Kylt®

For *in vitro* Veterinary Diagnostics only.

# Kylt® ORT

DNA-Extraction & Real-Time PCR Detection Kit
for *Ornithobacterium rhinotracheale*

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## DNA-Extraction & Real-Time PCR Detection Kit for *Ornithobacterium rhinotracheale*

25 / 100 reactions
*in vitro* Diagnosticum for poultry

### A. Introduction

- This Real-Time PCR test detects bacterial DNA of the species *Ornithobacterium rhinotracheale* (ORT). Following DNA extraction a qualitative testing for ORT is conducted by species-specific and real-time detected amplification of an ORT target gene.
- The validated test spectrum includes swab sampling material and tissue samples from poultry as well as pure and mixed cultural material originating from birds.
- This kit was developed for use by trained laboratory personnel following standardized procedures. This Direction For Use must be followed strictly.

## B. Reagents and Materials

- Kylt® ORT contains the following reagents:

| Reagent | Color code of lid | Quantity in kit with 25 / 100 reactions | Storage conditions |
|---|---|---|---|
| DNA Extraction-Mix II | ○ white | 1 x 20 ml | +2 °C to +8 °C |
| Reaction-Mix | ○ transparent | 1 x / 4 x 500 µl | +2 °C to +8 °C |
| Positive Control | ● red | 2 x / 4 x lyophilisate (final 20 µl each) | +2 °C to +8 °C lyophilized<br>+2 °C to +8 °C short term rehydrated<br>-18 °C to -20 °C long term rehydrated |
| Negative Control (Nuclease-free water) | ● blue | 1 x 1 ml | +2 °C to +8 °C |

- The kit and its components are stored at +2 °C to +8 °C. **Alternatively, for long term storage the kit can be stored at -18 °C to -20 °C.** Avoid repeated freezing and thawing of the kit or its components. If occasional processing of few samples is expected, you may prepare aliquots of the Reaction-Mix before storage at -18 °C to -20 °C. Reaction-Mix has to be stored dark, do not expose to direct (sun-)light!
- Before first use, rehydrate the lyophilized Positive Control: add 20 µl of Negative Control (Nuclease-free water), incubate briefly at room temperature and mix thoroughly by repeated vortexing. Storage of aliquots with 5 µl to 10 µl volume (depending on the expected number of Positive Control reactions per kit) at -18 °C to -20 °C is recommended.
- The reagents are to be used within the indicated shelf life.
- This kit can be used on all commercially available Real-Time PCR thermal cyclers that are able to detect the emitted fluorescence of fluorescent dyes FAM and HEX. The following Real-Time PCR thermal cyclers have been validated for routine diagnostics with this kit: Mastercycler® RealPlex2 (Eppendorf), Rotor-Gene® 3000, 6000 & Q (Corbett / QIAGEN), Chromo4™ & CFX96 Touch™ (Bio-RAD), Applied Biosystems® 7500 & 7500 Fast (Life Technologies), Mx3005P (Stratagene / Agilent Technologies), Eco™ (Illumina®), LightCycler® 96 & 480 and LightCycler® 2.0 (Roche).
- We recommend to exclusively use certified Nuclease-free materials and powder-free protective gloves. Pipette tips have to be changed between samples to avoid cross-contamination. Gloves have to be changed frequently, especially after spillage or other suspected contamination.
- Apart from the disposables, the following devices are needed (not included in this kit):
  - Table top microcentrifuge
  - Dry heating block (+100 °C ± 3 °C)
  - Vortex
  - Magnet stirrer
  - Pipetting devices 1 - 10 / 20 µl, 10 - 100 µl, 100 - 1000 µl
  - Centrifuge for PCR tubes or plates
  - Real-Time PCR thermal cycler

## C. Control Reactions

- The Positive Control included in this kit allows for control of the specificity and efficiency of the reagents used and the Real-Time PCR reaction itself. The Negative Control included in the kit allows exclusion of contaminations. The test is only valid if both, Positive and Negative Controls, are used and verified for validity in every Real-Time PCR run.
- An Internal Amplification Control is included in the Reaction-Mix in defined copy number; it is co-amplified (channel HEX) with every single reaction to detect possible inhibitory effect of the DNA preparation and to verify true-negative results.
- If appropriate sampling is unsure we recommend to analyze in parallel with Real-Time (RT-)PCR specific for house-keeping genes of the species, such as the Kylt® Host Cells Real-Time RT-PCR Detection Kit.

## D. Protocol *(see also "Protocol At A Glance" at the end of this Direction For Use)*

- The overall protocol of this ORT-analysis consists of the following main workflow:
  1. sample preparation
  2. DNA extraction
  3. reaction setup and amplification (Real-Time PCR)
  4. data analysis – validity and qualitative result

### 1. Sample Preparation

- We recommend pooling of not more than five individual samples or samples of more than five individuals, respectively, per DNA preparation.
- Pool swabs in adequate volume of sterile buffer (usually 1 ml; alternatively Normal Saline or 0.1 x TE) , let the swabs soak for an adequate period of time and finally wash out the swabs by thoroughly pulse-vortexing them. Immediately transfer the entire washed out supernatant to a conical screw cap tube.
- Alternatively, you may skip this procedure and extract the DNA directly out of the swab (only recommended for single small-sized swabs).
- Pure or mixed cultural material is directly transferred into conical screw cap tubes.
- Tissue samples are thoroughly homogenized and a suitable volume is utilized for DNA preparation using appropriate kits.

### 2. DNA Extraction

- Pre-heat heating-block to set temperature of +100 °C, the block must have an actual temperature of +100 °C ± 3 °C at first use.
- The swab washout or cultural material in the conical screw cap tube is pelleted by centrifugation at 10.000 g to 12.000 g for 10 min.

- Remove the supernatant using a 1000 µl pipette tip (not by decantation) and discard it.
- The DNA Extraction-Mix II is stirred on a magnetic stirrer, it must be used as a homogenous suspension. The pellet is resuspended by repeated up-and-down pipetting with 20 µl to 200 µl of DNA Extraction-Mix II. Avoid formation of bubbles and aerosols. The volume of DNA Extraction-Mix II for resuspension of the pellet should be chosen as small as possible, depending on the size of the pellet (e.g. for hardly visible pellets of tracheal swabs use 50 µl), to minimize dilution effects.
- Screw cap tight, vortex thoroughly and incubate for 10 min to 15 min at +100 °C ± 3 °C.
- Vortex sample thoroughly and centrifuge at 10.000 g to 12.000 g for 5 min; the supernatant is the DNA-Extract and can be used for Real-Time PCR immediately. Short-term storage (few hours) of the DNA-Extract at +2 °C to +8 °C is possible. For long-term storage of the DNA-Extract at -18 °C to -20 °C, take supernatant and transfer to new (screw cap) tube. Before next use in Real-Time PCR incubate DNA-Extracts that were stored at -18 °C to -20 °C for few minutes at +100 °C ± 3 °C, vortex and spin down.
- For preparation of DNA from homogenates of tissue samples we recommend to use appropriate commercial systems, such as the Kylt® RNA/DNA Isolation Kit.

## 3. PCR Setup and Amplification

- Reaction-Mix and Negative Control are vortexed and spun down before each use.
- Determine the number of needed PCR-reactions: number of samples and add two more for the Negative Control and the Positive Control.
- Pipette 18 µl of Reaction-Mix to each PCR reaction tube / each PCR plate well (»cavity«). Keep exposure of Reaction-Mix to light as short as possible!
- Add 2 µl of Negative Control to the corresponding cavity and cap it.
- Add 2 µl of prepared DNA (DNA-Extract or eluted DNA) to the corresponding cavities and cap them. Solely use the clear supernatant of the DNA extraction. Avoid carry-over of particles from the pellet.
- Once all sample cavities are sealed, 2 µl of the Positive Control is added to corresponding cavity and sealed. The Positive Control is vortexed and spun down before each use.
- Place PCR cavities into the Real-Time PCR thermal cycler and start amplification, using the following parameters:

| Step | Temperature | Duration | |
|---|---|---|---|
| Activation Polymerase | 95 °C | 10 min | |
| Denaturation | 95 °C | 15 sec | 42 cycles |
| Annealing & Extension | 60 °C | 1 min | |
| Fluorescence Detection | channels FAM and HEX | | |

- Please follow the specific instructions of your Real-Time PCR thermal cycler as recommended by the manufacturer.
- The amplification parameters above allow for combination of this Kylt® ORT with other Kylt® Real-Time PCRs for detection of bacteria. When combining Kylt® detection kits for different pathogens, make sure all necessary channels are used!

## 4. Data Analysis – Validity and Qualitative Results

### General

- The data of the amplification reactions can be automatically processed using specific software for the Real-Time PCR thermal cycler. Alternatively a threshold can be set manually, with following considerations: The threshold should cross the HEX-curve of the Negative Control and the FAM-curve of the Positive Control, respectively, in the linear area of its slope. By setting the thresholds, 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.
- 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 logarithmic scaling), should be considered as positive.
- For the test analysis the validity of the run is determined with the Negative and Positive Controls. After that the validity of each single sample is tested by Internal Amplification Controls and the ORT-specific status is checked.

## Test Evaluation

- The **Real-Time PCR run** is only **valid** if the FAM-curve of the Negative Control is negative, if the HEX-curve of the Negative Control is positive and if 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 ≤ 38 and the HEX-Ct-value of the Negative Control has to be > 15 and ≤ 40.

| | | | | |
|---|---|---|---|---|
| HEX-curve positive | yes | yes | no | no |
| FAM-curve positive | no | yes | yes | no |
| **The sample is ORT** | **negative** | **positive** | **positive** | **inhibited** |

- The **sample** is **ORT-negative** when its HEX-curve is positive (Ct ≤ 40), but its FAM-curve is negative.
- The **sample** is **ORT-positive** when its FAM-curve is positive (10 < Ct ≤ 42), independent of the HEX-curve.
- The **sample** is **inhibited** when neither the HEX-curve nor the FAM-curve are positive.
- **Recommendation:** In case of an inhibited sample the test should be repeated with a dilution of the DNA preparation at 1:10 (9 volumes Negative Control + 1 volume DNA-Extract or eluted DNA). The Negative Control is used as the diluting agent. Alternatively, the original sample or the DNA-Extract can be utilized for DNA preparation using appropriate alternative systems, such as the Kylt® RNA/DNA Isolation Kit.
- Convenient and reliable sample data entry, start of the Real-Time PCR run, final qualitative analysis and documentation can be conducted with the Kylt® Software package, please inquire.

Production:
AniCon Labor GmbH | Muehlenstr. 13a | D-49685 Hoeltinghausen | Germany | www.anicon.eu | www.kylt.eu



For veterinary use only. For *in vitro* use only. Regulatory requirements vary by country, this product may not be available in your geographic area.

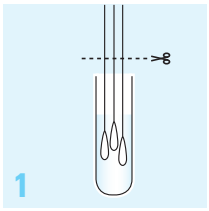
# PROTOCOL AT A GLANCE

## DNA Extraction and Real-Time PCR

**Swab samples (e.g. tracheal or joint swabs, swabs from lung and air sacs) and pure and mixed cultural material**

### 1. Sample handling

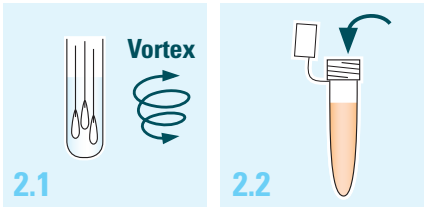
Pool max. 5 samples in tube with saline (0.9%) or 0.1 x TE




### 2. Harvest

2.1 wash by vortexing

2.2 transfer total supernatant




### 3. DNA Extraction

3.1 10.000-12.000 g 10 min

3.2 Discard supernatant

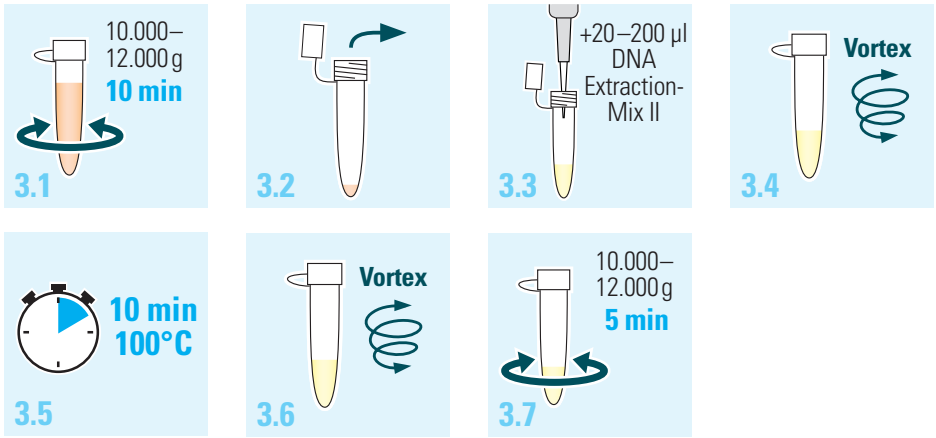
3.3 Add 20–200 µl DNA Extraction-Mix II

3.4 Mix by vortexing

3.5 Incubation 10 min 100°C

3.6 Mix by vortexing

3.7 10.000–12.000 g 5 min

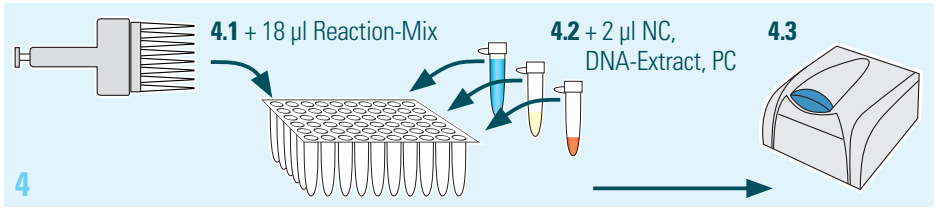



### 4. PCR Setup

4.1 Mix Reaction-Mix and dispense

4.2 Add 2 µl NC, DNA-Extract, PC

4.3 Seal cavities and amplify




### 5. Analysis

Set threshold and analyse samples