



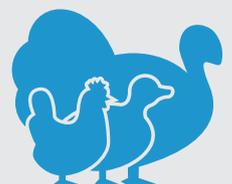
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Kylt[®]

Kylt[®] Riemerella anatipestifer

Real-Time PCR Detection

www.kylt.eu



Kylt® *Riemerella anatipestifer*

Real-Time PCR Detection

A. General

- Kylt® *Riemerella anatipestifer* products are intended for the specific detection of bacterial DNA of *Riemerella anatipestifer*. The products are suitable for the analysis of samples from birds such as swab samples (e.g. tracheal or from lungs), tissues and organs and pure or mixed colony material / isolates derived from cultural processes of the aforementioned samples.
- The qualitative testing with Kylt® *Riemerella anatipestifer* products is based on a duplex Real-Time PCR: In one reaction setting, the target genes for *Riemerella anatipestifer* as well as for the Internal Control 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 *Riemerella anatipestifer* and the Internal Control target genes are labeled with fluorescent dyes FAM and HEX, respectively, and their emitted fluorescence is separately optically measured by the Real-Time PCR thermal cycler. By means of both individual analyses in one reaction vessel per sample and the Negative Control and Positive Control per run the *Riemerella anatipestifer*-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 products 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® *Riemerella anatipestifer* products are available and comprise the following reagents:

Reagent	Colour of Lid	Quantity in Kit with 100 / 25 Reactions		Store at
		Article No 31032 / 31033	Article No [#] 31457 / 31458	
Reaction-Mix	● orange	4 x / 1 x 450 µl	-/-	≤ -18 °C
Detection-Mix	● yellow	-/-	4 x / 1 x lyophilizate (final 150 µl each)	≤ -18 °C
Positive Control	● red	4 x / 2 x lyophilizate (final 50 µl each)	4 x / 2 x lyophilizate (final 50 µl each)	≤ -18 °C
Negative Control	● blue	1 x 1 ml	1 x 1 ml	≤ -18 °C

To conduct an analysis for *Riemerella anatipestifer* an additional, at least 2x concentrated qPCR-Mix is required, please refer to chapter C. "Equipment and Reagents not Included" or inquire for recommendations.

- 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.
- The components are to be used within the indicated shelf life (see label on the outer packing). The components of different batches may not be mixed.
- Before its first use, rehydrate the Positive Control: 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.
- The Detection-Mix needs to be stored protected from abundant light. Do not expose to direct (sun) light. Before first use, rehydrate the lyophilized Detection-Mix: add 150 µl of the Negative Control per vial of Detection-Mix, briefly incubate at room temperature and mix by pulse-vortexing. Generate aliquots of suitable volumes and store them at ≤ -18 °C.

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 and HEX (emission 520 and 550, respectively). Note that default normalization option against ROX (e.g. using ABI cyclers) must be deactivated.
- Apart from the disposables, the following further devices are needed and are not included in the Kylt® *Riemerella anatipestifer* 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
- For Kylt® products supplied with Detection-Mix only, at least 2x concentrated qPCR-Mix is needed (e.g. BCD 2x qPCR-Mix).
- 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 Control allows 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 or rehydrated Detection-Mix and Negative Control.
- To determine the total number of reactions needed, count the number of samples and add two more for the Negative Control and the Positive Control.
- In case of products supplied with Reaction-Mix this is ready-to-use, add 16 µl to each of the PCR tubes or plate wells (“cavities”).
- In case of products supplied with Detection-Mix:
 - Prepare the Master-Mix using the components listed below. It is strongly recommended to prepare a Master-Mix, consisting of qPCR-Mix (e.g. BCD 2x qPCR-Mix) and Detection-Mix, at least per PCR setup.
 - When calculating volumes for preparation of the Master-Mix, consider the concentration (e.g. “2x”) of the qPCR-Mix used. For more information about the qPCR-Mix please refer to the Direction For Use of the respective manufacturer.
 - Alternatively, when using the recommended BCD 2 x qPCR Mix a larger volume of a ready to use working Master-Mix can be prepared and stored at ≤ -18 °C for convenient use over a longer period of time up to the expiry date given on the label. In case of frozen storage the working Master-Mix should be aliquoted in such a way that freeze-thaw-cycles are reduced to a maximum of three.
 - Vortex, spin down and add 16 µl of the finalized Master-Mix to each of the PCR tubes or plate wells (“cavities”).

Example Calculation for 2x concentrated qPCR-Mix:	Volume (µl)	
	per Reaction	e.g. n=7
qPCR-Mix (e.g. BCD 2x qPCR-Mix)	10 µl	70 µl
Detection-Mix	6 µl	42 µl
Total Master-Mix	16 µl	112 µl dispense 16 µl per reaction
DNA (Negative Control / sample DNA / Positive Control)	4.0 µl	
Total Reaction	20.0 µl	

- Keep exposure of the Reaction-Mix, Detection-Mix and prepared Master-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 the Master-Mix, 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 Control 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 Control (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 [Kylt® Profile II](#) as given below.
- In case Kylt® products supplied with Detection-Mix are used in combination with BCD 2x qPCR Mix, preferably the given Kylt® Profile II is applied. For other qPCR Mixes, please refer to the respective Direction For Use, check for appropriateness and adapt duration or temperature of individual steps, if necessary.

Kylt® 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 and HEX		

- Kylt® Profile II allows for combined run of this and most other Kylt® qPCR detection methods.
- Alternatively, the [Kylt® Profile I](#) given below can be applied. Kylt® Profile I allows for combined run of this and most other Kylt® qPCR detection methods as well as Kylt® RT-qPCR detection products that need Reverse Transcription, such as those for detection of viral RNA.
- In case of Kylt® products supplied with Detection-Mix please refer to the according Direction For Use of the qPCR-Mix used to check if using combined Kylt® Profile I is feasible.

Kylt® 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 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

- The amplification data can be processed automatically using the specific software tool of your Real-Time PCR thermal cycler. Alternatively, the threshold can be set manually considering the following directions: The threshold should cross the FAM-curve 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 HEX- and FAM-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 *Riemerella anatipestifer*-specific status of each sample is analyzed (FAM).

Test Evaluation

- The **Real-Time PCR test run** is only **valid** if the FAM-curve of the Negative Control is negative, the HEX-curve of the Negative Control is positive and the FAM-curve of the Positive Control is positive. For a valid test the FAM-Ct-value of the Positive Control has 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	negative
<i>Riemerella anatipestifer</i>	FAM	negative	positive	negative
The sample is <i>Riemerella anatipestifer</i>		negative	positive	inhibited

- A **sample** is **negative for *Riemerella anatipestifer*** if its HEX-curve is positive ($Ct \leq 40$), but its FAM-curve is negative.
- A **sample** is **positive for *Riemerella anatipestifer*** if its FAM-curve is positive ($Ct \leq 42$), independent of the HEX-curve.
- A **sample** is **inhibited** if neither the FAM-curve nor the HEX-curve are 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® Host Cells	31106 / 31107	100 / 25	Kit to detect animal host cells; to verify sample taking process
BCD 2x qPCR-Mix	31129	100	2-fold concentrated Real-Time PCR mix

Production:

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

Kylt® is a registered trademark.



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PROTOCOL AT A GLANCE

Real-Time PCR Setup

Kylt® Products supplied with
Reaction Mix

Kylt® Products supplied with
Detection Mix

1



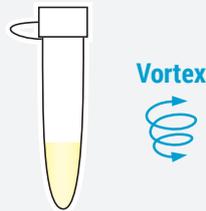
Prepare a Master-Mix*



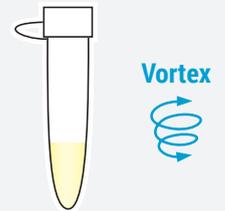
* please refer to chapter 3

2

2.1 Thaw, if applicable
 2.2 Pulse-vortex and spin down

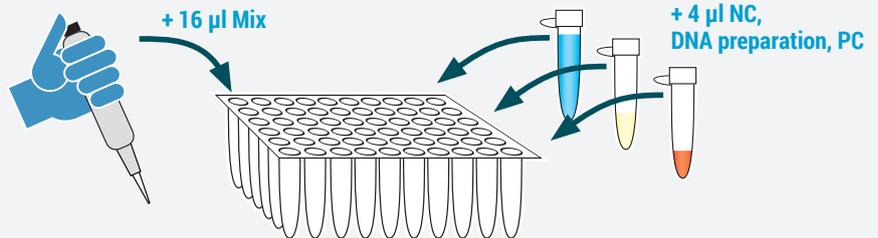


Pulse-vortex and spin down



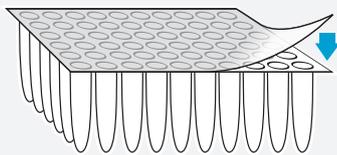
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Dispense Reaction- / Master-Mix and add 4 µl NC, DNA preparation, PC



4

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



5

Analysis

