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Kylt® Salmonella spp. 2.0

(DNA Extraction and) Real-Time PCR Detection Kit for Salmonella spp.

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Art. No.	Reactions	Title
31001	100	Kylt® Salmonella spp. 2.0 DNA Extraction and Real-Time PCR Detection Kit
31302	100	Kylt® Salmonella spp. 2.0 Real-Time PCR Detection Kit

A. General

- Kylt® Salmonella spp. 2.0 (DNA Extraction and) Real-Time PCR Detection Kit (in the following Kylt® Salmonella) detects Salmonella spp. in feed and food samples, environmental samples and primary production samples including veterinary samples such as boot swabs, feces, dust samples, tissue samples from affected organs, swabs from such tissues and sampling material derived from cultural processes with the aforementioned samples (e.g. cultural harvest) of poultry, swine and cattle.
- Kylt® Salmonella comprises all reagents necessary for sample preparation from cultured materials (optionally) as well as all reagents and controls for subsequent detection of bacterial DNA of Salmonella spp.: After bacterial pre-enrichment and DNA preparation, Salmonella spp. is qualitatively detected via specific and real-time detected amplification of a Salmonella spp. target gene. This way, results can be achieved within a few hours after bacterial enrichment.
- The qualitative testing of Kylt® Salmonella is based on a duplex Real-Time PCR system: In one reaction setting the target genes for Salmonella spp. as well as for the included Internal Amplification 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 Salmonella spp. and Internal Amplification Control target genes are labeled with FAM 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 and Positive Control per run the Salmonella spp.-specific state of a sample can be evaluated in the end.
- This kit was developed for use by trained laboratory personnel following standardized procedures.

B. Reagents and Materials

- Kylt® Salmonella spp. 2.0 (DNA Extraction and) Real-Time PCR Detection Kit contains the following reagents:

Reagent	Colour of Lid	Quantity in kit with 100 reactions	Store conditions
DNA Extraction-Mix II*	○ white	1 x 20 ml	+2 °C to +8 °C
Reaction-Mix	● yellow	4 x 450 µl	+2 °C to +8 °C
Positive Control	● red	2 x lyophilizate (final 50 µl each)	+2 °C to +8 °C lyophilised +2 °C to +8 °C rehydrated (max. 2 days) ≤ -18 °C rehydrated (max. 6 months)
Negative Control	● blue	1 x 1 ml	+2 °C to +8 °C

*DNA Extraction-Mix II is not included in Art. No 31302

- All reagents are stored at +2 °C to +8 °C. The kit is to be used within the indicated shelf life. If occasional processing of few samples is expected, you may prepare aliquots of the Reaction-Mix before storage at -18 °C to -20 °C. Avoid repeated freezing and thawing of the kit or its components. Reaction-Mix has to be stored dark, do not expose to direct sunlight!
- Before the first use the lyophilized Positive Control is rehydrated: 50 µl of the Negative Control are added to the Positive Control, briefly incubated at room temperature and mixed thoroughly by repeated vortexing. Storage of aliquots with 5 - 10 µl volume (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 are able to detect the emitted fluorescence of fluorescent dyes FAM (emission 520 nm) and HEX (also JOE/VIC, emission 550 nm). Note that default normalization option against ROX (e.g. using ABI cyclers) must be deactivated.
- We recommend to exclusively using certified Nuclease-free materials and powder free protective gloves. Pipette tips have to be changed between samples to avoid cross contamination. Gloves have to be changed frequently, especially after spillage or other suspected contamination.
- Apart from the disposables, the following devices are needed (not included in this kit):
  - Incubators for sample pre-enrichment (+37 ± 1 °C & +41.5 ± 1 °C)
  - DNA(/RNA) preparation kit incl. respective accessories (optionally, e.g. in case DNA Extraction-Mix II is not used)
  - Table top microcentrifuge
  - Dry heating block (+100 °C ± 3 °C)
  - Vortex
  - Magnetic stirrer
  - Micropipettes volume range 1 – 1000 µl
  - Centrifuge for PCR tubes or plates
  - Real-Time PCR thermal cycler

C. Control Reactions

- The Positive Control included in this kit allows for control of the specificity and efficiency of the reagents and the reaction itself. The Negative Control included in the kit allows exclusion of contaminations. The test is only valid if both, Positive and Negative Controls, are used and verified for validity in every Real-Time PCR run.
- An Internal Amplification Control is included in the Reaction-Mix in defined copy number; it is co-amplified (fluorescent dye HEX) in every single reaction to detect possible inhibitory effects of the DNA preparation and to verify true negative results.

D. Protocol *(see also „Protocol At A Glance" at the end of this Direction For Use)*

- The overall protocol of this Salmonella spp. analysis consists of the following steps: cultural pre-enrichment, DNA preparation, Real-Time PCR and final analysis.

1. Cultural Pre-Enrichment

- The sample preparation and handling should follow the rules of Good Laboratory Practice with sterile instruments to avoid external contamination. Country specific regulations regarding sampling, pooling and subsampling should be followed. Pre-enrichment should be performed according to ISO 6579, FDA-BAM or USDA. In general 25 g or 375 g of sample is analyzed in a 1:9 sample/broth ratio (25 g in 225 ml broth or 375 g in 3375 ml broth, e.g. Buffered Peptone Water, BPW). Pre-enrichment of environmental samples and primary production samples should be performed according to ISO 6887. To enable sufficient propagation of potentially present Salmonellae during incubation time, the used pre-enrichment medium has to be pre-warmed to 37°C 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 material for future microbiological or biomolecular analysis at least 3 ml of the 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 °C to +8 °C for at most 72 hours. Storage in test tubes is just a recommendation, the 1 ml sample for DNA extraction may also be transferred directly from the initial vessel into a conical screw cap tube and the vessel 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 should be used for pre-enrichment.
- 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 initial vessel (e.g. Stomacher bag) can be stored for potential subsequent cultural examination.
- Certain sample matrices, such as turf- or soil-containing sock swabs and feces with a high concentration of humic acids or polyphenolic substances (e.g. feces from cattle), are known to have a potential inhibitory effect on the Real-Time PCR. In such cases a second enrichment step is performed with the pre-enrichment and the entire process of DNA extraction and Real-Time PCR is repeated (see details below). Or, 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 in between.

- For the second enrichment step the selective medium Rappaport-Vassiliadis-Soy Broth (RVS) is used. The readily incubated preenrichment (sample in e.g. BPW) is diluted at a ratio of 1:100 in RVS (e.g. 100 µl in 10 ml). It is incubated for at least 5 hours at + 41.5 ± 1 °C without agitation. Longer incubation times can be implemented if required or if more suitable for the daily lab routine.

2. Colony Material from Solid Media

- 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.
  - The DNA Extraction-Mix II is stirred on a slow moving magnetic stirrer, so that aliquots of a homogenous suspension can be taken. The pellet is resuspended by repeated up-and-down pipetting in 500 µl of DNA Extraction-Mix II. Use a 1000 µl pipette with filtered tips. Avoid formation of bubbles and aerosols and proceed with step 5 in 3a. »DNA Preparation Using the optionally included DNA-Extraction Mix- II«.
  - Alternatively the purification of the isolate can be carried out using Kylt® RNA/DNA Purification (see also 3b. »DNA Preparation Using Kylt® RNA/DNA Purification«) or using manually or (semi-)automated DNA-purification systems (see also 3c. »DNA Preparation Using Manual or (Semi-)Automated DNA-Purification Systems«).

3. DNA preparation

- DNA preparation can be carried out using either the optionally included DNA Extraction-Mix II (proceed with chapter 3a), manual DNA-purification using Kylt® RNA/DNA Purification (proceed with chapter 3b), or other manual or (semi-) automated DNA-purification systems in combination with respective DNA-purification kits (proceed with chapter 3c).

3a. DNA Preparation Using the Optionally Included DNA Extraction-Mix II

1. Pre-heat the heating block to set temperature of +100 °C, the block must have an actual temperature of +100 ± 3 °C at use.
2. The aliquoted pre-enrichment in the conical screw cap tube is pelleted by centrifugation at 10.000 g to 12.000 g for five minutes.
3. Discard the supernatant using a 1000 µl pipette tip, not by decantation.
4. The DNA Extraction-Mix II is stirred on a slow moving magnetic stirrer, so that aliquots of a homogenous suspension can be taken. The pellet is resuspended by repeated up-and-down pipetting in 200 µl of DNA Extraction-Mix II. Use a 1000 µl pipette with filtered tips. Avoid formation of bubbles and aerosols.
5. Screw cap tight, vortex thoroughly and incubate for 10 min to 15 min at +100 ± 3 °C.
6. Vortex sample thoroughly and centrifuge at 10.000 g to 12.000 g for five minutes; 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 longterm storage of the DNA extract at -18 °C to -20 °C, transfer supernatant to a 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 ± 3 °C, vortex and spin down by pulse-centrifugation.

3b. DNA Preparation Using Kylt® RNA/DNA Purification

- For detailed information, please refer to the Direction For Use of Kylt® RNA/DNA Purification.

3c. DNA Preparation Using Manual or (Semi-)Automated DNA-Purification Systems

- The DNA preparation from samples can also be performed manually or on (semi-)automated DNA-purification systems using commercially available DNA-purification kits. For the specific purification protocols please refer to the manual of the respective DNA purification kit appropriate for the sample matrix which has to be analyzed.

4. PCR Setup and Amplification

- Reaction-Mix and Negative Control are vortexed and pulse-centrifuged before each use.
- Determine the number of PCR-reactions needed: number of samples + Positive and Negative Control.
- Pipette 16 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 4 µl of Negative Control to corresponding cavity and seal.
- Add 4 µl of sample DNA preparation to the corresponding cavity and seal. In case of manually prepared DNA-extract solely use clear supernatant, avoid transferring debris.
- Once all sample cavities are sealed, 4 µl of the Positive Control are added to the corresponding cavity and sealed. Vortex and pulsecentrifuge the rehydrated Positive Control before each use.
- Avoid formation of bubbles when pipetting Reaction-Mix, samples and controls. It is recommended to always briefly pulse-centrifuge cavities before the PCR run.
- Place cavities in Real-Time PCR thermal cycler and amplify using the following parameters:

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 and HEX		

- Alternatively, the following standard Kylt® Profile I can be used to combine Kylt® Salmonella spp. 2.0 with other Kylt Real-Time-(RT-) PCR Detection products.

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 and HEX		

- These amplification parameters allow the combination of this Kylt® Salmonella spp. 2.0 Real-Time PCR with other Kylt® Real-Time PCRs for detection of bacteria or viruses. When combining several detection tests, make sure all necessary channels are used!
- Please follow the specific instructions of your Real-Time PCR thermal cycler as recommended by the manufacturer.

5. Analysis

General

- The data of the amplification reactions can automatically be processed using specific software for the Real-Time PCR thermal cycler. Alternatively, the thresholds can be set manually using either the linear or the logarithmic view. The threshold should cross the HEX-curve of the Negative Control and the FAM curve of the Positive Control, respectively, in the exponential area of its slope (see exemplary figures below). By setting the thresholds, the crossing points with the HEX- and FAM-curves determine the respective "cycle of threshold" (Ct), which is negatively correlated with the initial concentration of copies of the target genes in the Real-Time PCR reaction.

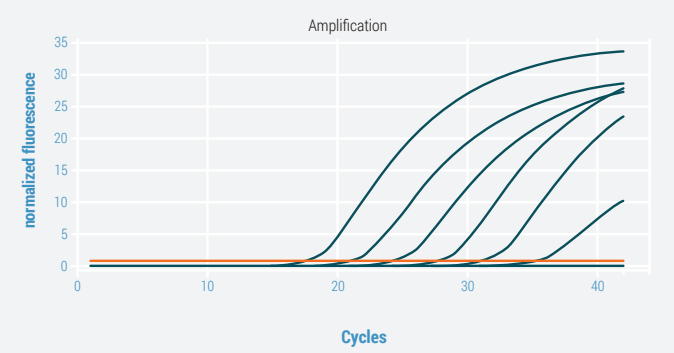

Figure 1: Linear view

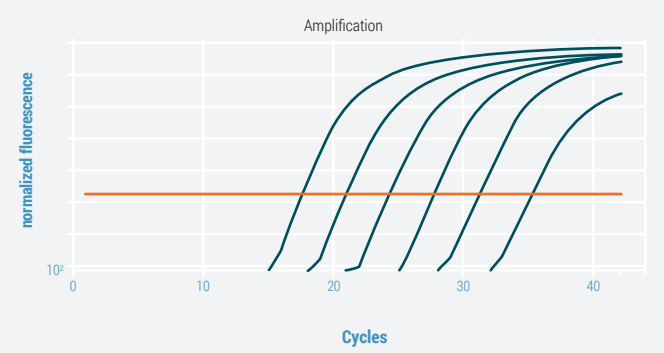

Figure 2: Logarithmic view

- Only curves with the typical exponential amplification, meaning the curve of the raw data shows a flat baseline at the beginning in the linear view, followed by a clear (exponential) slope in fluorescence and possibly reaching a plateau-phase, should be regarded as positive.
- For the test analysis the validity of the run is determined with the Negative and Positive Controls. After that the validity of each single sample is verified by the Internal Amplification Control reaction and finally their Salmonella spp.-specific status is checked.

Test Evaluation

- The **test** 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. For a valid test the FAM-Ct-value of the Positive Control should be > 15 and ≤ 35 and the HEX Ct-value of the Negative Control should be ≤ 40.

Target	Channel	Signal			
Internal Control	HEX	positive	positive	negative	negative
Salmonella spp.	FAM	negative	positive	positive	negative
The sample is *Salmonella spp.*		**negative**	**positive**	**positive**	**inhibited**

- A **sample** is **negative for *Salmonella spp.*** if its HEX-curve is positive, but its FAM-curve is negative.
- A **sample** is **positive for *Salmonella spp.*** if its FAM-curve is positive, independent of the HEX-curve.
- A **sample** is **inhibited** if neither the FAM-curve nor the HEX-curve are positive.
- **Recommendation:** Recommendation: In case of an inhibited sample the PCR can be repeated with a 1/10 dilution of the DNA extract. If the sample is still inhibited after running the PCR with the diluted sample or as a direct alternative, the pre-enrichment may be incubated for at least 5 hours in RVS (for details see chapter D.1. »Cultural Pre-Enrichment«). By this step potentially present ingredients in the preenrichment that have inhibitory effects on the effectiveness of the Real-Time PCR are diluted and potentially present Salmonellae are selectively propagated. After the second enrichment the entire process of DNA preparation and Real-Time PCR is repeated (see chapters D.3. »DNA Preparation« and D.4. »PCR Setup and Amplification«).
- Convenient and reliable sample data entry, Real-Time PCR start, final qualitative analysis and documentation can be conducted with the Kylt® PCR Software package, please inquire.
- Presumptive results should be confirmed by methods other than the Kylt® Salmonella spp. 2.0 (DNA Extraction and) Real-Time PCR Detection kit. It is recommended to use the cultural reference method for Salmonella spp. (ISO 6579) to confirm the results. Briefly, a subculture of the pre-enrichment is prepared in RVS broth or on MSRV agar as well as in MKTTn broth, depending on the sample type. Subsequently, the incubated samples are streaked on XLD agar and one additional selective agar. Typical colonies are confirmed by serological or biochemical methods. For details please refer to the current version of ISO 6579.

E. Supplementary information

- The components of the Kylt® Salmonella spp. 2.0 products listed in the table on page 2 are equivalent reagents.
- Kylt® Salmonella spp. 2.0 has been validated in a harmonized MicroVal/AOAC RI/Nordval validation in combination with the included DNA Extraction-Mix II as well as with Kylt® RNA/DNA Purification (Art. No. 31314/31315) as an alternative DNA purification method. The validation covers samples from raw meat and ready to cook meat products (25 g and 375 g), raw poultry and ready to cook poultry products (25 g and 375 g), environmental samples (such as process water or swab samples) as well as primary production samples (such as boot socks, faeces or rectal swabs). The performance of the kit was tested with the following Real-Time PCR instruments: ABI 7500 platform (Life Technologies), CFX 96 and CFX 384 (BioRad), Rotor-Gene Q (Qiagen), Rotor-Gene 3000 and 6000 (Corbett Research), AriaMx Pro (Agilent), LightCycler® 96 and 480 (Roche), Mic (BMS).
- For more information please refer to MicroVal Certificate No. 2017LR78.

Production:

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Development, manufacturing and distribution of Kylt® *In-Vitro* Diagnostica is certified according to ISO 9001:2015.

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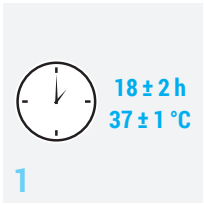
qPCR.Salm.02, Rev004, April 2018

PROTOCOL AT A GLANCE

Real-Time PCR Setup

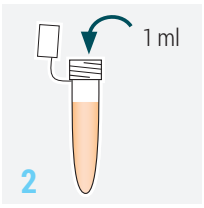
1. Pre-Enrichment of *Salmonella* spp.

Buffered Peptone Water
18 ± 2 h at 37 ± 1 °C



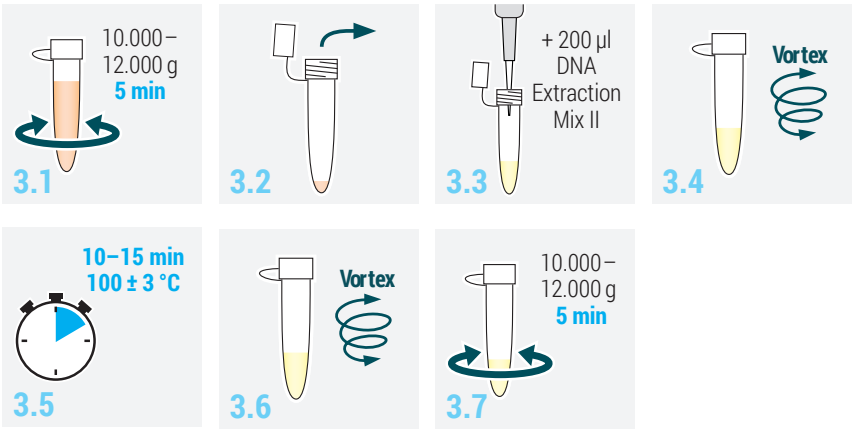
2. Bacterial Harvest

Transfer 1 ml pre-enrichment



3. DNA Extraction

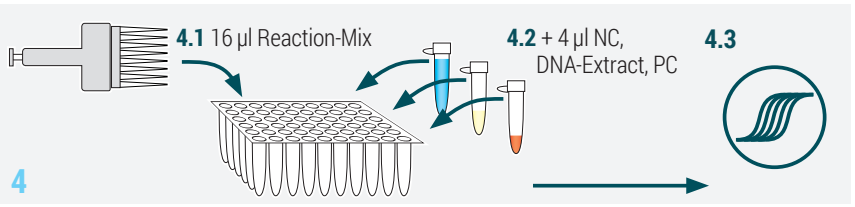
3.1 10.000-12.000 g 5 min
3.2 Discard supernatant
3.3 Add 200 µl DNA-Extraction Mix II
3.4 Mix by vortexing
3.5 Incubation 10–15 min 100 ± 3 °C
3.6 Mix by vortexing
3.7 10.000-12.000 g 5 min



Step 2. and 3. may alternatively be processed manually or (semi-) automated with appropriate DNA-purification kits; volume of pre-enrichment transferred may have to be adjusted.

4. PCR Setup

4.1 Mix Reaction-Mix and dispense
4.2 Add 4 µl NC, DNA-Extract, PC
4.3 Seal cavities and amplify



5. Analysis

Set threshold and analyse samples