Kylt®

V For *in vitro* Veterinary Diagnostics only.

Kylt® Influenza A

Real-Time RT-PCR Detection Kit
for Influenza Virus Type A

www.kylt.eu

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25 / 100 reactions,
in vitro Diagnosticum for birds and swine

A. General

- Kylt® Influenza A Real-Time RT-PCR Detection Kit is for detection of Influenza Virus Type A in samples from birds and swine, such as tissues and organs (e.g. trachea, lung, cecal tonsils), swab samples (e.g. nasal, tracheal or cloacal swabs), saliva- and BAL-samples (BALF / bronchoalveolar lavage fluid, swine only) and sampling material derived from cultural processes with the aforementioned samples (e.g. cell culture supernatant, allantoic fluid).
- Kylt® Influenza A comprises all reagents and controls for detection of viral RNA of Influenza Virus Type A. The qualitative testing of Kylt® Influenza A is based on a duplex Real-Time RT-PCR system: In one reaction setting the target genes for Influenza Virus Type A as well as for the spiked Kylt® Internal Control RNA (IC-RNA) are reverse transcribed (Reverse Transcription (RT)) and amplified in parallel by respective primer pairs in the subsequent Polymerase Chain Reaction (PCR). Amplified target gene fragments are detected via fluorescently labeled probes during the PCR reaction in real-time (Real-Time RT-PCR). The probes specific for detection of amplified Influenza Virus Type A and IC-RNA 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 IVA Positive Control per run the Influenza A-specific status of a sample can be evaluated in the end. This way, results can be achieved within few hours after sample receipt.
- 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® Influenza A contains the following reagents:

Reagent	Color code of lid	Quantity in kit with 25 / 100 reactions	Storage conditions
2x RT-qPCR-Mix	○ transparent	1 x / 4 x 280 µl	-18 °C to -20 °C
IVA Detection-Mix	● orange	1 x / 4 x lyophilisate (final 150 µl each)	-18 °C to -20 °C
IVA Positive Control (PC)	● red	2 x / 4 x lyophilisate (final 50 µl each)	-18 °C to -20 °C
Negative Control (NC)	● blue	2 x 1 ml	-18 °C to -20 °C
Kylt® Internal Control RNA (IC-RNA)	● black	1 x / 2 x lyophilisate (final 500 µl each)	-18 °C to -20 °C

- After receipt the kit and its components are immediately stored at -18 °C to -20 °C. Avoid repeated freezing and thawing of the kit or its components and keep them thawed as short as possible. If needed, generate appropriate aliquots upon its first thawing. The kit and its components are to be used within the indicated shelf life (see label on the outer packing), if stored properly. The components of different batches may not be mixed.
- The IVA Detection-Mix needs to be stored protected from light at -18 °C to -20 °C. Do not expose it to direct sunlight. Before first use the lyophilized IVA Detection-Mix is rehydrated: add 150 µl of Negative Control per vial of IVA Detection-Mix, briefly incubate at room temperature and mix by repeated vortexing. Generate aliquots of suitable volumes and store them at -18 °C to -20 °C.
- Before its first use rehydrate the IC-RNA by adding 500 µl of Negative Control per vial, briefly incubate at room temperature and mix by repeated vortexing. Generate aliquots of suitable volumes and store them at -18 °C to -20 °C.
- Before its first application rehydrate the lyophilized IVA Positive Control by adding 50 µl of Negative Control per vial, briefly incubate at room temperature and mix by repeated vortexing. Generate aliquots of suitable volumes and store them at -18 °C to -20 °C.
- The kit can be used on all commercially available Real-Time PCR thermal cyclers that detect the emitted fluorescence of the fluorescent dyes FAM and HEX (except glass-capillary based thermal cyclers). The following Real-Time PCR thermal cyclers e.g. have been verified 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®), qTOWER *rapid*PCR & qTOWER 2.0 (Analytik Jena), LightCycler® 96 (Roche).
- 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. Pipette tips must be changed between each sample to avoid cross-contaminations.

- Apart from the disposables, the following equipment is needed (not included in this kit):
  - RNA extraction kit / protocol (e.g. Kylt® RNA-/DNA-Isolation Kit)
  - Table top microcentrifuge
  - Vortex
  - Micropipettes covering volumes of 1 µl to 1000 µl
  - Centrifuge for PCR tubes or plates
  - Real-Time PCR thermal cycler

C. Control Reactions

- The Negative Control included in this kit provides the ability to exclude possible contaminations of the reagents. The IVA Positive Control included in this kit provides the ability to monitor the specificity and efficiency of the reagents used, i.e. the performance of RT and Real-Time PCR itself and of the Real-Time PCR thermal cycler, respectively. The sample testing is only valid if both the Negative Control and the IVA Positive Control reactions are included in every Real-Time RT-PCR run and used to verify the validity of such run.

- The IC-RNA is added to the respective lysis buffer in standardized copy number before RNA preparation and co-purified with each sample. In case of a successful RNA preparation and the absence of RT- and Real-Time PCR-inhibitors the IC-RNA can be detected in the Internal Control channel (HEX). This channel then is used to confirm true-negative test results by verifying successful RNA preparation and by excluding the presence of factors in the RNA preparation that are inhibitory to Real-Time RT-PCR.

- It is recommended to run one or more of a RNA Isolation Control (RIC) per set of RNA preparations, depending on the total number of samples processed at once. The RIC is a "mock sample" composed of the plain sterile buffer used for raw sample processing. It is randomly placed between the samples, processed like a normal sample and enables for detection of potential contaminations of the reagents used (additionally to the Negative Control reaction) as well as for detection of potential carryover contaminations between individual samples during the RNA preparation process.

- If proper sampling is unsure you may further analyze the sample in parallel with an appropriate Real-Time (RT-)PCR specific for housekeeping 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 the IVA analysis consists of the following main workflow:
  1. sample processing
  2. RNA preparation
  3. reaction setup, Reverse Transcription and amplification (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 RNA preparation at -18 °C to -20 °C or -70 °C to -80 °C until further processing of the IVA detection. Avoid repeated freezing and thawing of the RNA preparations.

1. Sample Processing

- For the sample processing the relevant EU regulations should be observed (e.g. EU 2005/734/EC and 2006/437/EC for avian samples). With the use of Kylt® Influenza A it could be demonstrated that a pooling of up to a maximum of ten samples or samples of ten individuals per RNA preparation is possibile without a reduction in sensitivity.

- Swabs are pooled in a sufficient volume of sterile buffer (e.g. Normal Saline or 0.1 x TE) and soaked for an adequate period of time. Then, the sample is washed out thoroughly by pulse-vortexing and the washed out supernatant is used for RNA preparation. Single small swabs may directly be immersed in lysis buffer.
- Tissue samples are homogenized thoroughly in sterile buffer (see above) and a suitable volume is administered to RNA preparation.
- Cultural material is directly processed with an appropriate RNA preparation protocol.

2. RNA Preparation

- Prepare the according lysis buffer of the RNA preparation kit to be ready for use. Add 5 µl of rehydrated IC-RNA (see also chapter B "Reagents and Materials") per sample preparation to according volume of lysis buffer before adding the processed sample material.
- **Recommendation:** Assemble respective lysis buffer with IC-RNA in larger volume, aliquot in respective final volumes for individual sample preparation (e.g. in microcentrifuge tubes) and store these aliquots at -18 °C to -20 °C upon thawing for direct usage in RNA preparation.
- RNA that was prepared with commercially available RNA preparation kits, such as the Kylt® RNA-/DNA-Isolation Kit or alternative kits is suitable for the application of Kylt® Influenza A. As a reference, the concentration of the readily prepared RNA should be between 1 pg/µl and 1 µg/µl. Ratio OD260/280 should be between 1.8 and 2.2 to ensure proper quality of the purified RNA.
- For detailed information on the RNA preparation process please refer to the manual of the respective kit.

3. Reaction Setup, Reverse Transcription and Amplification

- Before each use, briefly vortex and spin down the 2x RT-qPCR-Mix and the rehydrated IVA Detection-Mix (see also chapter B "Reagents and Materials").
- To determine the total number of reactions needed, count the number of samples (including the RIC(s), if processed) and add two more for the Negative Control and the IVA Positive Control.
- Prepare a Master-Mix containing the 2x RT-qPCR-Mix and the IVA Detection-Mix for the appropriate number of reactions. Then add 16 µl of the Master-Mix to each of the PCR plate wells / cavities. The Real-Time RT-PCR is set up in the given order:

Reagent	Volume (µl)	
	Per reaction	e.g. n=7
2x RT-qPCR-Mix	10.0	70.0
IVA Detection-Mix	6.0	42.0
Total Master-Mix	**16.0**	**112, dispense 16 µl per reaction**
RNA (Negative Control / sample (/ RIC) / IVA Positive Control)	4.0	
Total Reaction	**20.0**	

- Expose the 2x RT-qPCR-Mix and the IVA Detection-Mix as short as possible to (sun)light and return both back to -18 °C to -20 °C 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 the sample RNA (final RNA preparations - including RIC(s), if processed) to the corresponding cavity and seal it individually, if possible.
- To minimize risk of potential cross-contaminations, 4 µl of the IVA 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 IVA Positive Control (see also chapter B "Reagents and Materials").
- If not already done, finally seal the cavities. It is recommended to spin them down before start of the Real-Time RT-PCR run.
- Place the cavities in the Real-Time PCR thermal cycler and run the test using the following parameters:

Step	Temperature	Duration	
Reverse Transcription	50 °C	10 min	
Activation of Polymerase	95 °C	1 min	
Denaturation	95 °C	10 sec	42 cycles
Annealing & Extension	60 °C	1 min	
Fluorescence Detection	channels FAM and HEX		

- Please follow the specified instructions of your Real-Time PCR thermal cycler as recommended by the manufacturer.
- These parameters enable the combination of Kylt® Influenza A with further Kylt® Real-Time RT-PCRs detecting viral RNA. If detection kits for different pathogens are combined, please make sure to detect all relevant channels.

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 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 RT-PCR reaction.
- The actual test analysis starts with the validity check of the entire Real-Time RT-PCR run. Afterwards, by means of the IC-RNA 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 IVA-specific status of each sample (and the RIC(s), if processed) is analyzed (FAM).
- The test certificate of the respective kit batch gives Ct-values for the detection of Positive Control (FAM) and Internal Control (HEX) for the listed specific Real-Time PCR thermal cyclers during quality testing. The experienced Ct-values should be achieved within a maximum deviation of ± 3. Otherwise the Ct-values for analyses must be adjusted to the specific conditions of laboratory and thermal cycler by verification according to the quality management system.

Test Evaluation

- The **Real-Time RT-PCR test run** is only **valid** if the FAM-curve of the Negative Control is negative (Ct > 42) and the FAM-curve of the IVA Positive Control is positive. For a valid test the FAM-Ct-value of the IVA Positive Control has to be within the acceptable range of the experienced Ct-value.
- If one or more of a RNA Isolation Control (RIC(s)) is processed, its FAM-curve must be negative (Ct > 42) and the HEX-Ct-value of the IC-RNA has to be within the acceptable range of the experienced Ct-value.

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

- A **sample** is IVA **negative** if its FAM-curve is negative (Ct > 40) and the HEX-curve is within the acceptable range of the experienced Ct-value.
- A **sample** is IVA **positive** if its FAM-curve is significant positive (10 ≤ Ct < 35), independent of the HEX-curve. A sample with a weak positive FAM-curve (35 ≤ Ct ≤ 40) has to be evaluated as **questionable**. The limits of Ct-values must be adjusted, if necessary, to the specific conditions of laboratory and thermal cycler by verification according to the quality management system.
- A **sample** is **inhibited** if neither the FAM-curve (Ct > 40) nor the HEX-curve is positive (deviation of the experienced Ct-value > 3).
- **Recommendation:** In the case of an inhibited sample you may repeat the test by using e.g. a 1:4 dilution of the respective RNA elution. The Negative Control serves as the diluting agent. Preferably, repeat the RNA preparation procedure from the start using less raw sample material. Afterwards, you may additionally conduct an ethanol precipitation to concentrate the RNA.
- Convenient and reliable sample data input, start of the Real-Time RT-PCR run as well as final qualitative test result analysis and documentation can be managed by the Kylt® PCR Software package. Please feel free to contact us.

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

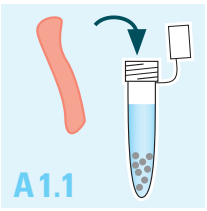
Sample Processing, RNA Preparation and Real-Time RT-PCR

A Organ / tissue samples, e.g. trachea, lung, cecal tonsil

1. Sample Handling

1.1 transfer tissue to tube with saline (0,9%) or 0.1 x TE

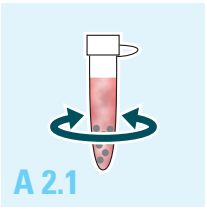
1.2 homogenisation

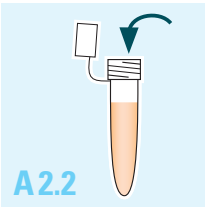


2. Harvest

2.1 centrifugation

2.2 transfer total supernatant

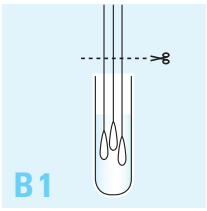




B Other samples e.g. tracheal swab, cloacal swab, BALF, cultural material

1. Sample Handling

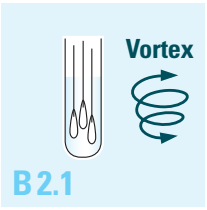
pool permissible number of samples in tube with saline (0.9%) or 0.1 x TE

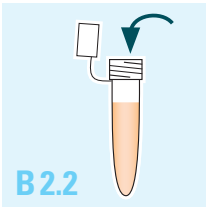


2. Harvest

2.1 wash by vortexing

2.2 transfer total supernatant



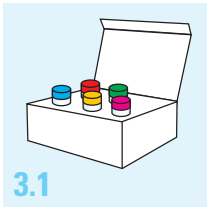


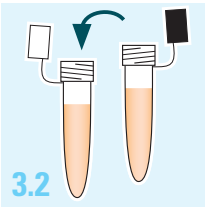
3. RNA Preparation

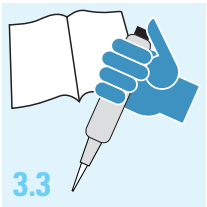
3.1 use a commercially available RNA preparation kit

3.2 add 5 µl of Kylt® IC-RNA per preparation to lysis buffer first

3.3 for further preparation refer to Direction For Use of respective kit





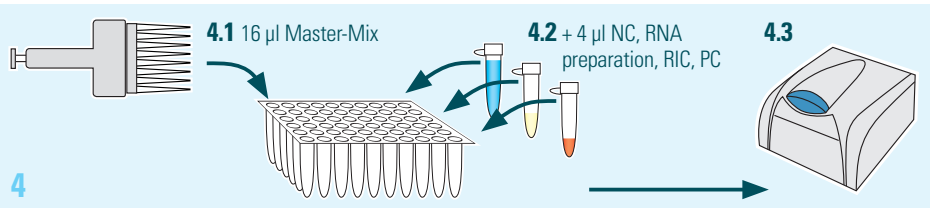


4. Real-Time RT-PCR Setup

4.1 prepare Master-Mix and dispense

4.2 add 4 µl NC, RNA preparation, RIC, PC

4.3 seal cavities and run reaction



5. Analysis

set threshold and analyze samples