Kylt® ASF/CSF Triplex

Real-Time RT-PCR for the detection of African Swine Fever Virus & Classical Swine Fever Virus

www.kylt.eu

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A. General

- Kylt® ASF/CSF Triplex kits are intended for the specific detection of viral DNA of African Swine Fever Virus (ASFV) as well as of viral RNA of Classical Swine Fever Virus (CSFV). The kits are suitable for the analysis of samples from swine and wildboar, such as blood, tissues, as well as swabs and preservative transport media.
- The qualitative testing with Kylt® ASF/CSF Triplex kits is based on a triplex Real-Time RT-PCR: In one reaction setting, the target genes for African Swine Fever Virus, Classical Swine Fever Virus 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 ASFV, CSFV 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 both individual analyses in one reaction vessel per sample and the Negative Control and Positive Control per run the ASFV- and CSFV-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® ASF/CSF Triplex kits are available and comprise the following reagents:

Reagent	Colour of Lid	100 Reactions Article No 31824	25 Reactions Article No 31825	Store at
2x RT-qPCR-Mix	transparent	4 x 280 µl	1 x 280 µl	≤ -18 °C
Detection-Mix	brown	4 x lyophilizate (final 150 µl each)	1x 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 Cy5, FAM and HEX (emission 670, 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® ASF/CSF Triplex kits:
  - DNA/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 the Real-Time RT-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 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. DNA/RNA Preparation
3. Reaction Setup 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 DNA/RNA preparation at ≤ -18 °C until further processing. Avoid repeated freezing and thawing of the DNA/RNA preparations.

1. Sample Preparation

- The kit is validated for pooling of at most 20 swab samples. However, we recommend pooling of at most five samples or samples from five individuals, respectively, per DNA/RNA preparation. Please consider your local regulatory guidelines for pooling as released by respective official institutions.
- 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/RNA preparation.
- Small swabs may directly be immersed in 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/RNA preparation.
- Material derived from cultural processes, i.e. cell culture supernatant, can be used directly for DNA/RNA preparation.

2. DNA Preparation

a) Kylt® RNA/DNA Purification products

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

b) Alternative methods

- All kinds of sample matrices, including blood, tissues, as well as swabs and preservative transport media may be processed with appropriate DNA/RNA preparation kits or appropriate in-house methods.
- For detailed information on the DNA/RNA 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 RT-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.
- 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 µl of the finalized Master-Mix to each of the PCR tubes or plate wells ("cavities").

Reagent	Volume (µl)	
	per Reaction	e.g. n=7
2x RT-qPCR-Mix	10 µl	70 µl
Detection-Mix	6 µl	42 µl
Total Master-Mix	**16 µl**	**112 µl** dispense 16 µl per reaction
RNA (Negative Control / sample RNA / RIC(s) / Positive Control)	4.0 µl	
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 DNA/RNA 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 I as given below.

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 Cy5, FAM and HEX		

- Kylt® Profile I allows a 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 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 Cy5-, FAM- and the HEX-curves in the linear increase of their slope (log scaling of the y-axis). By setting the threshold, the crossing points with the Cy5-, FAM- and the HEX-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 ASFV,- CSFV-specific status of each sample is analyzed (Cy5 and FAM).

Test Evaluation - Control Reactions

- The **Real-Time RT-PCR test run** is only **valid** if the curves of the control reactions can be evaluated as follows:

Control Reactions	Channel		
	HEX	Cy5	FAM
Negative Control	negative	negative	negative
Positive Control	**positive**	**positive**	**positive**

- For a valid test the Ct-values (HEX, Cy5 and FAM) of the Positive Control have to be > 15 and ≤ 35.
- The Ct-values of the Negative Control are negativ (Ct > 35).
- If one or more of a RNA Isolation Control (RIC(s)) is processed, its HEX-, Cy5- and FAM-curves must be negative (Ct > 35 for HEX-curves).

Test Evaluation - Samples

Target	Channel	Signal			
Internal Control	HEX	**positive**	positive / negative	positive / negative	negative
ASFV	Cy5	negative	**positive**	negative	negative
CSFV	FAM	negative	negative	**positive**	negative
The sample is African Swine Fever Virus		**negative**	**positive**	**negative**	**inhibited**
The sample is Classical Swine Fever Virus		**negative**	**negative**	**positive**	

- A **sample** is **negative for African Swine Fever Virus (ASFV) and for Classical Swine Fever Virus (CSFV),** if its HEX-curve is positive (Ct ≤ 35), but its Cy5- and FAM-curves are negative.
- A **sample** is **positive for African Swine Fever Virus (ASFV),** if its Cy5-curve is positive (Ct ≤ 42), independent of the FAM- and HEX-curve.
- A **sample** is **positive for Classical Swine Fever Virus (CSFV),** if its FAM-curve is positive (Ct ≤ 42), independent of the Cy5- and HEX-curve.
- A **sample** is **inhibited,** if neither the HEX-curve nor the Cy5- and FAM-curves are positive.
- **Recommendation:** In the case of an inhibited sample the test may be repeated with a dilution of the DNA/RNA preparation at e.g. 1:10 (9 volumes Negative Control + 1 volume DNA/RNA Extract or eluted DNA/RNA). The Negative Control is used as the diluting agent. Preferably, the entire DNA/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	Content / Reactions	Description
Kylt® RNA / DNA Purification	31315	50	Combined RNA and DNA purifi cation from veterinary samples (spin-column based).
Kylt® RNA / DNA Purification HTP	31826	4 x 96	Magnetic bead based combined RNA and DNA purification kit for veterinary diagnostic samples. Suitable for Kylt® Purifier and Kylt® Purifier 48.
Kylt® Purifier	31436	1 unit	Purification system for magnetic bead based kits. Up to 96 samples are processed in under 30 minutes. Intended for high-throughput laboratories.
Kylt® Purifier 48	31436	1 unit	Purification system for magnetic bead based kits. Up to 48 samples are processed in under 30 minutes. Intended for low to medium throughput laboratories.
Kylt® Purifier Spin Tips	31434	5 Sets	Plate with 96 separate spin tips, used by the Kylt® Purifer to mix the well contents by stirring. Sufficient for 480 samples.
Kylt® Purifier Plates	31435	20 Plates	Plates to be used for the several reactions and reagents during automated nucleic acid purification. Sufficient for 320 to 480 samples (depending on device and protocol) .

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 catalogue number
- Quantity and size of products
- Indicate if your account is VAT exempt

Production:

AniCon Labor GmbH | Muehlenstr. 13 | D-49685 Hoeltinghausen | Germany | www.kylt.eu | info@kylt.eu

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

Management System ISO 9001:2015
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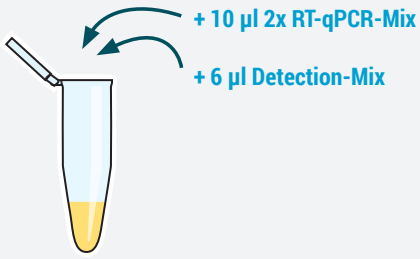
www.tuv.com ID 9000003434

RT-qPCR.CASFV.lyo.02, Rev001, June 2022

PROTOCOL AT A GLANCE
Real-Time RT-PCR Setup

1

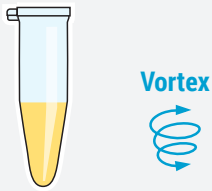
Prepare a Master-Mix*



* please refer to chapter 3

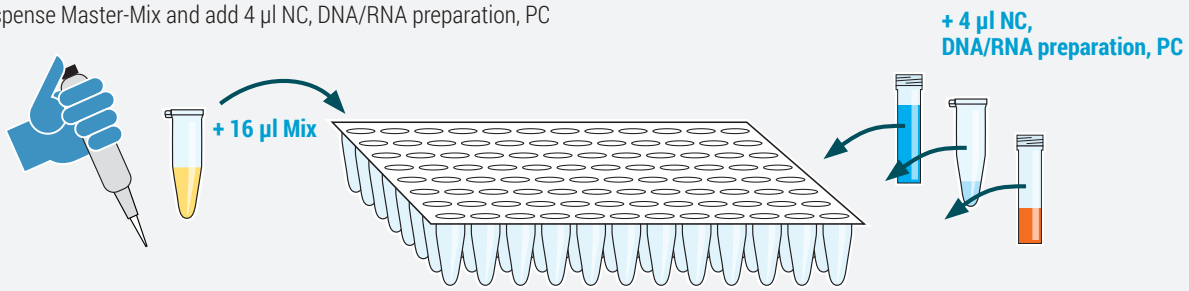
2

Pulse-vortex and spin down



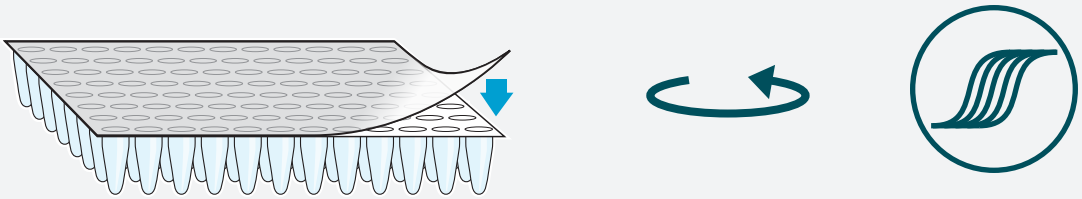
3

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



4

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



5

Analysis