Kylt®

For *feed and food applications* only.

Kylt® ST

Real-Time PCR Detection Kit for detection of *Salmonella* Typhimurium

www.kylt.eu

Art. No. 31207/31208

Kylt® ST

Real-Time PCR Detection Kit for detection of *Salmonella* Typhimurium

100 /25 reactions

in vitro Diagnosticum for feed and food applications only

A. Introduction

- Kylt® ST Real-Time PCR Detection Kit is for serovar-specific detection of *Salmonella* Typhimurium in feed and food samples.
- For conduction of the protocol described in this manual it is obligatory to combine the test kit with the Kylt® Salmonella spp. DNA-Extraction and Real-Time PCR Detection kit.
- Kylt® ST comprises all reagents and controls for detection of DNA from *Salmonella* Typhimurium. The qualitative testing of Kylt® ST is based on a duplex Real-Time PCR system: the target genes specific for *ST* 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. 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 *Salmonella* Typhimurium and the Internal Amplification Control are labeled with fluorescent dyes FAM and HEX, respectively, and their emitted fluorescence is separately optically measured by the Real-Time PCR thermal cycler. By means of two individual analyses in one reaction vessel per sample and the Negative Control and two Positive Controls per run the *ST*-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® ST contains the following reagents:

Reagent	Colour code of lid	Quantity in kit with 25 / 100 reactions	Storage conditions
DNA-Extractions-Mix II	green	1 x 20 ml	+2 °C to +8 °C
Reaction-Mix	violett	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 lyophilized -18 °C to -20 °C rehydrated
Negative Control	blue	1 x 1 ml	+2 °C to +8 °C

- The kit and its components are 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 sunlight!

- Before its first application rehydrate the 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 and HEX (emission: 520 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
  - Vortex
  - Micropipettes covering volumes of 1 µl to 1000 µl
  - Centrifuge for PCR tubes or plates
  - Real-Time PCR thermal cycler

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 provide 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 sample testing is only valid if 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. Cultural pre-enrichment
  2. DNA extraction
  3. reaction setup and amplification (Real-Time PCR)
  4. data analysis – validity and qualitative result

1.1 Cultural Pre-Enrichment

- Before testing for 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.

- A1 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. »Analysis«). 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

- To analyze pure or mixed cultures on solid media, a small portion of an individual colony or mixed colony material can be transferred to a sterile, conical screw cap tube.
- For DNA Extraction resuspend the colony material in 500µl DNA-Extraction mix II and proceed with Step 4 in Section 2. (DNA-Extraction).
- When analyzing colony material with Kylt® ST 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® ST.

2.DNA Extraction

- Kylt® ST should only be used after the samples were initially analyzed with Kylt® *Salmonella* spp (Art. No. 31019), the following steps should therefore already been applied for analyzing the sample with Kylt® *Salmonella* spp.
- 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 aliquoted pre-enrichment in the conical screw cap tube is pelleted by centrifugation at 10.000 g to 12.000 g for 5 min.
- Remove the supernatant by using a 1000 µl pipette tip (not by decantation) and discard it.
- The DNA Extraction-Mix II is stirred on a magnet stirrer, it must be used as a homogenous suspension. The pellet is resuspended by repeated up-and-down pipetting with 200 µl of DNA Extraction-Mix II. Use a 1000 µl pipette with filtered tips. 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 is 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.

- Initially screen the DNA extract for Salmonella spp. with Kylt® *Salmonella* spp. Samples with a positive result in *Salmonella* spp. screening (Ct<35) are suitable for the analysis with Kylt® ST. In case of samples with a positive result Ct>35 in *Salmonella* spp. screening a second enrichment step is performed. Perform the second pre-enrichment as described above (page 5).
- When analyze isolates from cultural processes we recommend to screen the DNA extract with Kylt® *Salmonella* spp. in parallel to Kylt® ST. It might be necessary to prepare a 1:100 and 1:1000 dilution of the DNA extracts, since undiluted samples might results in PCR inhibition due to high content of DNA. 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.

3.Reaction Setup 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 for ST and Positive Control for ST and Negative Control.
- Pipette 18 µl of Reaction-Mix to each PCR reaction tube / 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 cavity and seal. Solely use clear supernatant of DNA extract, avoid transferring debris.
- Once all sample cavities are sealed, 2 µl of the Positive Control is added to the corresponding cavity and sealed. The Positive Controls are vortexed and spun down before each use.
- Avoid formation of bubbles when pipetting Reaction-Mix, 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
Annealing & Extension	60 °C	60 sec	
Fluorescence Detection	Channels FAM 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® ST Real-Time PCR with other Kylt® Real-Time PCRs, e.g. for detection of *Salmonella* spp.. 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 data of the amplification reactions can automatically be processed using specific software for the Real-Time PCR thermal cycler. Alternatively a threshold can be set manually. The threshold should cross the HEX-curve of the Negative Control and the FAM-curve of the Positive Control, respectively, in the linear area of its slope. By setting the thresholds, the crossing points with the HEX- 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 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, the *ST* -specific status of each sample is analyzed (channel FAM).

Test Evaluation

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

HEX-curve	positive	positive/negative	negative
FAM-curve	negative	positive	negative
The sample is	**ST not detectable**	**ST positive**	**inhibited**

- A **sample** is **negative for *ST*** if its FAM- curve is negative and the HEX-curve is positive.
- A **sample** is **positive for *ST*** if its FAM-curve is positive, independent of the HEX-curve.
- A **sample** is **inhibited** if neither the FAM- curve nor the HEX-curve is positive.
- **Recommendation**: In the case of an inhibited sample the test should be repeated with a dilution of the DNA extract at 1:10 (9 volumes Negative Control + 1 volume eluted DNA). The Negative Control serves as the diluting agent. Alternatively, the original sample or the DNA elution can be utilized for DNA purification using appropriate alternative procedures, such as repetition of purification with less starting material or purification with Kylt® RNA/DNA Purification Kit.
- 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:
AniCon Labor GmbH | Muehlenstr. 13 | D-49685 Hoeltinghausen | Germany | www.anicon.eu | www.kylt.eu