label. Repeated thawing and freezing should be avoided. Please protect kit components from light.

5. 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, VIC/HEX and Cy5 channel
- Appropiate 96 well reaction plates or reaction tubes with corresponding (optical) closing material

6. 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 *Mycoplasma* 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.

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
	M. gallisepticum & *M. synoviae* Assay Mix	1.0 µl
	CR-3 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 H_2O has to be changed accordingly.

Positive Control: As positive control use 1 µl of the *M. gallisepticum* & *M. synoviae* 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 *Mycoplasma gallisepticum*
VIC-TAMRA for detection of *Mycoplasma synoviae*
Cy5-NONE for detection of internal positive control

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: 3 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) *M. gallisepticum*	Ct/Cp (VIC/HEX channel) *M. synoviae*	Ct/Cp (Cy5 channel) IPC target	Interpretation
Negative control	Negative	Negative	36 ± 2	Valid
Positive controls (undiluted, á 1µl/PCR)	28-31	28-31	36 ± 2	Valid
Extraction negative control (optional)	Negative	Negative	36 ± 2	Valid
Negative sample	Negative	Negative	36 ± 2	Valid
Positive sample European foulbrood	Positive	Negative	Pos./Neg.	Valid
Positive sample American foulbrood	Negative	Positive	Pos./Neg.	Valid

For analysis of PCR data please proceed as follows:
For analysis of PCR results gained with BactoReal® Kit *Mycoplasma gallisepticum & synoviae* please select fluorescence display options FAM channel for the *M. gallisepticum* taget, VIC/HEX channel for the *M. synoviae* target and Cy5 channel 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.

Once the analysis is completed, the following results are possible:

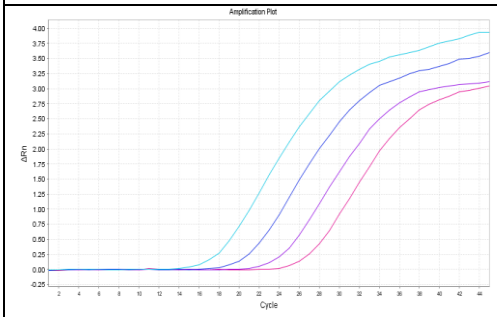
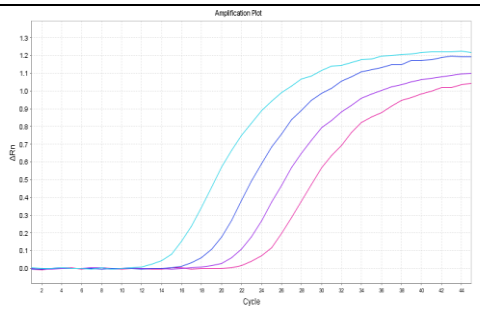
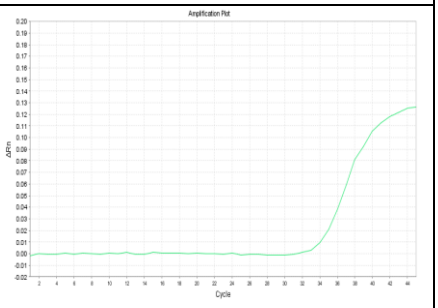
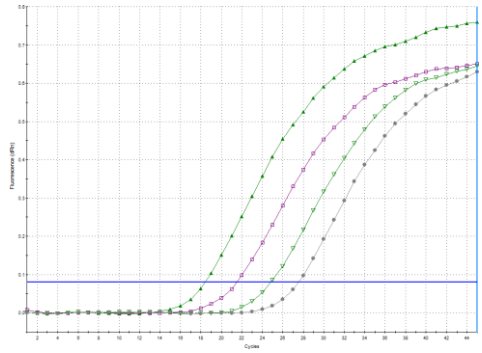
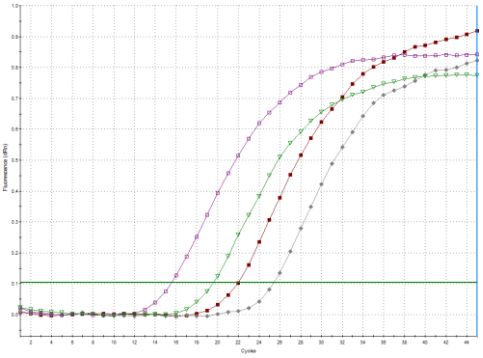
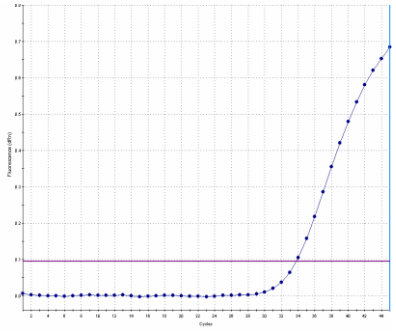
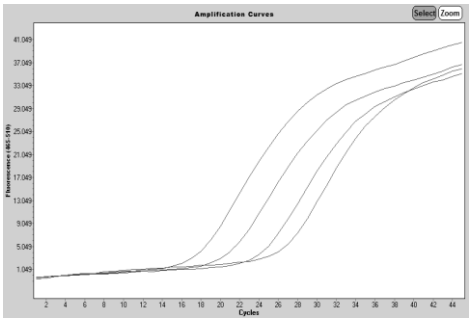
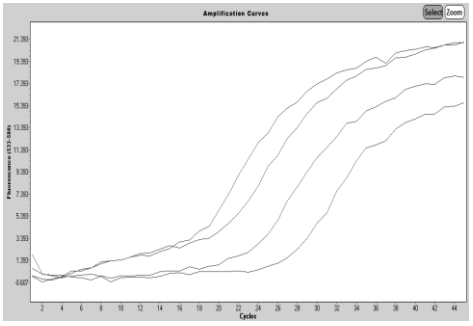
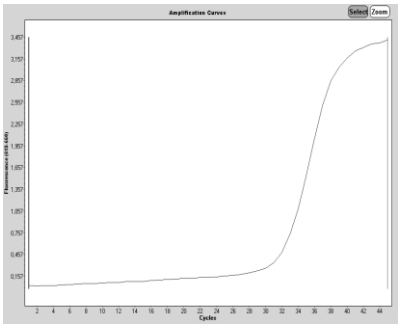
1. Signal in FAM channel, no signal in VIC/HEX channel:
→ DNA of *Mycoplasma gallisepticum* was amplified. The sample has to be interpreted as positive.
Mycoplasma DNA can lead to a reduced or absent fluorescence signal of the internal positive control (competition of PCR).

2. Signal in VIC/HEX channel, no signal in FAM channel:
→ DNA of *Mycoplasma synoviae* was amplified. The sample has to be interpreted as positive.
Mycoplasma DNA can lead to a reduced or absent fluorescence signal of the internal positive control (competition of PCR).

3. No signal in FAM or VIC/HEX channel:
→ No DNA of *M. gallisepticum* or *M. synoviae* is detectable in the sample. The sample has to be interpreted as negative. An inhibition of PCR cannot be excluded.

3a. No signal in FAM or VIC/HEX channel but signal of the internal positive control:
→ No DNA of *M. gallisepticum* or *M. synoviae* 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.

3b. No signals in FAM or VIC/HEX 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 ***M. gallisepticum***	Detection of ***M. synoviae***	Detection of internal positive control **CR-3**
		
ABI Prism® 7500: FAM channel, 530 nm 1:10 serial dilution of *M. gallisepticum* DNA	**ABI Prism® 7500:** VIC channel, 554 nm 1:10 serial dilution of *M. synoviae* DNA	**ABI Prism® 7500:** Cy5 channel, 667 nm Internal positive control
		
Mx3005P®: FAM channel 1:10 serial dilution of *M. gallisepticum* DNA	**Mx3005P®:** HEX channel 1:10 serial dilution of *M. synoviae* DNA	**Mx3005P®:** CY5 channel Internal positive control
		
LightCycler® 480: FAM channel 1:10 serial dilution of *M. gallisepticum* DNA	**LightCycler® 480:** VIC / HEX / Yellow555 channel 1:10 serial dilution of *M. synoviae* DNA	**LightCycler® 480:** Cy5 channel Internal positive control

9. Troubleshooting

1. No *M. gallisepticum* or *M. synoviae* specific signal with positive controls:

- 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.

2. No signal with the internal positive control and no *M. gallisepticum* or *M. synoviae* specific signal with the 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 H_2O).
- Incorrect PCR conditions.
  → Check the PCR conditions and repeat the PCR, if necessary.

3. *M. gallisepticum* or *M. synoviae* specific signal with the 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.

4. *M. gallisepticum* or *M. synoviae* specific signal with the 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

BactoReal® Kit *Mycoplasma gallisepticum & synoviae* was evaluated with the ABI PRISM® 7500 (Fast) instrument (Life Technologies), with the LightCycler® 480 (Roche) and the Mx3005P® (Agilent).

10.1. Analytical sensitivity

The analytical sensitivity for *M. gallisepticum* is 10 target copy/PCR reaction. The limit of detection (LoD95 = smallest number of copies of target DNA which can be detected in 95% of cases) is 27 target copies/reaction and was determined by several replicates around the detection limit.
The analytical sensitivity for *M. synoviae* is 1 target copy/PCR reaction. The limit of detection (LoD95 = smallest number of copies of target DNA which can be detected in 95% of cases) is 16 target copies/reaction and was determined by several replicates around the detection limit.
The presence of high concentrations of *M. synoviae* or *M. gallisepticum* DNA has no influence on amplification of *M. gallisepticum* or *M. synoviae* DNA. Due to the high sensitivity of the test, sample pools with up to 20 samples per pool can be analyzed.

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 *M. gallisepticum* and *M. synoviae* strains.
Specificity was tested on isolates of *M. gallisepticum, M. meleagridis* and *M. synoviae*. No cross reactions were observed. *Mycoplasma imitans*, a *Mycoplasma* species that is serologically related to *M. gallisepticum* and that presents the same biochemical properties is not detected by this test.
26 field samples were tested using the *Mycoplasma synoviae* assay and correctly analysed. Eight field samples were tested using the *Mycoplasma gallisepticum* assay and correctly analysed.

11. Annex – symbols

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