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Kylt[®]

Kylt[®] Influenza A - H5N1

Real-Time RT-PCR Detection

www.kylt.eu



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Real-Time RT-PCR Detection

| Revision No. | Amendments |
|--------------|----------------------------|
| 002 | Change of fluorescent dyes |

A. General

- Kylt® Influenza A - H5N1 kits are intended for the specific detection of viral RNA of Influenza Virus Type A subtype H5N1. The kits are suitable for the analysis of samples from birds such as swab samples (e.g. cloacal, choanal or tracheal), tissues and organs (e.g. trachea, lung, cecal tonsils) and samples from cultural processes with the aforementioned sample material.
- The qualitative testing with Kylt® Influenza A - H5N1 kits is based on a triplex Real-Time RT-PCR: In one reaction setting, the RNA target sequences for Influenza Virus Type A subtype H5N1 as well as for the endogenous control are reverse transcribed (Reverse Transcription (RT)) and amplified in parallel with 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 Influenza Virus Type A subtype H5, Influenza Virus Type A subtype N1 and the endogenous control target genes are labeled with fluorescent dyes Cy5, FAM and HEX, respectively, and their emitted fluorescence is separately optically measured by the Real-Time PCR thermal cycler. By means of all three individual analyses in one reaction vessel per sample and the Negative Control and Positive Control per run the Influenza Virus Type A subtype H5N1-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® Influenza A - H5N1 kits are available and comprise the following reagents:

| Reagent | Colour of Lid | 100 Reactions Article No 31778 | 25 Reactions Article No 31779 | Store at |
|------------------|---------------|---|---|----------|
| 2x RT-qPCR-Mix | ○ transparent | 4 x 280 µl | 1 x 280 µl | ≤ -18 °C |
| Detection-Mix | ● violet | 4 x lyophilizate (final 150 µl each) | 1 x lyophilizate (final 150 µl each) | ≤ -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.
- 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 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, HEX and Cy5 (emission 520, 550 and 670 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® Influenza A - H5N1 kits:
 - RNA 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 RT and 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 RNA, which is ubiquitous in the cells of the host that the sample is derived from. The beta-Actin RNA/endogenous RNA 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 RT-PCR run itself.
- It is recommended to run one or more of a RNA Isolation Control (RIC) per set of RNA preparation, 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 allows to detect potential contaminations of the reagents used (additionally to the Negative Control reaction) as well as for the detection of potential carryover contaminations between individual samples, e.g. during the RNA preparation process.

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. RNA Preparation
 3. Reaction Setup, Reverse Transcription and Amplification (Real-Time RT-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 $\leq -18\text{ °C}$ or $\leq -70\text{ °C}$ until further processing. Avoid repeated freezing and thawing of the RNA preparations.

1. Sample Preparation

- We recommend pooling of at most five samples or samples from five individuals, respectively, per RNA 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 RNA preparation.
- Tissue and organ samples are homogenized thoroughly in sterile buffer (see above) and a suitable volume is used for the RNA preparation.
- Material derived from cultural processes, i.e. cell culture supernatant or allantoic fluid, can be used directly for RNA preparation.

2. RNA Preparation

a) Kylt® RNA Preparation (requires Kylt® RNA / DNA Purification products (available separately))

- For detailed information on the RNA preparation process please refer to the manual of Kylt® RNA / DNA Purification products.

b) RNA Preparation by other Methods

- Other kits or in-house methods to purify RNA may be used, as long as the quality leads to satisfactory amplification and detection of the Internal Control RNA.

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

- Before each use, briefly vortex and spin down the 2x RT-qPCR-Mix, 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 (and RIC(s), if processed).
- Prepare the Master-Mix using the components listed below. A larger volume of a ready to use 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 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 μ l of the finalized Master-Mix to each of the PCR tubes or plate wells ("cavities").

| Reagent | Volume (μ l) | |
|---|-------------------------------|--|
| | per Reaction | e.g. n=7 |
| 2x RT-qPCR-Mix | 10 μ l | 70 μ l |
| Detection-Mix | 6 μ l | 42 μ l |
| Total Master-Mix | 16 μl | 112 μl dispense 16 μ l per reaction |
| RNA (Negative Control / sample RNA / RIC(s) / Positive Control) | 4.0 μ l | |
| Total Reaction | 20.0 μl | |

- Keep exposure of the 2x RT-qPCR-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 RNA preparation (including RIC(s), if processed) 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 KyIt® Profile I as given below.

| KyIt® 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 | | |

- KyIt® Profile I allows for combined run of this and most other KyIt® RT-qPCR detection methods as well as KyIt® PCR detection products.
- 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, the Cy5-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-, Cy5- 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.
- 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 RT-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 Influenza Virus Type A subtype H5N1-specific status of each sample is analyzed (FAM and Cy5).

Test Evaluation

- The **Real-Time RT-PCR test run** is only **valid** if the FAM- and Cy5-curves of the Negative Control are negative and the FAM- and Cy5-curves of the Positive Control are positive. For a valid test the FAM- and Cy5-Ct-values of the Positive Control have to be > 15 and ≤ 35 .
- The HEX-curve of the Negative Control is negative ($Ct > 35$) and the HEX-curve of the Positive Control is positive ($Ct \leq 35$).
- If one or more of a RNA Isolation Control (RIC(s)) is processed, its FAM- and HEX-curves must be negative ($Ct > 35$ for HEX-curves).

| Target | Channel | Signal | | | | |
|---|---------|-----------------|---------------------|---------------------|---------------------|------------------|
| Internal Control | HEX | positive | positive / negative | positive / negative | positive / negative | negative |
| Influenza A - H5 | Cy5 | negative | positive | negative | positive | negative |
| Influenza A - N1 | FAM | negative | negative | positive | positive | negative |
| The sample is Influenza A - H5 | | negative | positive | negative | positive | inhibited |
| The sample is Influenza A - N1 | | negative | negative | positive | positive | |
| The sample is Influenza A - H5N1 | | negative | negative | negative | positive | |

- A **sample is negative for Influenza Virus Type A subtype H5N1**, if its HEX-curve is positive ($Ct \leq 35$), but its FAM- and Cy5-curves are negative.
- A **sample is positive for Influenza Virus Type A subtype H5**, if its Cy5-curve is positive ($Ct \leq 42$), independent of the HEX- and FAM-curves.
- A **sample is positive for Influenza Virus Type A subtype N1**, if its FAM-curve is positive ($Ct \leq 42$), independent of the HEX- and Cy5-curves.
- A **sample is positive for Influenza Virus Type A subtype H5N1** if its FAM- and Cy5-curves are positive ($Ct \leq 42$), independent of the HEX-curve.
- A **sample is inhibited** if neither the FAM-curve nor the Cy5- and HEX-curves are positive.
- **Recommendation:** In the case of an inhibited sample you may repeat the test by using e.g. 1:4 dilution of the respective RNA preparation. The Negative Control is used as the diluting agent. Preferably, the entire RNA 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 veterinary samples |
| Kylt® RNA / DNA Purification HTP | 31826 | 4x96 | Combined, magnetic beads-based purification of RNA and DNA from veterinary samples, suitable for automated high throughput processing |
| Kylt® Influenza Virus Type A | 31068 / 31069 | 100 / 25 | Kit to detect Influenza Virus Type A (artificial Internal Control) |
| Kylt® IVA beta | 31163 / 31164 | 100 / 25 | Kit to detect Influenza Virus Type A (beta Actin as Internal Control) |

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.

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.

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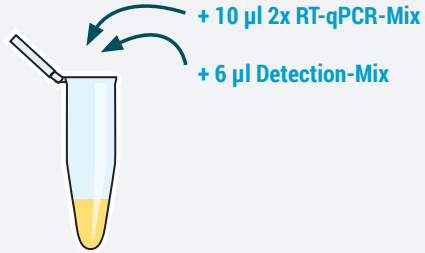


PROTOCOL AT A GLANCE

Real-Time RT-PCR Setup

1

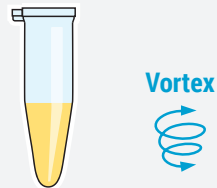
Prepare a Master-Mix*



* please refer to chapter 3

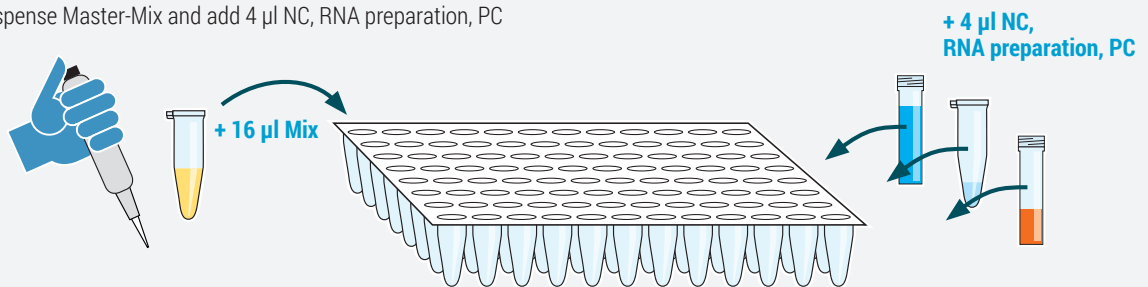
2

Pulse-vortex and spin down



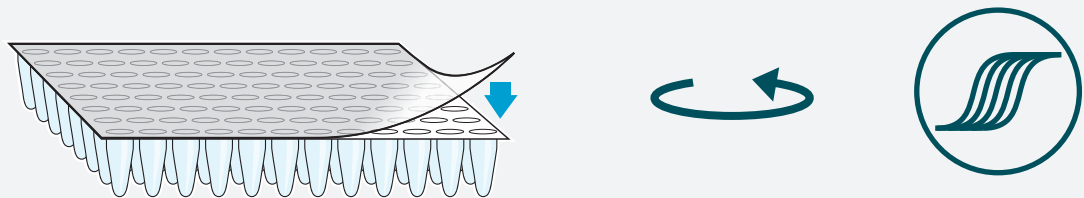
3

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



4

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



5

Analysis

