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Kylt® E. coli Stx1, Stx2, eae

Real-Time PCR Detection

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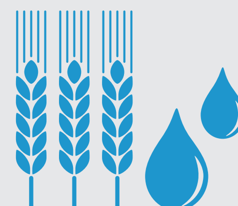

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Revision No.	Amendments
002	New Layout, Color of lid of Reaction-Mix changed to green (from Batch 20STEC:01 on), Storage temperature of reagents ≤ -18 °C

A. General

- Kylt® E. coli Stx1, Stx2, eae kits are intended for the detection of bacterial DNA of *E. coli* virulence factors Stx1 (Shigatoxin 1), Stx2 (Shigatoxin 2, including Stx2f) and eae (*E. coli* intimin). The kits are suitable for the analysis of isolates derived from cultural processes or pre-enriched samples with suitable sample material.
- The qualitative testing with Kylt® E. coli Stx1, Stx2, eae kits is based on a multiplex Real-Time PCR: In one reaction setting, the target genes specific for the different *E. coli* virulence factors as well as for the exogenous control (Internal Amplification Control (IAC)) 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 target genes of amplified Stx1, Stx2, eae and the exogenous control are labeled with fluorescent dyes FAM, Texas Red (TXR), Cy5 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 *E. coli* Stx1, Stx2 and eae-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® E. coli Stx1, Stx2, eae kits are available and comprise the following reagents:

Reagent	Colour of Lid	100 Reactions Article No 31734	25 Reactions Article No 31735	Store at
Reaction-Mix	green	4 x 500 µl	1 x 500 µl	≤ -18 °C
Positive Control	red	4 x lyophilizate (final 20 µl each)	2x lyophilizate (final 20 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 20 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 nm, 550 nm, 670 nm and 603 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® E. coli Stx1, Stx2, eae kits:
  - DNA preparation kit (e.g. Kylt® RNA / DNA Purification or Kylt® DNA Extraction-Mix II)
  - Table top microcentrifuge
  - Vortex
  - Micropipettes volume range 1 - 1000 µl
  - Centrifuge for PCR tubes or plates
  - Real-Time PCR thermal cycler
- 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 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 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 on the Real-Time PCR itself and thus to verify true-negative results.

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. DNA Extraction
  2. Reaction Setup and Amplification (Real-Time PCR)
  3. 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. DNA Extraction

- DNA that was prepared with commercially available DNA preparation kits, such as the Kylt® RNA/DNA Purification or alternative kits is suitable for the application of Kylt® E. coli Stx1, Stx2, eae. For detailed information on the DNA preparation process, please refer to the Direction For Use or Standard Operating Procedure of the respective kit or inhouse method, respectively.
- DNA–Extraction from pure colony material can be performed using Kylt® DNA-Extractionmix II (Art. No.: 31398). For further information please refer to the Direction For Use.

2. 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 and add three more for the Negative Control and the Positive Control.
- The Reaction-Mix is ready-to-use, add 18 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 2 µl of the Negative Control to the corresponding cavity and seal it individually, if possible.
- Add 2 µl of each DNA preparation to the corresponding cavities and seal them individually, if possible.

- To minimize risk of potential cross-contaminations, 2 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 Controls (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 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, HEX, Cy5 and TXR		

- 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, HEX, Cy5 and TXR		

- 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.

3. 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-curves 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 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 specific status of each sample with regard to *E. coli* virulence factors Stx1, Stx2 and eae is analyzed (channels FAM, TXR and Cy5, respectively).

Test Evaluation

- The **Real-Time PCR test run** is only **valid** if the FAM-, Cy5- and TXR-curves of the Negative Control are negative, the HEX-curve of the Negative Control is positive and the FAM-, HEX-, 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 and the HEX-Ct-value of the Negative Control has to be ≤ 40.

Target	Channel	Signal					
Internal Control	HEX	positive	positive/ negative	positive/ negative	positive/ negative	positive/ negative	negative
Stx1	FAM	negative	**positive**	negative	negative	**positive**	negative
Stx2	TXR	negative	negative	**positive**	negative	**positive**	negative
eae	Cy5	negative	negative	negative	**positive**	**positive**	negative
The sample is Stx1		**negative**	**positive**	**negative**	**negative**	**positive**	**inhibited**
The sample is Stx2		**negative**	**negative**	**positive**	**negative**	**positive**	
The sample is eae		**negative**	**negative**	**negative**	**positive**	**positive**	

- A **sample** is **negative for Stx1, Stx2 and eae,** if its HEX-curve is positive Ct ≤ 40, but its FAM-, Cy5, and TXR-curves are negative.
- A **sample** is **positive for Stx1,** if its FAM-curve is positive (Ct ≤ 42), independent of the HEX-, TXR- and Cy5-curves.
- A **sample** is **positive for Stx2,** if its TXR-curve is positive (Ct ≤ 42), independent of the HEX-, FAM- and Cy5-curves.
- A **sample** is **positive for eae,** if its Cy5-curve is positive (Ct ≤ 42), independent of the HEX-, FAM- and TXR-curves.
- A **sample** is **inhibited,** if neither the FAM-, TXR- and Cy5-curves nor the HEX-curve are positive.
- **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 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® 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 feed and food samples
Kylt® RNA / DNA Purification HTP	31826	4x96	Combined, magnetic beads-based purification of RNA and DNA from feed and food 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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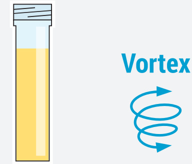
FS.qPCR.STEC.02, Rev002, April 2021

PROTOCOL AT A GLANCE
Real-Time PCR Setup

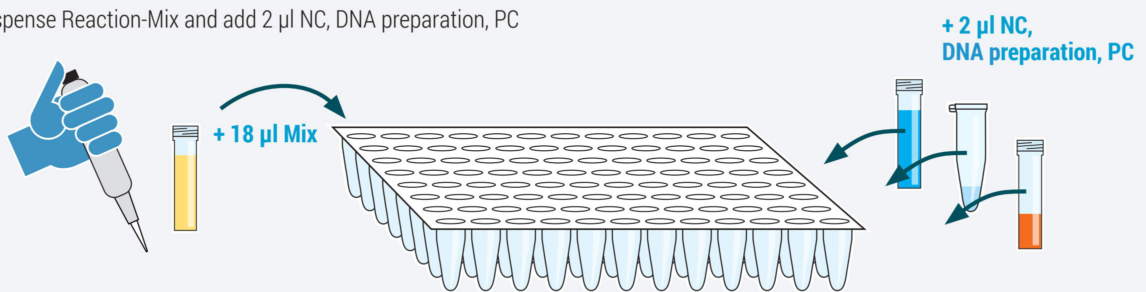
1

Pulse-vortex and spin down



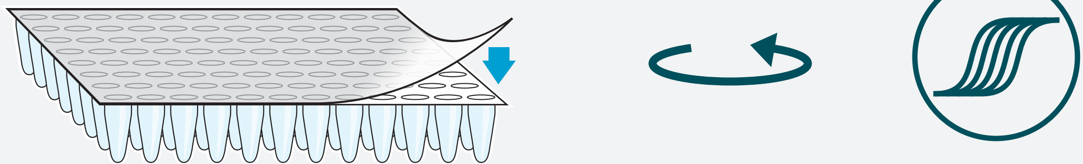
2

Dispense Reaction-Mix and add 2 µl NC, DNA preparation, PC



3

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



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Analysis