Kylt®

V For *in vitro* Veterinary Diagnostics only.

Kylt® *Salmonella* spp.

DNA Extraction and Real-Time PCR Detection Kit for *Salmonella* spp. suitable for sock swabs, feces and dust samples from chickens and turkeys as well as feces, rectal swabs, organ and tissue samples from swine and cattle

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Kylt® *Salmonella* spp.

Kylt® *Salmonella* spp. DNA Extraction and Real-Time PCR Detection Kit

100 Reactions, *in vitro* Diagnosticum
for chickens, turkeys, swine and cattle

A. General

- Kylt® *Salmonella* spp. DNA Extraction and Real-Time PCR Detection Kit detects *Salmonella* spp. in sock swabs, feces and dust samples from chickens and turkeys as well as in feces, rectal swabs, organ and tissue samples from swine and cattle.
- Kylt® *Salmonella* spp. comprises all reagents necessary for sample preparation from cultured materials as well as all reagents and controls for subsequent detection of bacterial DNA of *Salmonella* spp.: After bacterial pre-enrichment and DNA extraction, *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* spp. 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.
- Kylt® Salmonella spp. is validated according to international standard DIN EN ISO 16140 for sock swabs, feces and dust samples from chicken and turkeys (Report 2389, DIL – Deutsches Institut für Lebensmitteltechnik e.V.).
- This kit was developed for use by trained laboratory personnel following standardized procedures.

B. Reagents and Materials

- Kylt® *Salmonella* spp. DNA Extraction & Real-Time PCR Detection Kit contains the following reagents:

Reagent	Color code of lid	Quantity in kit with 100 reactions	Storage conditions
DNA Extraction-Mix II	○ white	1 x 20 ml	+2 °C to +8 °C
Reaction-Mix	● yellow	4 x 500 µl	+2 °C to +8 °C
Positive Control	● red	2 x lyophilisate / 2 x 20 µl rehydrated	+2 °C to +8 °C lyophilized
			+2 °C to +8 °C rehydrated (max. 2 days)
			-18 °C to -20 °C rehydrated (max. 6 months)
Negative Control	● blue	1 x 1 ml	+2 °C to +8 °C

- All reagents are stored at +2 °C to +8 °C. Reaction-Mix has to be stored dark, do not expose to direct sunlight! The reagents are to be used within the indicated shelf life.
- Before the first use the lyophilized Positive Control is rehydrated: 20 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 and HEX (emission at 520 and 550 nm, respectively).
- We recommend to exclusively use 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)
  - 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 the 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 extraction, 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 microbial contamination from external sources.

Chicken and turkey:

Sampling, pooling and subsampling as well as pre-enrichment is described in respective EU legislation. Pooled sock swabs, subsamples of feces or dust and surface dust sampling swabs are pre-enriched according to the respective EU legislation in the given volume of Buffered Peptone Water (BPW). For instance, pools of (two) sock swab 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).

Swine and cattle:

The sample preparation and pre-enrichment is carried out according to DIN EN ISO 6579-1:2017.

General:

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.

- At least 3 ml of supernatant of the pre-enrichment are transferred to a sterile test tube with sterile transfer pipettes and the Stomacher bag is discarded.
- **Note:** 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.
- An 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 for potential subsequent cultural examination.
- 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.
- Certain sample matrices, such as turf- or soil-containing sock swabs and feces (not including rectal swabs) from cattle with a high 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 is 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 in 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 of 1:100 in RVS (e.g. 100 µl in 10 ml). It is incubated for 5 ± 1 hours at +41,5 ± 1 °C without agitation.

2. DNA Extraction

- Pre-heat heating block to set temperature of +100 °C, the block must have an actual temperature of +100 ± 3 °C at use.
- 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.
- Discard the supernatant using a 1000 µl pipette tip, not by decantation.
- 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.
- Screw cap tight, vortex thoroughly and incubate for 10 min to 15 min at +100 ± 3 °C.
- 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 long-term 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.
- **Note:** The DNA-Extraction can alternatively be performed with other manual or automated methods. It is recommended to comparatively verify alternative processes in accordance with the responsible laboratory manager and in consideration of the current state of science and technology for the matrices to be examined.

3. PCR Setup and Amplification

- Reaction-Mix and Negative Control are vortexed and centrifuged before each use.
- Determine the number of PCR-reactions needed: number of samples + Positive 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 sample DNA extract to the corresponding cavity and seal. Solely use clear supernatant, avoid transferring debris.
- Once all sample cavities are sealed, 2 µl of the Positive Control are added to the corresponding cavity and sealed. Vortex and centrifuge the rehydrated Positive Control before each use.

- Avoid formation of bubbles when pipetting Reaction-Mix, samples and controls. It is recommended to always centrifuge cavities before the PCR run.
- Place cavities in Real-Time PCR thermal cycler and amplify using the following parameters:

Step	Temperature	Duration	
Activation Polymerase	95 °C	10 min	
Denaturation	95 °C	15 sec	42 cycles
Annealing & Extension	60 °C	1 min	
Fluorescence detection	Channels HEX and FAM		

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

4. Analysis

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, with following considerations: The threshold should be at least a ten-fold standard deviation above the average fluorescence value of cycles 3 to at least 15. 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 of 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 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 has to be > 15 and ≤ 35 and the HEX Ct-value of the Negative Control has to be > 10 and ≤ 40.

HEX-curve positive	yes	yes	no	no
FAM-curve positive	no	yes	yes	no
The sample is *Salmonella* spp.	**negative**	**positive**	**positive**	**inhibited**

- The **sample** is **negative** if its HEX-curve is positive (10 < Ct ≤ 40), but its FAM-curve is negative.
- The **sample** is **positive** if its FAM-curve is positive (10 ≤ Ct ≤ 42), independent of the HEX-curve.
- The **sample** is **inhibited** if neither the HEX-curve nor the FAM-curve is 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 pre-enrichment 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«).
- 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.

Production:
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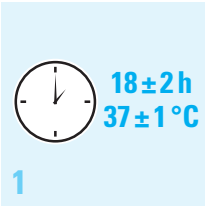
PROTOCOL AT A GLANCE

Pre-Enrichment, DNA Extraction and Real-Time PCR

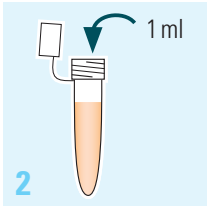
1. Pre-Enrichment of *Salmonella* spp.

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



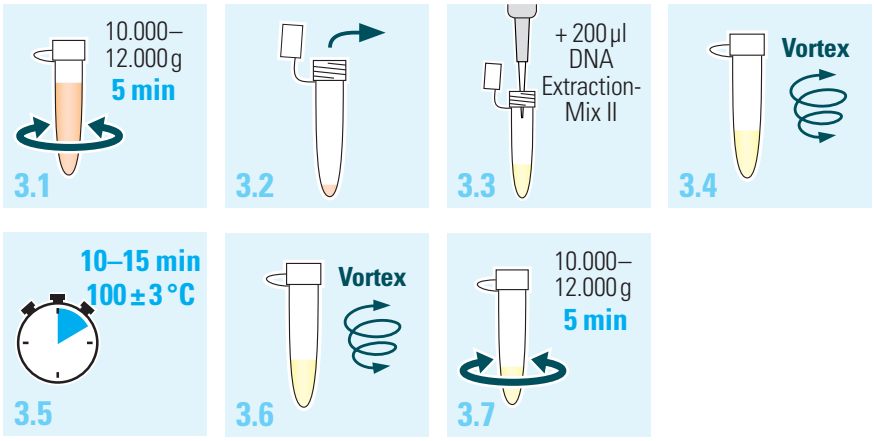
2. Bacterial Harvest

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

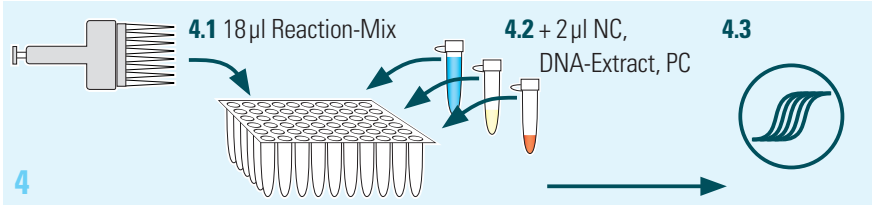


4. PCR Setup

4.1 Mix Reaction-Mix and dispense

4.2 Add 2 µl NC, DNA-Extract, PC

4.3 Seal cavities and amplify



5. Analysis

Set threshold and analyse samples