For *in vitro* Veterinary Diagnostics only.

Kylt®

Kylt® Clostridium perfringens

Real-Time PCR Detection

www.kylt.eu

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Revision No.	Amendments
008	also suitable for the analysis of samples from swine and ruminants
007	Deletion of alternative storage at +2°C to +8°C , Amendmendt in A. General: cpb is a Major toxin beta
006	Reduced number of Positive Controls in kit for 100 reactions (from batch 20CLM:02 on)
005	New Layout

A. General

- Kylt® Clostridium perfringens kits are intended for the specific detection of bacterial DNA of *Clostridium perfringens* Major (alpha, beta, epsilon, iota) and Minor (b2, entero, netB) Toxin Genes. The kits are suitable for the analysis of pure or mixed colony material / isolates derived from cultural processes from birds, swine and ruminants.
- The qualitative testing with Kylt® Clostridium perfringens kits is based on a multiplex Real-Time PCR: In two reaction settings (Multiplex 1 and Multiplex 2), the target genes for the different Major and Minor Toxin Genes of *Clostridium perfringens* as well as for the Internal Control 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 target genes of netB (Minor Toxin netB), cpen (Minor Toxin entero) and cpa (Major Toxin alpha) are labeled with fluorescent dyes Cy5, FAM and Texas Red (TXR) (Multiplex 1), respectively. The probes specific for detection of amplified target genes of amplified cpb2 (Minor toxin beta 2), cpep (Major toxin epsilon), cpb (Major toxin beta) and cpi (Major toxin iota) are labeled with fluorescent dyes Cy5, FAM, TXR and HEX (Multiplex 2), respectively. The emitted fluorescence is separately optically measured by the Real-Time PCR thermal cycler. By means of the individual analyses in two reaction vessels per sample and the Negative Control and Positive Controls per run the Major and Minor Toxin Gene 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® Clostridium perfringens kits are available and comprise the following reagents:

Reagent	Colour of Lid	100 Reactions Article No 31034	25 Reactions Article No 31035	Store at
Reaction-Mix Multiplex 1	brown	4 x 450 µl	1 x 450 µl	≤ -18 °C
Reaction-Mix Multiplex 2	green	4 x 450 µl	1 x 450 µl	≤ -18 °C
Positive Control Multiplex 1	red	2 x lyophilizate (final 50 µl each)	1 x lyophilizate (final 50 µl each)	≤ -18 °C
Positive Control Multiplex 2	red	2 x lyophilizate (final 50 µl each)	1 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 Controls Multiplex 1 and Multiplex 2: 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, HEX, Cy5 and TXR (emission 520, 550, 670 and 620 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® Clostridium perfringens kits:
  - DNA preparation kit / protocol (e.g. Kylt® DNA Extraction-Mix II or 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 Controls 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 product contains separate Positive Controls with specific DNA standards for the different Major and Minor Toxin Genes (Positive Control Multiplex 1: netB (Cy5), cpen (FAM) and cpa (TXR), respectively, Positive Control Multiplex 2: cpb2 (Cy5), cpep (FAM), cpb (TXR) and cpi (HEX), respectively).
- 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 gene encoding the Major toxin gene alpha (cpa) is present in all strains of *Clostridium perfringens*. Therefore, the detection of cpa also functions as an Internal Control reaction.

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

- Material derived from cultural processes, i.e. colony material, is directly transferred into respective tubes for Kylt® DNA Extraction (please refer to 2 "DNA Preparation"), 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.

2. DNA Preparation

a) Kylt® DNA Extraction (requires Kylt® DNA Extraction-Mix II)

- For detailed information, please refer to the Direction For Use of Kylt® DNA Extraction-Mix II.

b) Kylt® RNA/DNA Purification products

- The isolates 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.

c) Alternative methods

- The isolates 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 (detection with Multiplex 1 and Multiplex 2 separately) and add two more for the Negative Control and the Positive Control Multiplex 1 or Multiplex 2.
- 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 Controls 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 Controls (see also chapter B "Reagents and Materials"). For Reaction-Mix Multiplex 1 the Positive Control Multiplex 1 is to be used. For Reaction-Mix Multiplex 2 the Positive Control Multiplex 2 is to be used.
- 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, Cy5, TXR 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, Cy5, TXR 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-, Cy5-, TXR- 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 FAM-, Cy5-, TXR- 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 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 detection of cpa (function as Internal Control) the validity of each sample reaction and its true test result can be verified according to the Ct-value of the cpa channel (TXR). Finally, the specific status of each sample with regard to *Clostridium perfringens* Major and Minor Toxin Genes is analyzed (channels FAM, Cy5 and TXR for Multiplex 1 and channels FAM, Cy5, TXR and HEX for Multiplex 2, respectively).

Test Evaluation

Multiplex 1

- The **Real-Time PCR test run** is only **valid** if the FAM-, Cy5- and TXR-curves of the Negative Control are negative and the FAM-, Cy5- and TXR-curves of the Positive Control are positive. For a valid test the FAM-, Cy5- and TXR-Ct-values of the Positive Control have to be > 15 and ≤ 35.

Target	Channel	Signal			
cpa	TXR	negative	positive	negative	negative
netB	Cy5	negative	negative	positive	negative
cpen	FAM	negative	negative	negative	positive
The sample is cpa		**negative**	**positive**	**negative**	**negative**
The sample is netB		**negative**	**negative**	**positive**	**negative**
The sample is cpen		**negative**	**negative**	**negative**	**positive**

- A **sample** is **negative for Toxin Genes cpen, netB and cpa** if its FAM-, Cy5- and TXR-curves are negative.
- A **sample** is **positive for Toxin Gene cpa** if its TXR-curve is positive (Ct ≤ 42). Cpa is present in all *Clostridium perfringens* Types. In case the sample is negative for cpa the analyzed sample does not contain a *Clostridium perfringens* strain and is therefore not suitable for analysis with Kylt® Clostridium perfringens.
- A **sample** is **positive for Toxin Gene netB** if its Cy5-curve is positive (Ct ≤ 42).
- A **sample** is **positive for Toxin Gene cpen** if its FAM-curve is positive (Ct ≤ 42).
- A **sample** is **positive for more than one Toxin Gene** if at least two channels (FAM and/or Cy5 and/or TXR) are positive.
- **Caution**: If the difference in Ct values for different plots of the same sample is greater than ten (10), the plot with the higher Ct value is to be considered as questionable.

Multiplex 2

- The **Real-Time PCR test run** is only **valid** if the FAM-, Cy5-, TXR- and HEX-curves of the Negative Control are negative and the FAM-, Cy5-, TXR- and HEX-curves of the Positive Control are positive. For a valid test the FAM-, Cy5-, TXR- and HEX-Ct-values of the Positive Control have to be > 15 and ≤ 35.

Target	Channel	Signal				
cpi	HEX	negative	positive	negative	negative	negative
cpep	FAM	negative	negative	positive	negative	negative
cpb2	Cy5	negative	negative	negative	positive	negative
cpb	TXR	negative	negative	negative	negative	positive
The sample is cpi		**negative**	**positive**	**negative**	**negative**	**negative**
The sample is cpep		**negative**	**negative**	**positive**	**negative**	**negative**
The sample is cpb2		**negative**	**negative**	**negative**	**positive**	**negative**
The sample is cpb		**negative**	**negative**	**negative**	**negative**	**positive**

- A **sample** is **negative for Toxin Genes cpi, cpep, cpb2 and cpb** if its FAM-, Cy5-, TXR- and HEX-curves are negative.
- A **sample** is **positive for Toxin Gene cpi** if its HEX-curve is positive (Ct ≤ 42).
- A **sample** is **positive for Toxin Gene cpep** if its FAM-curve is positive (Ct ≤ 42).
- A **sample** is **positive for Toxin Gene cpb2** if its Cy5-curve is positive (Ct ≤ 42).
- A **sample** is **positive for Toxin Gene cpb** if its TXR-curve is positive (Ct ≤ 42).
- A **sample** is **positive for more than one Toxin Gene** if at least two channels (HEX and/or FAM and/or Cy5 and/or TXR) are positive.
- **Caution**: If the difference in Ct values for different plots of the same sample is greater than ten (10), the plot with the higher Ct value is to be considered as questionable.
- **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: in case of inhibited DNA Extracts derived from Kylt® DNA Extraction-Mix II, the original sample or the DNA Extract can be utilized for DNA preparation using appropriate alternative systems, such as Kylt® RNA/DNA Purification.
- 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® DNA Extraction-Mix II	31398	100	Simplified and economic DNA extraction
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® Purifier	31436	--	Purification system for magnetic beads. Up to 96 samples in under 30 minutes.
Kylt® Purifier Spin Tips	31434	5	Plate with 96 separate spin tips, used by the Kylt® Purifer to mix the well contents by stirring. One set used per run.
Kylt® Purifier Plates	31435	20	Plates to be used for the several reactions and reagents in a nucleic acid purification kit. 4 - 5 plates used per run.

G. Ordering information

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

- Delivery and 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
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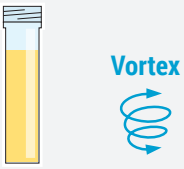
qPCR.CP-MPX.02, Rev008, September 2021

PROTOCOL AT A GLANCE
Real-Time PCR Setup

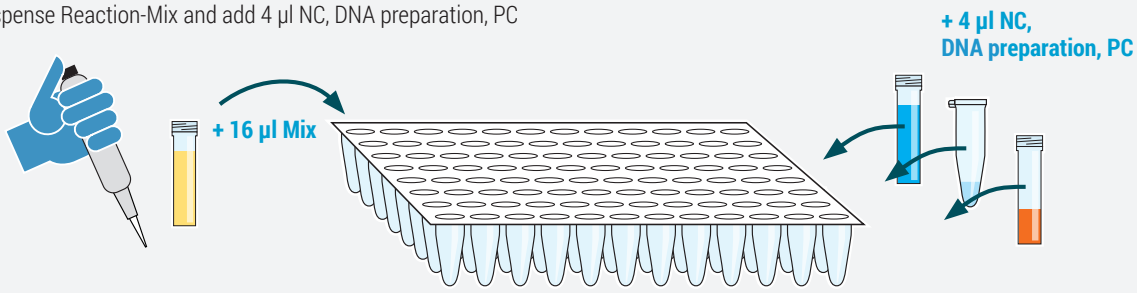
1

Pulse-vortex and spin down



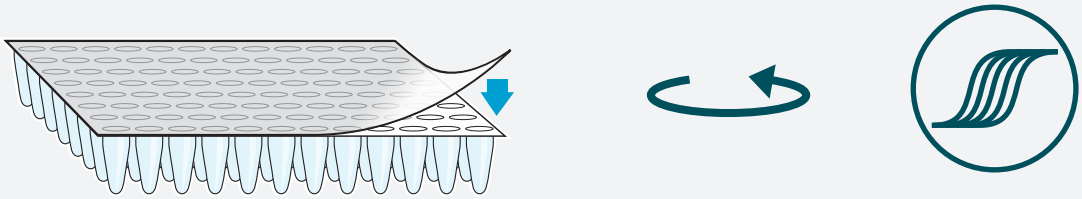
2

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



3

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



4

Analysis