



V For *in vitro* Veterinary
Diagnostics only.

Kylt[®]

Kylt[®] MS-H DIVA

Real-Time PCR Detection

www.kylt.eu



Kylt® MS-H DIVA

Real-Time PCR Detection

Revision No.	Amendments
009	New color of Lid of MSH-MS Positive Control
008	Layout

A. General

- Kylt® MS-H DIVA specific kits are intended for the differentiation of bacterial DNA of *Mycoplasma synoviae* (MS) field strains (MSf) and live vaccine strain MS-H. The kits are suitable for the analysis of samples from birds such as swab samples (e.g. nasal, choanal or tracheal, from tonsils), tissues and organs (e.g. trachea, lung, cecal tonsils) and pure or mixed colony material / isolates derived from cultural processes of the aforementioned samples.
- The qualitative testing with Kylt® MS-H DIVA kits is based on a triplex Real-Time PCR: In one reaction setting, the target genes for MSf and vaccine strain MS-H as well as for the exogenous control (Internal Amplification Control (IAC)) are amplified in parallel by respective primer pairs in the Polymerase Chain Reaction (PCR). Amplified target gene fragments are detected via fluorescently labeled probes during the PCR reaction in real-time (Real-Time PCR). The probes specific for detection of amplified MSf, vaccine strain MS-H and the exogenous control target genes are labeled with fluorescent dyes FAM, Cy5 and HEX, respectively, and their emitted fluorescence is separately optically measured by the Real-Time PCR thermal cycler. By means of all individual analyses in one reaction vessel per sample and the Negative Control and Positive Control per run the MSf- or MS-H-specific status of a sample can be evaluated in the end. This way, results can be achieved within a few hours after sample receipt.
- These kits were developed for use by trained laboratory personnel following standardized procedures. This Direction For Use must be followed strictly.

B. Reagents and Materials

- The following Kylt® MS-H DIVA kits are available and comprise the following reagents:

Reagent	Colour of Lid	100 Reactions	25 Reactions	Store at
		Article No 31134	Article No 31135	
Reaction-Mix	● orange	4 x 450 µl	1 x 450 µl	≤ -18 °C
MS-Positive Control (MSH-MS PC)	● red/white	2 x lyophilizate (final 50 µl each)	1 x lyophilizate (final 50 µl each)	≤ -18 °C
MS-Positive Control (MSH-MSH PC)	● red	2 x lyophilizate (final 50 µl each)	1 x lyophilizate (final 50 µl each)	≤ -18 °C
Negative Control	● blue	1 x 1 ml	1 x 1 ml	≤ -18 °C

- After receipt, the components are immediately stored at ≤ -18 °C. Avoid repeated freezing and thawing of all the reagents and keep them thawed as short as possible. If occasional processing of few samples only is expected you may prepare appropriate aliquots of reagents before storage at ≤ -18 °C. Prepare aliquots in such a way that freeze-thaw-cycles are reduced to a maximum of three. The Negative Control can alternatively be stored at +2°C to +8°C.
- Alternatively, it is possible to store the Reaction-Mix at +2°C to +8°C. For further information regarding the recommended storage temperature of the respective Reaction-Mix please see label on the tube.
- The components are to be used within the indicated shelf life (see box label). The components of different batches may not be mixed.
- Before its first use, rehydrate the Positive Controls: add 50 µl of Negative Control per vial, briefly incubate at room temperature and mix thoroughly by repeated vortexing. It is recommended to generate aliquots of suitable volumes and store them at ≤ -18 °C.
- The Reaction-Mix needs to be stored protected from abundant light. Do not expose to direct (sun)light.

C. Equipment and Reagents not included

- This detection method can be used on all commercially available Real-Time PCR thermal cyclers that detect the emitted fluorescence of the fluorescent dyes FAM, Cy5 and HEX (emission 520, 670 and 550, respectively), except glass-capillary based thermal cyclers. Note that default normalization option against ROX (e.g. using ABI cyclers) must be deactivated.
- For the following Real-Time PCR thermal cycler specific instructions are given:
 - Applied Biosystems® 7500 & 7500 Fast: Before starting the Real-Time PCR run deselect the passive reference dye ROX and select NFQ-MGB as quencher of the probe.
 - Rotor-Gene® 3000, 6000 & Q: In the case of combining Kylt® MS-H DIVA with other Kylt® Real-Time PCRs for detecting bacterial DNA please set the MS-H setup as first parameter in the Real-Time PCR run. For a combined Real-Time PCR run the gain setting have to be changed manually as follows: gain setting for channel FAM has to be measured at the position of the MS-Positive Control, gain setting for channel Cy5 has to be measured at the position of the MS-H-Positive Control and gain setting for channel HEX has to be measured at the position of the Negative Control.
- Apart from the disposables, the following further devices are needed and are not included in the Kylt® MS-H DIVA products:
 - DNA preparation kit / protocol (e.g. Kylt® DNA Extraction-Mix II or Kylt® RNA / DNA Purification)
 - Table top microcentrifuge
 - Vortex
 - Micropipettes covering volumes of 1 µl to 1000 µl
 - Centrifuge for PCR tubes or plates
- Accessory Kylt® products: see chapter F “Related and Accessory Products”.
- We recommend the exclusive use of certified Nuclease-free disposables as well as powder-free protective gloves. Please wear gloves during the entire experimental procedure. Gloves need to be changed frequently, especially after spillage or suspected contaminations.

D. Control Reactions

- The Positive Controls allow for control of the specificity and efficiency of the reagents and the reaction itself, including the performance of the Real-Time PCR and of the Real-Time PCR thermal cycler.
- The Negative Control allows for exclusion of contaminations. The sample testing is only valid if both, Positive and Negative Controls, are used and verified for validity in every Real-Time PCR run.
- The Internal Amplification Control is included in the Reaction-Mix or Detection-Mix, depending on the product used, in a defined copy number; it is co-amplified (channel HEX) with every single reaction to detect possible inhibitory effects of the DNA preparation on the Real-Time PCR itself and thus to verify true-negative results.
- If appropriate sampling is unsure we recommend to analyze the samples in parallel with Kylt® Host Cells Real-Time RT-PCR Detection for presence of amplifiable nucleic acids derived from host cell material, see chapter F “Related and Accessory Products”.

E. Protocol (see also „Protocol At A Glance“ at the end of this Direction For Use)

- The overall protocol of the analysis consists of the following main workflow:
 1. Sample Preparation
 2. DNA Preparation
 3. Reaction Setup and Amplification (Real-Time PCR)
 4. Data Analysis – Validity and Qualitative Result
- We recommend proceeding through the protocol without interruption to avoid potential degradation of the processed samples and reagents. If necessary, you may store the final DNA preparation at ≤ -18 °C until further processing. Avoid repeated freezing and thawing of the DNA preparations.

1. Sample Preparation

- We recommend pooling of at most five samples or samples from five individuals, respectively, per DNA preparation.
- Pool swabs in a sufficient volume of sterile buffer (e.g. 1 ml of Normal Saline or 0.1 x TE), let the swabs soak for an adequate period of time and finally wash out the swabs by thorough pulse-vortexing. The washed out supernatant is used for DNA preparation. For Kylt® DNA Extraction the supernatant is (fully) transferred to a conical screw cap tube (please refer to 2 "DNA Preparation"). Small swabs may directly be immersed in Kylt® DNA Extraction-Mix II or lysis buffer, if applicable.
- Tissue and organ samples are homogenized thoroughly in sterile buffer (see above) and a suitable volume is used for the DNA preparation.
- Material derived from cultural processes, i.e. colony material, is directly transferred into respective tubes for Kylt® DNA Extraction (please refer to 2 "DNA Preparation"), such as conical screw cap tube; therefore a little amount of a single colony is picked with a sterile loop wire or sterile pipette tip and transferred to the tube.

2. DNA Preparation

a) Kylt® DNA Extraction (requires Kylt® DNA Extraction-Mix II)

- For detailed information, please refer to the Direction For Use of Kylt® DNA Extraction-Mix II.

b) DNA Preparation by other Methods

- All kinds of sample matrices, including pure isolates, swabs, tissues and organs may be processed with appropriate DNA preparation kits, such as Kylt® RNA/DNA Purification (please refer to chapter F "Related Products") or appropriate in-house methods.
- For detailed information on the DNA preparation process, please refer to the Direction For Use or Standard Operating Procedure of the specific kit or in-house method, respectively.

3. Reaction Setup and Amplification (Real-Time PCR)

- Before each use, briefly vortex and spin down the Reaction-Mix and Negative Control.
- To determine the total number of reactions needed, count the number of samples and add three more for the Negative Control and the Positive Controls.
- The Reaction-Mix is ready-to-use, add 16 µl to each of the PCR tubes or plate wells (“cavities”).
- Keep exposure of the Reaction-Mix to (sun)light as short as possible and return it back to appropriate storage temperature right after application. Avoid the formation of bubbles when pipetting samples and controls.
- Add 4 µl of the Negative Control to the corresponding cavity and seal it individually, if possible.
- Add 4 µl of each DNA preparation to the corresponding cavities and seal them individually, if possible.
- To minimize risk of potential cross-contaminations, 4 µl of the Positive Controls are added to the corresponding cavity after all previous samples and control reactions are set up. Before each use, briefly vortex and spin down the rehydrated Positive Controls (see also chapter B “Reagents and Materials”).
- If not already done, finally seal the cavities. It is recommended to briefly spin them down before the start of the Real-Time PCR run.
- Place the cavities in the Real-Time PCR thermal cycler and run the test with Kylyt® Profile II as given below.

Kylyt® Profile II				
Step No	Description	Temperature	Duration	
1	Activation of Polymerase	95 °C	10 min	
2	Denaturation	95 °C	15 sec	} 42 cycles
3	Annealing & Extension	60 °C	1 min	
4	Fluorescence Detection	channels FAM, Cy5 and HEX		

- Kylyt® Profile II allows for combined run of this and most other Kylyt® qPCR detection methods.
- Alternatively, the Kylyt® Profile I given below can be applied. Kylyt® Profile I allows for combined run of this and most other Kylyt® qPCR detection methods as well as Kylyt® RT-qPCR detection products that need Reverse Transcription, such as those for detection of viral RNA.

Kylyt® Profile I				
Step No	Description	Temperature	Duration	
1	Reverse Transcription	50 °C	10 min	
2	Activation of Polymerase	95 °C	1 min	
3	Denaturation	95 °C	10 sec	} 42 cycles
4	Annealing & Extension	60 °C	1 min	
5	Fluorescence Detection	channels FAM, Cy5 and HEX		

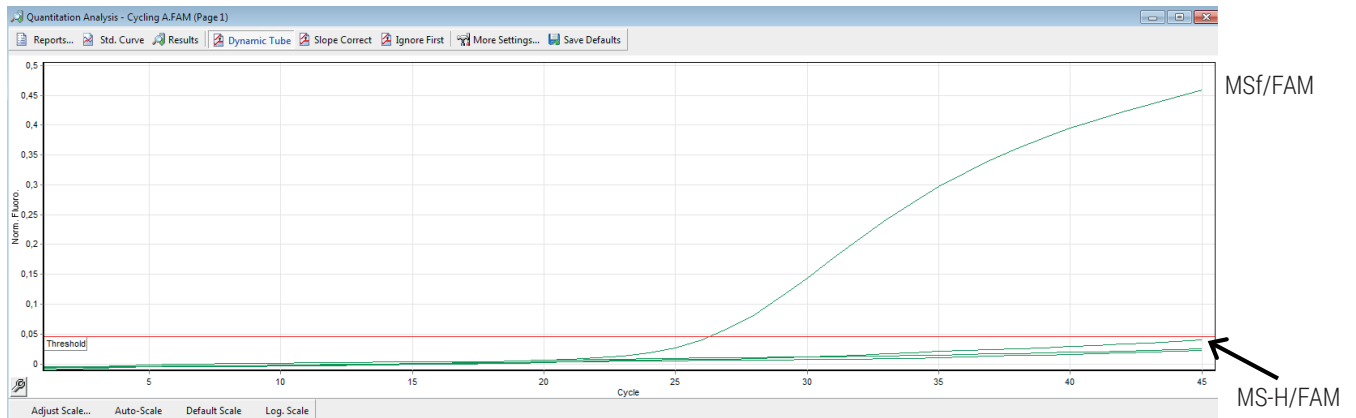
- In the event of a combined Real-Time (RT)-PCR run, make sure all necessary channels are detected.
- Please follow the specified instructions of your Real-Time PCR thermal cycler as recommended by the manufacturer.

4. Data Analysis – Validity and Qualitative Result

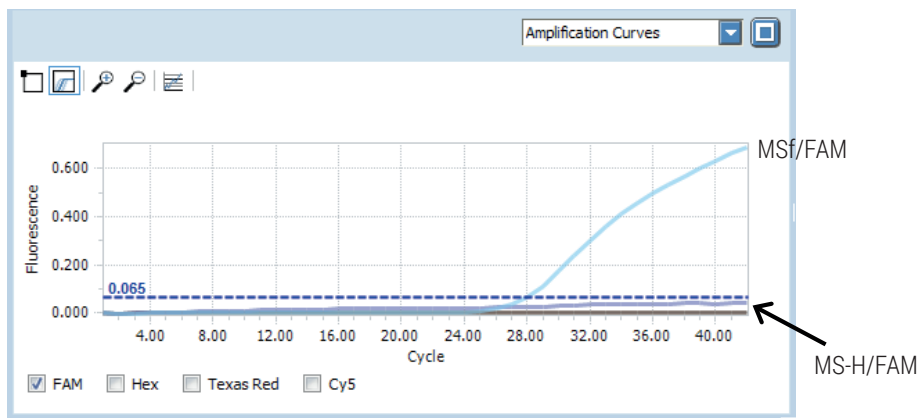
General

- For evaluating the amplification data the threshold has to be set manually, preferably in linear scale of x- and y-axis of the amplification plot. For the amplification plot of the MS-Positive Control reaction a strong increase in fluorescence in the FAM-channel for MSf is expected. In addition a minimal increase in background fluorescence in the FAM-channel from MS-H-Positive Control might be determined, depending on the Real-Time PCR thermal cycler used. Set the threshold for the FAM-channel above the background fluorescence of the MS-H-Positive Control.

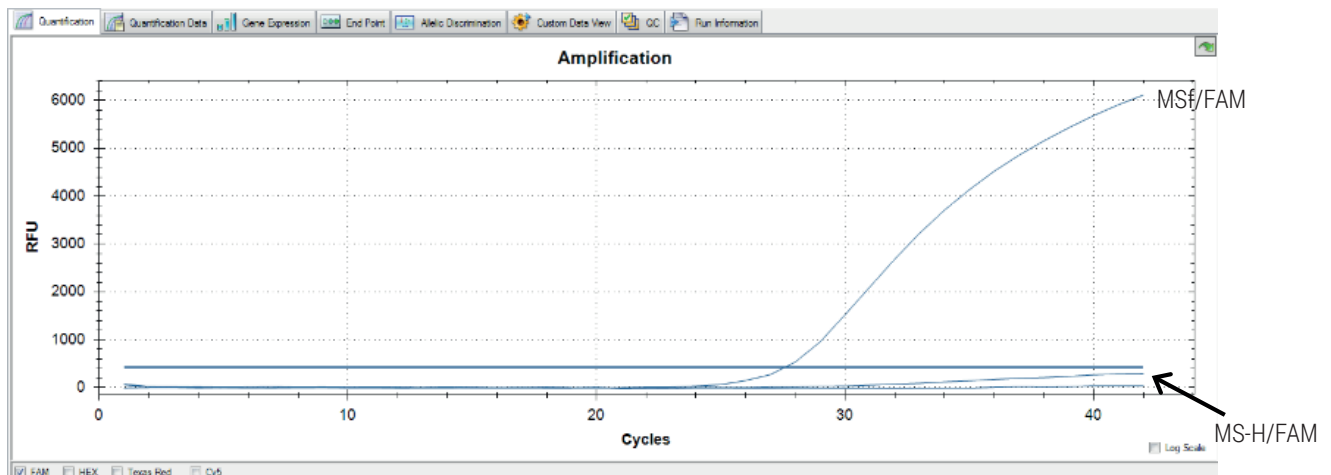
plot (linear scale) for MS-Positive Control (FAM) generated on Rotorgene® 6000



plot (linear scale) for MS-Positive Control (FAM) generated on LightCycler® 96

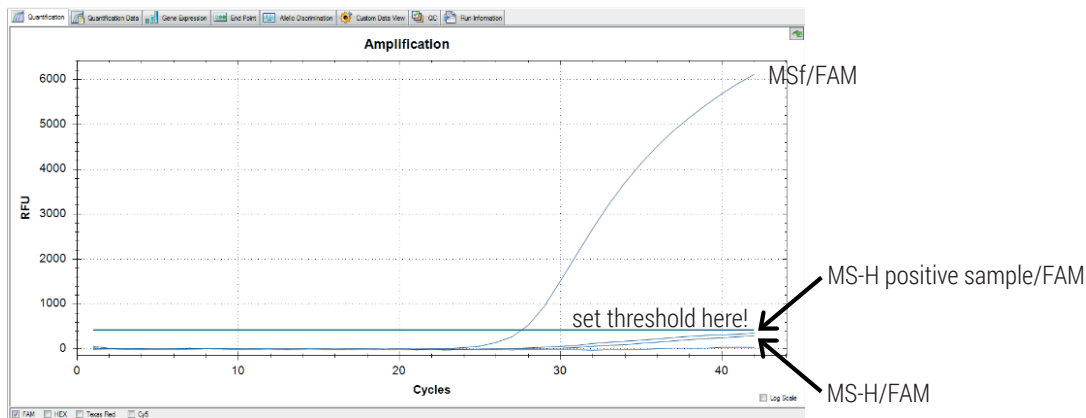


plot (linear scale) for MS-Positive Control (FAM) generated on CFX96 Touch™



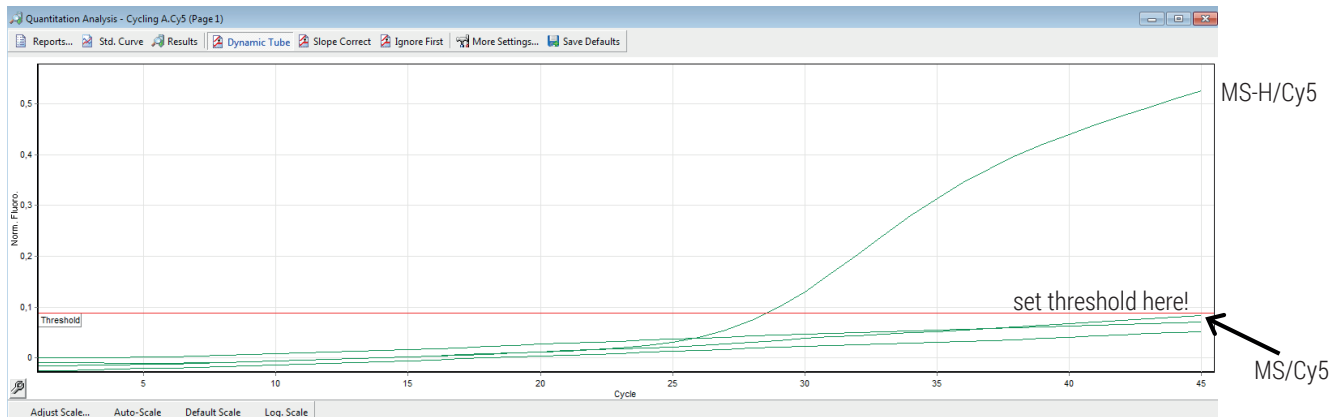
- MS-H positive samples with a high Ct-value (in Cy5-channel) may cause a higher increase in background fluorescence in the FAM-channel as observed for the MS-H Positive Control. In this case set the threshold for the FAM-channel above the background fluorescence of the sample which is strongly positive for MS-H. The unspecific minimal increase of the MS-H Positive Control and MS-H positive samples in the FAM-channel can be clearly distinguished by the lower slope of the fluorescence curve and the lower endpoint fluorescence when compared to the MS-field Positive Control and MS field positive samples:

plot (linear scale) for MS-Positive Control (FAM) and MS-H positive sample generated on CFX96 Touch™

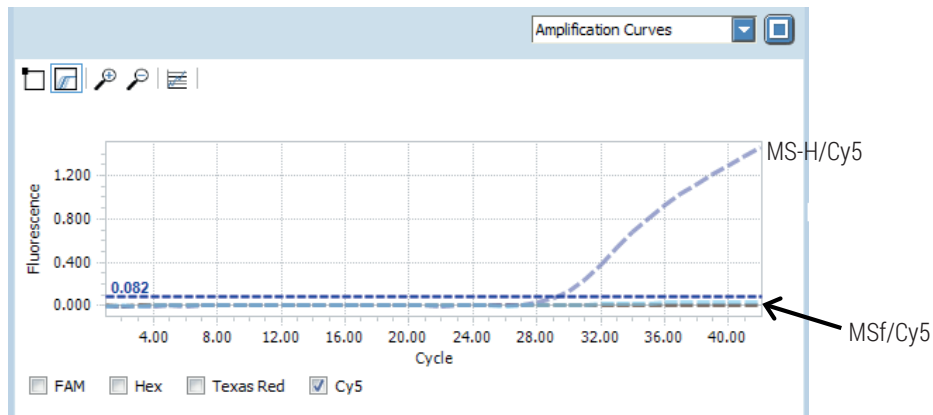


- For the amplification plot of the MS-H-Positive Control reaction a strong increase in fluorescence in the Cy5- channel for the MS-H vaccine is expected. In addition a minimal increase in background fluorescence in the Cy5- channel from MS-Positive Control might be determined, depending on the Real-Time PCR thermal cycler used. Set the threshold for the Cy5-channel above the background fluorescence of the MS-Positive Control.

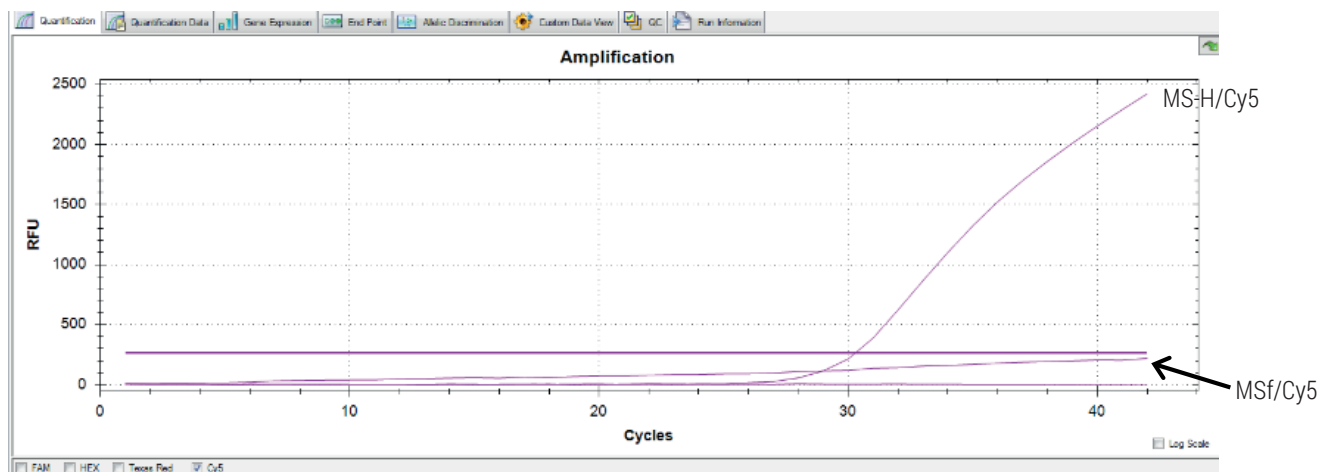
plot (linear scale) for MS-H-Positive Control (Cy5) generated on Rotorgene® 6000



plot (linear scale) for MS-H-Positive Control (Cy5) generated on LightCycler® 96



plot (linear scale) for MS-H-Positive Control (Cy5) generated on CFX96 Touch™



- MSf positive samples with a high Ct-value (in FAM-channel) may cause a higher increase in background fluorescence in the Cy5-channel as observed for the MS-Positive Control. In this case set the threshold for the Cy5-channel above the background fluorescence of the sample which is strongly positive for MSf. The unspecific minimal increase of the MSf Positive Control and MSf positive samples in the Cy5-channel can be clearly distinguished by the lower slope of the fluorescence curve and the lower endpoint fluorescence when compared to the MS-H Positive Control and MS-H positive samples. Please refer to the amplification plot for MS-Positive Control (FAM) and MS-H positive sample generated on CFX96 Touch™ on page 8.
- The threshold should cross the FAM-, Cy5- and the HEX-curve in the linear increase of their slope (log scaling of the y-axis). By setting the threshold, the crossing points with the FAM-, Cy5- and HEX-curves determine the respective cycle threshold (Ct), which is negatively correlated with the initial concentration of copies of the target genes in the Real-Time PCR reaction.
- Only curves with the typical exponential amplification, meaning the curve of the raw data shows a flat baseline at the beginning, followed by a clear (exponential) slope in fluorescence and possibly reaching a plateau-phase (y-axis in log scaling), should be regarded as positive.
- The actual test analysis starts with the validity check of the entire Real-Time PCR run. Afterwards, by means of the Internal Control the validity of each sample reaction and its true test result can be verified according to the Ct-value of the Internal Control channel (HEX). Finally, the MSf and MS-H specific status of each sample is analyzed (FAM and Cy5, respectively).

Test Evaluation

- The **Real-Time PCR test run** is only **valid** if the FAM- and Cy5-curves of the Negative Control are negative, the HEX-curve of the Negative Control is positive and the FAM- and Cy5-curves of the Positive Controls are positive. For a valid test the FAM- and Cy5-Ct-value of the Positive Controls have to be > 15 and ≤ 35 and the HEX-Ct-value of the Negative Control has to be ≤ 40 .

Target	Channel	Signal				
Internal Control	HEX	positive	positive / negative	positive / negative	positive / negative	negative
MSf	FAM	negative	positive	negative	positive	negative
MS-H	Cy5	negative	negative	positive	positive	negative
The sample is MSf		negative	positive	negative	positive	inhibited
The sample is MS-H		negative	negative	positive	positive	

- A **sample is negative for MSf and MS-H vaccine strain** if its HEX-curve is positive ($Ct \leq 40$), but its FAM- and Cy5-curve are negative.
- A **sample is positive for MSf** if its FAM-curve is positive ($Ct \leq 42$), independent of the HEX-curve.
- A **sample is positive for MS-H vaccine strain** if its Cy5-curve is positive ($Ct \leq 42$), independent of the HEX-curve.
- A **sample is positive for MSf and MS-H vaccine strain** if its FAM- and Cy5-curve are positive ($Ct \leq 42$), independent of the HEX-curve.
- A **sample is inhibited** if neither the FAM-curve nor the Cy5- and HEX-curve is positive.
- **Recommendation:** In the case of an inhibited sample the test may be repeated with a dilution of the DNA preparation at e.g. 1:10 (9 volumes Negative Control + 1 volume DNA Extract or eluted DNA). The Negative Control is used as the diluting agent. Preferably, the entire DNA preparation process is repeated: in case of inhibited DNA Extracts derived from Kylt® DNA Extraction-Mix II, the original sample or the DNA Extract can be utilized for DNA preparation using appropriate alternative systems, such as Kylt® RNA/DNA Purification.
- Convenient and reliable sample data entry, Real-Time PCR start, final qualitative analysis and documentation can be conducted with the Kylt® Software, please inquire.

F. Related and Accessory Products

Product	Article No	Reactions	Description
Kylt® DNA Extraction-Mix II	31398	100	Simplified and economic DNA extraction
Kylt® RNA / DNA Purification	31314 / 31315	250 / 50	Combined RNA and DNA purification from veterinary samples
Kylt® RNA / DNA Purification HTP	31826	4 x 96	Combined, magnetic beads-based purification of RNA and DNA from veterinary samples, suitable for automated high throughput processing
Kylt® Host Cells	31106 / 31107	100 / 25	Kit to detect animal host cells; to verify sample taking process

Production:

AniCon Labor GmbH | Muehlenstr. 13 | D-49685 Hoeltinghausen | Germany | www.kylt.eu | info@kylt.eu

Development, manufacturing and distribution of Kylt® *In-Vitro* Diagnostica is certified according to ISO 9001:2015.



Kylt® is a registered trademark.

For veterinary use only. For *in vitro* use only. Regulatory requirements vary by country, not all of the products described herein may be available in your geographic area.

© 2019 AniCon Labor GmbH. All rights reserved. The trademark mentioned herein is the property of AniCon Labor GmbH or their respective owners.

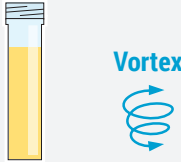
qPCR.MS-H DIVA.02, Rev.009, November 2019

PROTOCOL AT A GLANCE

Real-Time PCR Setup

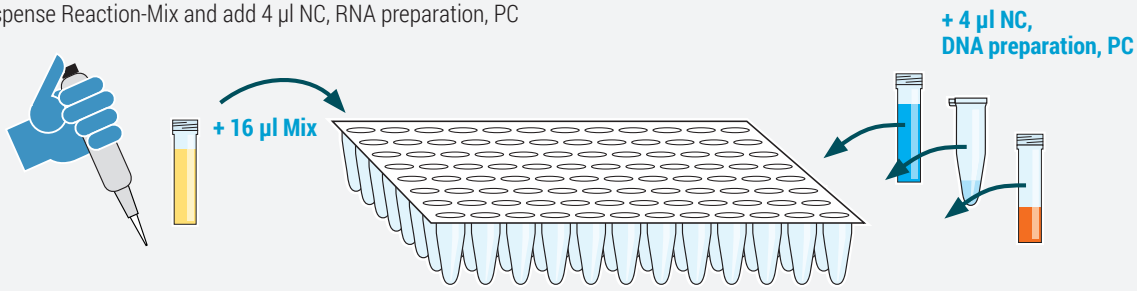
1

Pulse-vortex and spin down



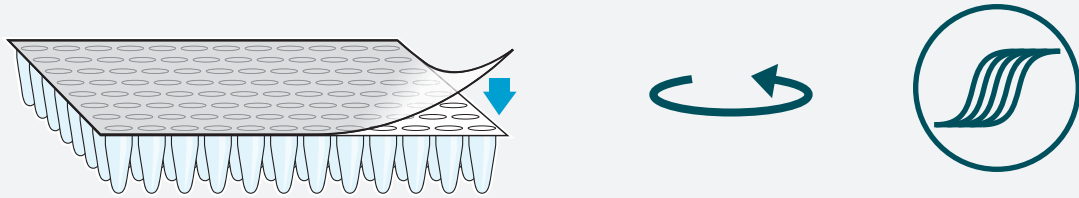
2

Dispense Reaction-Mix and add 4 µl NC, RNA preparation, PC



3

Seal cavities, spin down (recommended), and start cycler



4

Analysis

