For *in vitro* Veterinary Diagnostics only.

Kylt®

Kylt® APP

Real-Time PCR Detection

www.kylt.eu

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Revision No.	Amendments
004	Positive Control includes beta-Actin (from batch 20APP:01 on)
003	New Layout

A. General

- Kylt® APP kits are intended for the specific detection of bacterial DNA of *Actinobacillus pleuropneumoniae*. The kits are suitable for the analysis of samples from swine, such as tissues and organs (e.g. lung, tonsils, oral fluids), swab samples of the aforementioned samples and pure or mixed colony material / isolates derived from cultural processes of the aforementioned samples.
- The qualitative testing with Kylt® APP kits is based on a duplex Real-Time PCR: In one reaction setting, the target genes for *Actinobacillus pleuropneumoniae* as well as for the endogenous control (beta-Actin) 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 *Actinobacillus pleuropneumoniae* and the endogenous 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 *Actinobacillus pleuropneumoniae*-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® APP kits are available and comprise the following reagents:

Reagent	Colour of Lid	100 Reactions Article No 31439	25 Reactions Article No 31440	Store at
Reaction-Mix	violet	4 x 450 µl	1 x 450 µl	≤ -18 °C
Positive Control	red	4 x lyophilizate (final 50 µl each)	2 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 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.

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 nm, 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® APPkits:
  - DNA preparation kit / protocol (e.g. Kylt® RNA / DNA Purification products)
  - 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 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 Control is based on detection of beta-Actin DNA, which is ubiquitous in the cells of the host that the sample is derived from. The beta-Actin DNA/endogenouse DNA target gene is co-amplified (channel HEX) with every single reaction and allows for evaluation of sufficient sampling, sample storage and shipment, sample preparation and the Real-Time PCR run itself.

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 supernatant is used for DNA preparation.
- Small swabs may directly be immersed in 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, 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.
- Material derived from cultural processes, i.e. cell culture supernatant or allantoic fluid, can be used directly for DNA preparation.

2. DNA Preparation

a) Kylt® RNA/DNA Purification products

- All kinds of sample matrices, including pure isolates, swabs, tissues and organs may be processed with Kylt® RNA/DNA Purification products (please refer to chapter F "Related Products").
- For detailed information on the DNA preparation process, please refer to the respective Direction For Use.

b) Alternative methods

- All kinds of sample matrices, including pure isolates, swabs, tissues and organs may be processed with appropriate DNA preparation kits 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 two more for the Negative Control and the Positive Control.
- 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 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.

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.

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 *Actinobacillus pleuropneumoniae*-specific status of each sample is analyzed (FAM).

Test Evaluation

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

Target	Channel	Signal		
Internal Control	HEX	positive	positive / negative	negative
Actinobacillus pleuropneumoniae	FAM	negative	positive	negative
The sample is *Actinobacillus pleuropneumoniae*		**negative**	**positive**	**inhibited**

- A **sample** is **negative for *Actinobacillus pleuropneumoniae,*** if its HEX-curve is positive (Ct ≤ 40), but its FAM-curve is negative.
- A **sample** is **positive for *Actinobacillus pleuropneumoniae,*** if its FAM-curve is positive (Ct ≤ 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 using Kylt® RNA/DNA Purification products or appropriate alternative.
- 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® RNA / DNA Purification	31314 / 31315	250 / 50	Combined RNA and DNA purification from all relevant sample matrices
Kylt® RNA / DNA Purification HTP	31826	4x96	Combined, magnetic beads-based purification of RNA and DNA from all relevant sample matrices, suitable for automated high throughput processing
Kylt® Purifier	31436	--	Purification system for magnetic beads. Up to 96 samples in under 30 minutes.

G. Ordering information

For a fast and efficient service please send your order to orders@kylt.eu and please provide the following information:

- Delivery address
- Invoice address
- Purchaser contact telephone number
- End user name and telephone number (if different)
- Purchase order number
- Product name and cataloge number
- Quantity and size of products
- Indicate if your account is VAT exempt

Production:

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

Management System ISO 9001:2015
TÜVRheinland CERTIFIED
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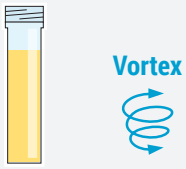
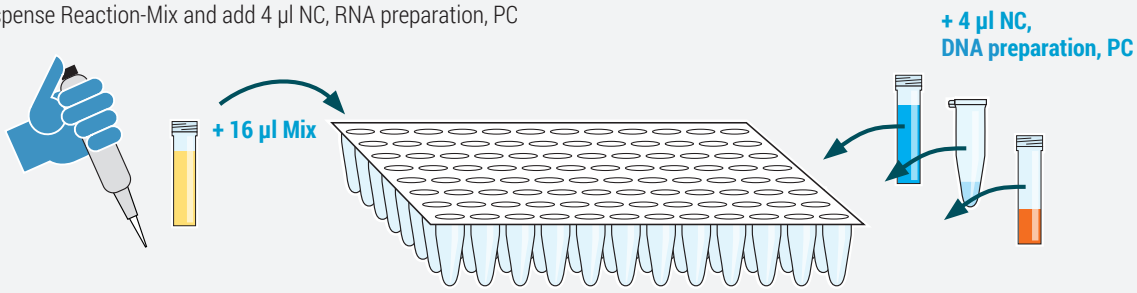
PROTOCOL AT A GLANCE
Real-Time PCR Setup

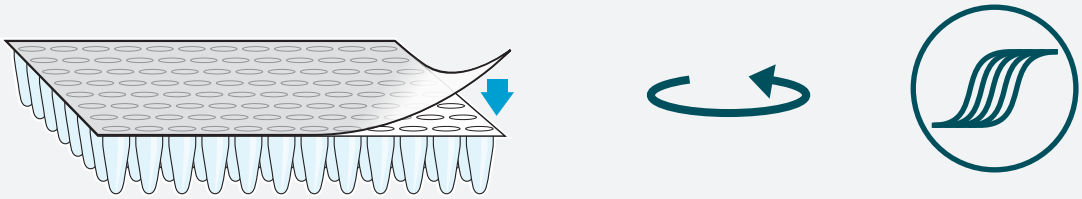
1 Pulse-vortex and spin down



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



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



4 Analysis