For in vitro Veterinary Diagnostics only.

KyIt® SE DIVA 2

DNA Extraction and Real-Time PCR for differentiation of *Salmonella* Enteritidis vaccine strain SM24/Rif12/Ssq from field strains

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Kylt® SE DIVA 2

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100 / 25 reactions

A. General

- Kylt® SE DIVA 2 DNA Extraction and Real-Time PCR is suitable for differentiation of Salmonella Enteritidis (SE) live vaccine strain Sm24/Rif12/Ssq (SEV2) from field strains (SEF). The live vaccine strain Sm24/Rif12/Ssq is present in the commercially available vaccine AviPro SALMONELLA VAC E. Kylt® SE DIVA 2 is intended to be used for Salmonella spp.-positive material from cultural processes according to DIN EN ISO 6579 or equivalent cultivation methods.

- With limitations the kit can also be used for Salmonella spp. positive pre-enrichments in Buffered Peptone Water. In this case only the detection of the vaccine strain is significant, any indication for an SE field strain has to be confirmed by cultural isolation methods e.g. ISO 6579 and Kauffmann-White Agglutination.

- If the kit is to be used for examination of flocks which have been vaccinated with the AviPro SALMONELLA DUO vaccine, the kit can only be used on Salmonella spp. positive colony material from cultural processes.

- Only samples which were positive for Salmonella spp. with a Ct value <25 in the Kylt® Salmonella spp. kit (Art. No. 31019) are suitable for analysis with Kylt® SE DIVA 2. This restriction applies regardless of the origin of the sample (e.g. colony material derived from cultural process or pre-enrichments in Buffered Peptone Water). In case of positive samples with Ct >25 in Salmonella spp. screening a second enrichment step should be performed. For detailed information see chapter D 2 »Analysis of pre-enrichments in Buffered Peptone Water«.

- Kylt® SE DIVA 2 comprises all reagents and controls for DNA extraction, detection and differentiation of bacterial DNA of SE. After bacterial pre-enrichment and DNA extraction the qualitative testing of Kylt® SE DIVA 2 is based on a triplex Real-Time PCR system: the target genes specific for serovar Salmonella Enteritidis (SE), field strains (SEF) of this serovar and live vaccine strain Sm24/Rif12/Ssq (SEV2) are amplified in parallel by respective primer pairs in the 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 SE, SEF and SEV2 are labeled with fluorescent dyes FAM, Texas Red and Cy5, respectively, and their emitted fluorescence is separately optically measured by the Real-Time PCR thermal cycler. By means of three individual analyses in one reaction vessel per sample and the Negative Control and Positive Controls per run the SE field strain- or SE vaccine strain Sm24/Rif12/Ssq-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® SE DIVA 2 contains the following reagents:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Color code of lid</th>
<th>Quantity in kit with 25 / 100 reactions</th>
<th>Storage conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Extraction-Mix II</td>
<td>white</td>
<td>1 x 7 ml / 1 x 20 ml</td>
<td>+2 °C to +8 °C</td>
</tr>
<tr>
<td>Reaction-Mix</td>
<td>violet</td>
<td>1 x / 4 x 500 µl</td>
<td>-18 °C to -20 °C</td>
</tr>
<tr>
<td>SEF-Positive Control (SEf PC)</td>
<td>red</td>
<td>1 x / 2 x lyophilisate (final 20 µl each)</td>
<td>+2 °C to +8 °C lyophilized -18 °C to -20 °C rehydrated</td>
</tr>
<tr>
<td>SEV2-Positive Control (SE Sm24/Rif12/Ssq PC)</td>
<td>red</td>
<td>1 x / 2 x lyophilisate (final 20 µl each)</td>
<td>+2 °C to +8 °C lyophilized -18 °C to -20 °C rehydrated</td>
</tr>
<tr>
<td>Negative Control</td>
<td>blue</td>
<td>1 x 1 ml</td>
<td>+2 °C to +8 °C</td>
</tr>
</tbody>
</table>

- After receipt the components of the kit should immediately be stored at the respective temperature listed in the table above. Avoid repeated freezing and thawing of the kit or its components and keep them thawed as short as possible. 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 sun(light). The kit and its components are to be used within the indicated shelf life (see label on the outer packing), if stored properly.

- Before its first application rehydrate both lyophilized SEF- and SEV2-Positive Controls 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 (Emission 520 nm), Texas Red (Emission 605nm) and Cy5 (Emission 670nm).

- 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 equipment is needed (not included in this kit):
  - Incubators for sample pre-enrichment (+37 ± 1 °C)
  - Table top microcentrifuge
  - Dry heating block (+100 °C ± 3 °C)
  - Magnetic stirrer
  - 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 Controls** 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 kit contains separate Positive Controls with SEf- or SE Sm24/Rif12/Ssq-specific DNA standards (channels FAM and TXR positive or channels FAM and Cy5 positive, respectively). The sample testing is only valid if both, the Negative Control and the two Positive Controls, are included in every Real-Time PCR run and used to verify the validity of such run.

- This kit does not include a primer-probe-setup/channel for detection of the **Internal Amplification Control (IAC)**. During initially screening of the DNA extracts with Kylt® *Salmonella* spp. it is possible to exclude potential inhibition of Real-Time PCR amplification and detection.

D. Protocol

1. **Analysis of isolates derived from cultural processes**

   - The main application of Kylt® SE DIVA 2 is the differentiation of field and vaccine strains using *Salmonella* spp. Isolates derived from cultural processes according to DIN EN ISO 6579.

   - The overall protocol of the analysis consists of the following main workflow:
     1. Cultural isolation according to DIN EN ISO 6579 or comparable processes
     2. DNA extraction (see chapter D 1.1 »DNA Extraction«)
     3. reaction setup and amplification (Real-Time PCR) (see chapter E 3 »PCR Setup and Amplification«)
     4. data analysis – validity and qualitative result (see chapter E 4 »Data Analysis«)

   - When analyzing colony material with SE DIVA 2 it is recommended to initially or in parallel screen the samples with Kylt® *Salmonella* spp.. Since high DNA concentrations can lead to PCR inhibition it 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 SE DIVA 2.

1.1 **DNA Extraction**

   - Kylt® SE DIVA 2 should only be used after the samples were initially analyzed with Kylt® *Salmonella* spp. (Art. No. 31019), the following steps should therefore have 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.

   - 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 magnet stirrer; it must be used as a homogenous suspension. For DNA Extraction resuspend the colony material in 500 µl DNA-Extraction-Mix II by repeated up-and-down pipetting. 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.

Proceed with chapter 3 »Polymerase chain reaction«.

2. Analysis of pre-enrichments in Buffered Peptone Water

- The overall protocol of the analysis consists of the following main workflow:
  1. Pre-enrichment in Buffered Peptone Water (see chapter D 2.1 »Cultural Pre-Enrichment«)
  2. DNA extraction (see chapter D 2.2 »DNA Extraction«)
  3. Reaction setup and amplification (Real-Time PCR) (see chapter E 3 »PCR Setup and Amplification«)
  4. Data analysis – validity and qualitative result (see chapter E 4 »Data Analysis«)

- Kylt® SE DIVA 2 can only be used directly on DNA extracts from Buffered Peptone Water enrichments if the following conditions are met:
  - Samples have been positive for Salmonella spp. using Kylt® Salmonella spp. Real-Time PCR with a CT <25.
  - Samples have been taken from birds which were vaccinated with AviPro Salmonella Vac E.
  - Kylt® SE DIVA 2 cannot be used if AviPro SALMONELLA DUO has been applied to the flock.

- In case the sample is positive for Salmonella spp. with a CT >25 a second enrichment in Selenite-Cystine medium is mandatory. Therefore the readily incubated pre-enrichment (sample in BPW) is diluted at a 1:10 ratio in Selenite-Cystine (e.g. 100 µl in 10 ml). It is incubated overnight at +37 ± 1 °C without agitation. After a second enrichment in Selenite-Cystine samples have to be screened for the presence of Salmonella spp. using Kylt® Salmonella spp. Only samples showing a CT <25 after a second enrichment in Selenite-Cystine are suitable for further testing using Kylt® SE DIVA 2

2.1 Cultural Pre-Enrichment

- 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 local legislation, such as EU legislation. Pooled sock swabs, subsamples of feces or dust and surface swabs are immersed according to the respective 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 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 several days. 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 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, 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 E 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.

### 2.2 DNA Extraction

- Kylt® SE DIVA 2 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.

### E. Polymerase chain reaction

### 3. PCR 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 + SEF-Positive Control, SEV2-Positive Control 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 SEF-Positive Control is added to the corresponding cavity and sealed. Afterwards, 2 µl of the SEV2-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:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Duration</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activation of Polymerase</td>
<td>95 °C</td>
<td>10 min</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>95 °C</td>
<td>15 sec</td>
<td></td>
</tr>
<tr>
<td>Annealing &amp; Extension</td>
<td>60 °C</td>
<td>1 min</td>
<td>42</td>
</tr>
<tr>
<td>Fluorescence Detection</td>
<td>channels FAM, TXR and Cy5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- 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® SE DIVA 2 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

- For evaluating the amplification data the threshold has to be set manually, preferably in linear scale of x- and y-axis of the amplification plot. For the amplification plot of the SEV2-Positive Control reaction a strong increase in fluorescence in the FAM- and Cy5-channel is expected. For the amplification plot of the SEf-Positive Control reaction a strong increase in fluorescence in the FAM- and TXR-channel for SE field strain is expected.

- The thresholds should cross the FAM-curve, TXR-curve and Cy5-curve in the linear increase of their slope (log scaling of the y-axis). By setting the thresholds, the crossing points with the FAM-, TXR- and Cy5-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. For detailed information about the threshold setting please refer to the manufacturer’s Direction For Use of the respective Real-Time PCR thermal cycler.

- 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. Finally, the SEf- or SE Sm24/Rif12/Ssq-specific status of each sample is analyzed (channels FAM- and TXR or FAM and Cy5, respectively).
Test Evaluation

- In order to correctly interpret the obtained results it is mandatory to know which vaccine has been applied in the flock.

- The **Real-Time PCR test run** is only **valid** if the following conditions are met:
  1. The Negative Control is negative in FAM-, TXR- and Cy5-channel.
  2. The SEV2-Positive Control is positive in FAM- and Cy5-channel.
  3. The SEf-Positive Control is positive in FAM- and TXR-channel.

- **Attention:** In samples with a positive TXR- and / or Cy5-channel and a negative FAM-channel, respectively, the Serovar *Salmonella* Enteritis and the SE vaccine strain Sm24/Rif12/Ssq could not be detected. However, another *Salmonella* serovar apart from *Salmonella* Enteritidis might be present in the sample.

- If the conditions mentioned above are met the following results are to be expected:

<table>
<thead>
<tr>
<th></th>
<th>FAM-curve (Serovar SE) is</th>
<th>TXR-curve (SEf) is</th>
<th>Cy5-curve (SEV2) is</th>
<th>The sample is <em>Salmonella</em> Enteritidis</th>
<th>The sample is SEf*</th>
<th>The sample is SEV2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>positive</td>
<td>positive</td>
<td>negative</td>
<td>positive</td>
<td>positive</td>
<td>negative</td>
</tr>
<tr>
<td></td>
<td>positive</td>
<td>negative</td>
<td>negative</td>
<td>positive / no further typing possible</td>
<td>negative</td>
<td>negative</td>
</tr>
</tbody>
</table>

*An indication for a SEf strain also includes detection of live vaccine strains which are not Sm24/Rif12/Ssq. In case of direct use of Buffered Peptone Water enrichments only the detection of the vaccine strain Sm24/Rif12/Ssq is a valid result. Any indication of a field strain has to be confirmed by cultural and biochemical methods.

- **A sample is positive for Serovar *Salmonella* Enteritidis** if the FAM-channel is positive, independent of the channels TXR and Cy5. In case the sample is positive in the FAM-channel and negative in the TXR- and Cy5-channel, the SE strain present in the sample could not be further differentiated. In this case the analysis should be repeated using fresh colony material from ISO 6579 derived processes.

- **A sample is positive for SEV2** if its FAM and Cy5-curve are positive.

- **A sample is positive for SEf** if its FAM- and TXR-curve are positive. **Attention:** An indication for a SEf strain also includes detection of live vaccine strains which are not Sm24/Rif12/Ssq.

- In case Kylt® SE DIVA 2 has been used directly on Buffered Peptone Water enrichments only the detection of a vaccine strain represents a valid result. Every indication of a SEf infection has to be confirmed by cultural and biochemical methods (e.g. ISO 6579 and Kauffmann White scheme).

- A potential **double infection with a SEV2 and SEf or with SEV2 and another *Salmonella* serovar is possible** if its FAM-, TXR- and Cy5-curves are positive. Double infections are to be confirmed by cultural and biochemical methods (e.g. ISO 6579 and Kauffmann White scheme).

- 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.