Kylt® SE/ST Triplex

Real-Time PCR Detection

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Revision No.	Amendments
005	valid from 01 August 2023: exclusion of Kylt® DNA Extraction-Mix II, new layout for test evaluation, changed storage temperatures.

A. General

- Kylt® SE/ST Triplex kits are intended for the serovar-specific detection of bacterial DNA of *Salmonella* Enteritidis and *Salmonella* Typhimurium. The kits are suitable for the analysis of feed and food samples, espescially of pre-enrichment samples positively tested for *Salmonella* spp. with a Ct value < 35 as well as *Salmonella* spp. positive colony material from cultural processes (e.g. DIN EN ISO 6579). For conduction of the protocol described in this manual, it is obligatory to combine the test kit with the Kylt® Salmonella spp. 2.0 Real-Time PCR Detection kit (Art. No. 31302). In case of samples with a positive result Ct > 35 in *Salmonella* spp. screening either a second enrichment step should be performed or the analysis should be repeated using colony material from microbiological culture.
- The qualitative testing with Kylt® SE/ST Triplex kits is based on a triplex Real-Time PCR: In one reaction setting, the target genes for *Salmonella* Enteritidis and *Salmonella* Typhimurium 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 amplified *Salmonella* Enteritidis and *Salmonella* Typhimurium and the exogenous control target genes are labeled with fluorescent dyes FAM, Cy5 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 *Salmonella* Enteritidis- and *Salmonella* Typhimurium-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® SE/ST Triplex kits are available and comprise the following reagents:

Reagent	Colour of Lid	100 Reactions Article No 31165	25 Reactions Article No 31166	Store at
Reaction-Mix	green	4 x 500 µl	1 x 500 µl	≤ -18 °C
Positive Control (SE)	red-white	2 x lyophilizate (final 20 µl each)	1x lyophilizate (final 20 µl each)	≤ -18 °C
Positive Control (ST)	red	2 x lyophilizate (final 20 µl each)	1 x 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 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® SE/ST Triplex kits:
  - DNA preparation kit / protocol (e.g. Kylt® DNA Extractionmix III)
  - 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 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. Cultural Pre-Enrichment
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 until further processing. Avoid repeated freezing and thawing of the DNA preparations.

1.1 Cultural Pre-Enrichment

- Before testing for SE/ST screen the samples with regard to the *Salmonella* spp. status. The sample preparation and handling for *Salmonella* spp. screening should follow the rules of Good Laboratory Practice with sterile instruments to avoid external contamination. Sampling, pooling and subsampling as well as pre-enrichment is described in the respective EU legislation. Pooled sock swabs, subsamples of feces or dust and surface swabs are immersed according to the respective EU legislation in the given volume of Buffered Peptone Water (BPW). For instance, pools of (two) sock swabs pairs from commercial layers, broilers or fattening turkeys must be fully immersed (in a Stomacher bag) in at least 225 ml BPW (Commission Regulation EC No 517/2011, 200/2012 and 1190/2012, respectively). To enable sufficient propagation of potentially present *Salmonellae* during incubation time, the BPW has to be pre-warmed to at least room temperature prior to the start of incubation time. The pre-enrichment is incubated at +37 ± 1°C for 18 ± 2 hours without agitation.
- **Recommendation:** To retain the sample for future microbiological analysis or biomolecular analysis at least 3 ml of supernatant of the pre-enrichment are transferred to a sterile test tube with sterile transfer pipettes and the initial vessel (e.g. Stomacher bag) is discarded. The 3 ml in the tube can be used for storing the sample at +2 to +8°C for several days. Storage in test tubes is just a recommendation, the 1 ml sample for DNA extraction may also be transferred directly from the Stomacher bag into a conical screw cap tube and the Stomacher bag may be retained.
- **Attention:** Mixing of the pre-enrichment after incubation by shaking or any other agitation must be avoided! Avoid transferring solid or greasy debris. An aliquot should be taken directly below the surface, avoiding floating particles. For samples that are too difficult to be separated from the debris after incubation, Stomacher bags with filters can be used for pre-enrichment in BPW.

- A 1 ml aliquot is transferred from the test tube into a sterile, conical screw cap microcentrifuge tube. The remaining pre-enrichment in the test tube or Stomacher bag can be stored or potential subsequent cultural examination.
- Certain sample matrices, such as turf- or soil-containing sock swabs and feces with a high concentration of humic acids, are known to potentially have an inhibitory effect on the Real-Time PCR. In case of inhibition of the Real-Time PCR, a second enrichment step if performed and the entire process of DNA extraction and Real-Time PCR is repeated (see also chapter D.4. »Data Analysis - Test Evaluation«). Alternatively, for samples known to have an inhibitory effect on the Real-Time PCR, the second enrichment step may directly be performed subsequently to the pre-enrichment step without Real-Time PCR testing between.
- For the second enrichment step the selective medium Rappaport-Vassiliadis-Soy Broth (RVS) is used. The readily incubated pre-enrichment (sample in BPW) is diluted at a ratio 1:100 in RVS (e.g. 100 µl in 10 ml). It is incubated for at least 5 ± 1 hours at +41,5 ± 1 °C without agitation. Use liquid RVS for the second pre-enrichment step.

1.2 Material from Cultural Processes

- The kit can also be used on material from cultural processes. Please choose an appropriate purification method and proceed according to the respective manual (see chapter 2. "DNA Extraction").
- When analyzing colony material with Kylt® SE/ST Triplex it is recommended to initially or in parallel screen the samples with Kylt® Salmonella spp.. Since high DNA concentrations can lead to PCR inhibition in can be reasonable to dilute DNA extracts 1:100 or 1:1000. A potential inhibitory effect can be addressed by analyzing the pure and diluted DNA extraction with Kylt® Salmonella spp. Determine the undiluted DNA extracts of the isolates as well as the dilutions, respectively, in both Real-Time PCR reactions. The screening of the DNA extracts and its dilutions with Kylt® Salmonella spp. gives information about the potential inhibition effect of the samples and the required dilution suitable for Kylt® SE/ST Triplex.

2. DNA Extraction

- Kylt® SE/ST Triplex should only be used after the samples were initially analyzed with Kylt® Salmonella spp., the DNA Extraction should therefore already been applied for analyzing the sample with Kylt® Salmonella spp.

a) Kylt® DNA Extraction (requires Kylt® DNA Extractionmix II)

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

b) Kylt® RNA/DNA Purification products

- Samples 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

- Samples 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 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 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").
- 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 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 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.

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-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 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 *Salmonella* Enteritidis and *Salmonella* Typhimurium-specific status of each sample is analyzed (FAM and Cy5).

Test Evaluation - Control Reactions

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

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

- The FAM- and Cy5-Ct-value of the Positive Control has to be > 15 and ≤ 35.
- The HEX-Ct-values of the Positive and Negtive Control have to be ≤ 40.

Test Evaluation - Samples

Target	Channel	Signal				
Internal Control	HEX	**positive**	positive / negative	positive / negative	positive / negative	negative
Salmonella Enteritidis	FAM	negative	**positive**	negative	**positive**	negative
Salmonella Typhimurium	Cy5	negative	negative	**positive**	**positive**	negative
The sample is *Salmonella* Enteritidis		**negative**	**positive**	**negative**	**positive**	**inhibited**
The sample is *Salmonella* Typhimurium		**negative**	**negative**	**positive**	**positive**	

- A **sample** is **negative for *Salmonella* Enteritidis and *Salmonella* Typhimurium,** if its HEX-curve is positive (Ct ≤ 40), but its FAM- and Cy5-curves are negative.
- A **sample** is **positive for *Salmonella* Enteritidis,** if its FAM-curve is positive (Ct ≤ 42), independent of the Cy5- and HEX-curves.
- A **sample** is **positive for *Salmonella* Typhimurium,** if its Cy5-curve is positive (Ct ≤ 42), independent of the FAM- and HEX-curves.
- A **sample** is **inhibited** if neither the FAM- and Cy5-curve nor the HEX-curve are positive.
- **Recommendation:** In case of an inhibited sample the pre-enrichment is incubated for additional 5 ± 1 hours in RVS (for details see chapter D.1. »Cultural Pre-Enrichment«). By that potentially present ingredients in the preenrichment that have inhibitory effects on the effectiveness of the Real-Time PCR are diluted. Also potentially present *Salmonellae* are selectively propagated. After the second enrichment the entire process of DNA extraction and Real-Time PCR is repeated (see chapters D.2. »DNA Extraction« and D.3. »PCR Setup and Amplification«).
- **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® Salmonella spp. 2.0	31302	100	Detection of *Salmonella* spp. in food and feed samples as well as in veterinary samples.
Kylt® DNA Extraction-Mix II	31398	100	Simplified and economic DNA extraction.
Kylt® RNA / DNA Purification	31315	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	1 unit	Purification system for magnetic beads. Up to 96 samples in under 30 minutes.
Kylt® Purifier 48	31748	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	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.
Kylt® Salmonella Purification HTP	31433	4x96	Combined, magnetic beads-based purification of DNA from *Salmonella* pre-enrichment samples, suitable for automated high throughput processing.

G. Ordering information

For a fast and efficient service please send your order to *orders.kylt-de@san-group.com* 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
- Catalogue number
- Quantity and size of products
- Indicate if your account is VAT exempt

Production:

SAN Group Biotech Germany GmbH | Muehlenstr. 13 | 49685 Hoeltinghausen | Germany
www.kylt.eu | kylt-de@san-group.com

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

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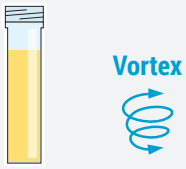
FS.DNA-DK.SE/ST.02, Rev005, July 2023

PROTOCOL AT A GLANCE
Real-Time PCR Setup

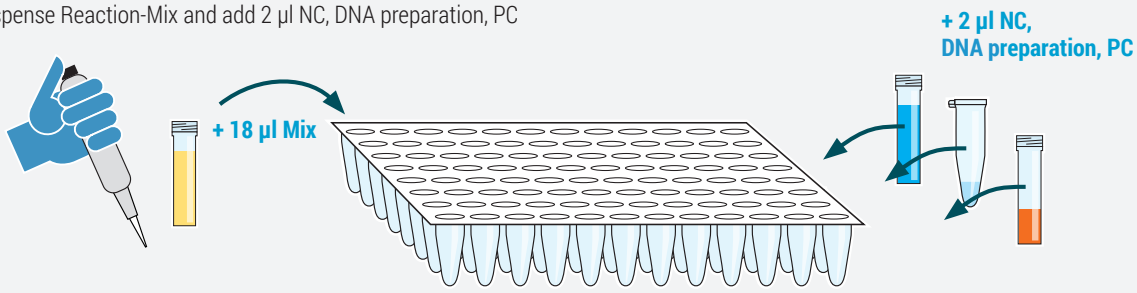
1

Pulse-vortex and spin down



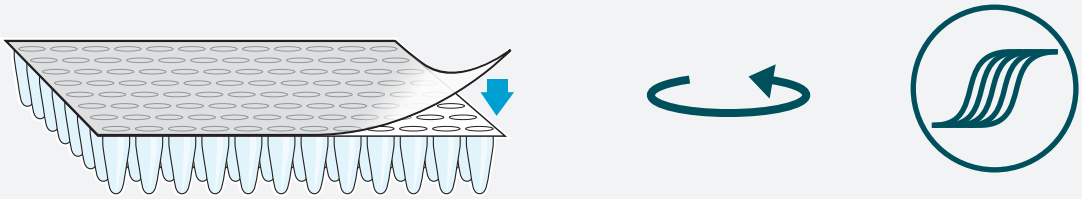
2

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



3

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



4

Analysis