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Kylt® Clostridium difficile Toxin A/B

Real-Time PCR Detection Kit
for detection and differentiation of
Clostridium difficile Toxin Genes A and B

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100 / 25 reactions
in vitro Diagnosticum

A. General

- Kylt® Clostridium difficile Toxin A/B Real-Time PCR Detection Kit is for detection of *Clostridium difficile* Toxin Genes A (Enterotoxin) and B (Cytotoxin) in samples from isolates derived from cultural processes with suitable sample material originating from swine. Kylt® Clostridium difficile Toxin A/B is only to be used on pure colonies from cultural processes.
- Kylt® Clostridium difficile Toxin A/B comprises all reagents and controls for detection and differentiation of Toxin A and B. It may be supplemented by Kylt® DNA Extraction Mix II (article no 31398) for economic and convenient DNA preparation from pure isolates. The qualitative testing of Kylt® Clostridium difficile Toxin A/B is based on a multiplex Real-Time PCR system: After DNA extraction the target genes specific for the Toxin Genes A and B as well as for the Internal Amplification Control are amplified in parallel by respective primer pairs in the subsequent Polymerase Chain Reaction (PCR) in one reaction vessel per sample. 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 target genes of amplified Toxin A, Toxin B and Internal Amplification Control are labeled with fluorescent dyes FAM, Cy5 and HEX respectively. The emitted fluorescence is separately optically measured by the Real-Time PCR thermal cycler. By means of the individual analyses in one reaction vessel per sample and the Negative Control and the Positive Controls per run the 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® Clostridium difficile Toxin A/B contains the following reagents:

Reagent	Colour code of lid	Quantity in kit with 25 / 100 reactions	Storage conditions
Reaction-Mix	green	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
Negative Control	blue	1 x 1 ml	+2 °C to +8 °C

- After receipt the kit and its components are immediately 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). 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.

- Before its first application rehydrate lyophilized Positive Control by adding 20 µl of Negative Control per vial, briefly incubate at room temperature and mix by repeated vortexing. Storage of aliquots with 5 µl to 10 µl (depending on the expected number of Positive Control reactions per kit) at -18 °C to -20 °C is recommended.

- This kit can be used on all commercially available Real-Time PCR thermal cyclers that detect the emitted fluorescence of the fluorescent dyes FAM, Cy5 and HEX (emission: 520 nm, 669 nm and 550 nm).

- 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 devices are needed (not included in this kit):
  - Table top microcentrifuge
  - Dry heating block (+100 °C ± 3 °C)
  - Magnetic stirrer
  - Micropipettes covering volumes of 1 µl to 1000 µl Centrifuge for PCR tubes or plates
  - Real-Time PCR thermal cycler

- Accessory Kylt® products (not included in this kit):
  - Kylt® DNA Extraction Mix II (article no 31398 for 100 preparations) for simplified and economic DNA extraction from pure bacterial isolates or bacterial enrichments.
  - Kylt® RNA/DNA Purification (articles no 31314 / 31315 for 250 / 50 preparations) for purification of high quality RNA and DNA from a vast variety of veterinary samples or from pure bacterial or viral culture isolates or enrichments.

C. Control Reactions

- The Negative Control included in this kit provides the ability to exclude possible contaminations of the reagents. The Positive Control included in this kit provides the ability to monitor the specificity and efficiency of the reagents used, i.e. the performance of Real-Time PCR itself and of the Real-Time PCR thermal cycler, respectively. The kit contains one Positive Control with specific DNA standards for the different Toxin Genes A and B. The sample testing is only valid if both, the Negative Control and the Positive Control, are included in every Real-Time PCR run and used to verify the validity of such run.

- The Internal Amplification Control is included in the Reaction-Mix in a defined copy number; it is co-amplified (channel HEX) with every single reaction to detect possible inhibitory effects of the DNA preparation and to verify true-negative results.

D. Protocol

- The overall protocol of the 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

- 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 to -20 °C (or -70 °C to -80 °C) until further processing of the Toxin A and B detection of *Clostridium difficile*, please also refer to chapter 2 “DNA Extraction”. Avoid repeated freezing and thawing of the DNA preparations.

1. Sample Preparation

- Pure or mixed cultural material is directly transferred into respective tube for DNA preparation, such as conical screw cap tube; therefore a little amount of a single colony is picked with a sterile loop whire or sterile pipette tip and transferred into the tube.

2. DNA Extraction (attention: requires Kylt® DNA Extraction Mix II (article no 31398 for 100 preparations)

- 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 DNA Extraction-Mix II is stirred on a magnet stirrer, it must be used as a homogenous suspension. Immerse the colony material in 500 µl of DNA Extraction-Mix II and resuspend the colony material carefully. Avoid formation of bubbles and aerosols.

- 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 in 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.

- All kind of sample matrices, including e.g. pure isolates, tissue or swab samples, may alternatively be processed with alternative DNA preparation kits, such as Kylt® RNA/DNA Purification kit (articles no 31314 / 31315 for 250 / 50 preparations).

3. Reaction Setup, Reverse Transcription and Amplification

- Before each use, briefly vortex and spin down the Reaction-Mix and Negative Control.
- Determine the number of needed PCR-reactions: number of samples + Positive Control and Negative Control.
- Pipette 18 µl of Reaction-Mix to the PCR reaction tubes / each PCR plate well (»cavity«). Keep exposure of Reaction-Mix to light as short as possible!
- Add 2 µl of Negative Control to corresponding cavity and seal.
- Add 2 µl of the prepared DNA (DNA extract or eluted DNA) of sample to corresponding cavities and seal. Solely use clear supernatant of DNA extract, avoid transferring debris.
- Once all sample cavities are sealed, 2 µl of the Positive Control are added to the corresponding cavity and sealed. The Positive Control is vortexed and spun down before each use.
- Avoid formation of bubbles when pipetting Reaction-Mixes, samples and controls. It is recommended to consistently spin down cavities before the PCR run.
- Place the cavities in the Real-Time PCR thermal cycler and run the test using the following parameters:

Step	Temperature	Duration	
Activation of Polymerase	95 °C	10 min	
Denaturation	95 °C	15 sec	42 cycles
Annealing & Extension	60 °C	1 min	
Fluorescence Detection	channels FAM, Cy5 and HEX		

- Please follow the specified instructions of your Real-Time PCR thermal cycler as recommended by the manufacturer.
- The amplification parameters above allow for combination of this Kylt® Clostridium difficile Toxin A/B Real-Time PCR with other Kylt® Real-Time PCRs. In the event of a combined Real-Time PCR run, make sure all necessary channels are used!

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 cycler. Alternatively, the threshold can be set manually considering the following direction: The threshold should cross the FAM-, Cy5- and HEX-curve in linear scale of x- and y-axis of the amplification plot. By setting the thresholds, the crossing points with the FAM-, Cy5- and HEX-curves, respectively, 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. For detailed information about the threshold setting please refer to the manufacturer's Direction For Use of the respective Real-Time PCR thermal cycler.

- 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.
- The actual test analysis starts with the validity check of the entire Real-Time PCR run. Afterwards, by means of the Internal Amplification 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, specific status of each sample with regard to Clostridium difficile Toxin A and B is analyzed (channels FAM and Cy5, respectively).

Test Evaluation

- The **Real-Time PCR test run** is only **valid** if the FAM- and Cy5-curves of the Negative Control are negative, if the HEX-curve of the Negative Control is positive and if the FAM- and Cy5-curves of the Positive Control are positive.

HEX-curve is positive	yes	yes / no	yes / no	yes / no	no
FAM-curve is positive	no	yes	no	yes	no
Cy5-curve is positive	no	no	yes	yes	no
The sample is Toxin A	**negative**	**positive**	**negative**	**positive**	**inhibited**
The sample is Toxin B	**negative**	**negative**	**positive**	**positive**	

- A **sample** is **negative for Toxin Genes A and B** if its FAM- and Cy5-curves are negative and the HEX-curve is positive.
- A **sample** is **positive for Toxin A** if its FAM-curve is positive, independent of the HEX-curve.
- A **sample** is **positive for Toxin B** if its Cy5-curve is positive, independent of the HEX-curve.
- A **sample** is **positive for Toxin A and B** if its FAM- and Cy5-curves are positive, 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 the test should be repeated with a dilution of the DNA extract at 1:10 or 1:100 (9 volumes Negative Control + 1 volume DNA extract or eluted DNA or 99 volumes Negative Control + 1 volume DNA extract or eluted DNA). The Negative Control serves 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 Purification (articles no 31314 / 31315 for 250 / 50 preparations).
- Convenient and reliable sample data input, start of the Real-Time PCR run as well as final qualitative test result analysis and documentation can be managed by the Kylt® Software package. Please feel free to contact us.

Production:

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