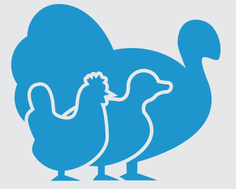
For *in vitro* Veterinary Diagnostics only.

Kylt®

Kylt® SE DIVA 2

Real-Time PCR Detection



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Revision No.	Amendments
011	addition of vaccine strain CAL 10 Sm+/Rif+/Ssq- (present in commercially available vaccine *Primun Salmonella E*), which will also be detected in the SEV2-channel (Cy5-channel) together with the *AviPro Salmonella vaccine,* Lid colour of SEf PC is red-white.
010	valid from 01 August 2023: exclusion of Kylt® DNA Extraction-Mix II, new layout for test evaluation.

A. General

- Kylt® SE DIVA 2 kits are intended for the differentiation of Salmonella Enteritidis (SE) from SE field strains (SEf) and the following live vaccine strains (hereinafter summarized as "SEV2"):
  - Name of the commercially available vaccine: ***AviPro SALMONELLA VAC E*** and ***AviPro SALMONELLA DUO***
  - Name of the strain: Sm24/Rif12/Ssq
  - Name of the manufacturer: Elanco (Lohmann Animal Health GmbH)

  - Name of the commercially available vaccine: ***Primun Salmonella E***
  - Name of the strain: CAL 10 Sm+/Rif+/Ssq-
  - Name of the manufacturer: CALIER
- The kits are suitable for the analysis of pre-enrichment samples positively tested for *Salmonella* spp. with a Ct value < 25 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 > 25 in *Salmonella* spp. screening either a second enrichment step should be performed or the analysis should be repeated using colony material from microbiological culture.
- **Attention**: If the kit is to be used for examination of flocks which have been vaccinated with the ***AviPro SALMONELLA DUO*** vaccine, the kit can <u>only</u> be used on *Salmonella* spp. positive colony material (isolates) from cultural processes.
- 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.

- The qualitative testing with Kylt® SE DIVA 2 kits is based on a triplex Real-Time PCR: In one reaction setting, the target genes for SE, SEf and SEV2 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 SE, SEf and SE vaccine strains are labeled with fluorescent dyes FAM, TXR and Cy5, 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 SEf or SEV2-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 DIVA 2 kits are available and comprise the following reagents:

Reagent	Colour of Lid	100 Reactions Article No 31159	25 Reactions Article No 31160	Store at
Reaction-Mix	violet	4 x 450 µl	1 x 450 µl	≤ -18 °C
SEf-Positive Control (SEf PC)	red-white	2 x lyophilizate (final 50 µl each)	1 x lyophilizate (final 50 µl each)	≤ -18 °C
SEV2-Positive Control (SEV2 PC)	red	2 x lyophilizate (final 50 µl each)	1 x lyophilizate (final 50 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 Controls SEf and SEV2: add 50 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, TXR and Cy5 (emission 520, 620 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 DIVA 2 kits:
  - DNA preparation kit / protocol (e.g. Kylt® RNA / DNA Purification products)
  - 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 Control 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.

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 & 2. Cultural Pre-enrichment & DNA Extraction

- Before using Kylt® SE DIVA 2 screen the samples with regard to the *Salmonella* spp. status, please refer to chapter F "Accessory products" and the according Directions For Use. Only confirmed *Salmonella* spp.-positive samples can be analyzed by Kylt® SE DIVA 2. Thus, cultural pre-enrichment and DNA extraction is to be done according to the Direction for Use for the *Salmonella* spp.-confirmatory PCR method applied. By this, the DNA extract to be applied for Kylt® SE DIVA 2 is also already proven to be of appropriate quality by the preceeding PCR methods.

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 two more for the Negative Control and the Positive Control.
- The Reaction-Mix is ready-to-use, add 16 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 4 µl of the Negative Control to the corresponding cavity and seal it individually, if possible.
- Add 4 µl of each DNA preparation to the corresponding cavities and seal them individually, if possible.
- To minimize risk of potential cross-contaminations, once all sample cavities are sealed, 4 µl of the **SEf**-Positive Control is added to the corresponding cavity and sealed. Afterwards, 4 µl of the **SEV2**-Positive Control is added to the corresponding cavity and sealed. 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.

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, TXR and Cy5		

- 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, TXR and Cy5		

- 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.

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 **SEf**-Positive Control reaction a strong increase in fluorescence in the **FAM- and TXR-channel** for SE field strain is expected. For the amplification plot of the **SEV2**-Positive Control reaction a strong increase in fluorescence in the **FAM- and Cy5-channel** 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 SEV2-specific status of each sample is analyzed (channels FAM and TXR or channels FAM and Cy5, respectively).

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		
	FAM	TXR	Cy5
Negative Control	negative Ct > 35	negative Ct > 35	negative Ct > 35
SEf-Positive Control	**positive** Ct ≤ 35	**positive** Ct ≤ 35	negative Ct > 35
SEV2-Positive Control	**positive** Ct ≤ 35	negative Ct > 35	**positive** Ct ≤ 35

- In order to correctly interpret the obtained results it is mandatory to know which vaccine has been applied to the flock.
- **Attention**: In samples with a positive TXR- and / or Cy5-channel and a negative FAM-channel, respectively, neither the Serovar *Salmonella* Enteritidis nor the SE vaccine strains Sm24/Rif12/Ssq and CAL 10 Sm+/Rif+/Ssq- could be detected. However, another *Salmonella* serovar might be present in the sample.

Test Evaluation - Samples

Target	Channel	Signal			
Salmonella Enteritidis (SE)	FAM	**positive**	**positive**	**positive**	negative
SE field strain (SEf)*	TXR	negative	**positive**	negative	positive / negative
SEV2 (SE vaccine strain Sm24/Rif12/Ssq or CAL 10 Sm+/Rif+/Ssq-)	Cy5	**positive**	negative	negative	positive / negative
The sample is *Salmonella* Enteritidis		**positive**	**positive**	**positive no further typing possible**	**negative**
The sample is SEf *		**negative**	**positive**	**negative**	**negative**
The sample is SE vaccine strain Sm24/Rif12/Ssq or CAL 10 Sm+/Rif+/Ssq-		**positive**	**negative**	**negative**	**negative**

* This also includes the detection of SE field strains as well as of live vaccine strains which are not Sm24/Rif12/Ssq or CAL 10 Sm+/Rif+/Ssq-. In case of direct use of Buffered Peptone Water enrichments only the detection of the vaccine strains Sm24/Rif12/Ssq and CAL 10 Sm+/Rif+/Ssq- are valid results. Any indication of a field strain has to be confirmed by cultural and biochemical methods.

- A **sample** is **negative for SE, SEf and SEV2** if its FAM-, Cy5- and TXR-curve are negative.
- A **sample** is **positive for Serovar *Salmonella* Enteritidis** if its FAM-curve is positive, independent of the Cy5- and TXR-curves. In case the sample is positive in the FAM channel and negative in the Cy5- and TXR-channel, the SE strain present in the sample could not be further differentiated. In this case the analysis should be repeated using colony material from ISO 6579 derived processes.
- A **sample** is **positive for SEV2 (vaccine strain Sm24/Rif12/Ssq or CAL 10 Sm+/Rif+/Ssq-)** if its FAM- and Cy5-curves are positive (Ct < 30).
- A **sample** is **positive for SEf** if its FAM- and TXR-curves are positive (Ct < 30).
  Attention: This result also includes the detection of SE field strains as well as of live vaccine strains other than SE Sm24/Rif12/Ssq or CAL 10 Sm+/Rif+/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 SEV2 and SEf or with SEV2 and another *Salmonella* serovar** is possible if its FAM-, TXR- and Cy5-curves are positive (CT<30). Double infections are to be confirmed by cultural and biochemical methods (e.g. ISO 6579 and Kaufmann-White scheme).
- 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 veterinary and in food and feed 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 veterinary samples (spin-column based).
Kylt® RNA / DNA Purification HTP	31575	4 x 96	Magnetic bead based combined RNA and DNA purification kit for veterinary diagnostic samples. Suitable for Kylt® Purifier and Kylt® Purifier 48.
Kylt® Purifier	31436	1 unit	Purification system for magnetic bead based kits. Up to 96 samples are processed in under 30 minutes. Intended for high-throughput laboratories.
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 Sets	Plate with 96 separate spin tips, used by the Kylt® Purifer to mix the well contents by stirring. Sufficient for 480 samples.
Kylt® Purifier Plates	31435	20 Plates	Plates to be used for the several reactions and reagents during automated nucleic acid purification. Sufficient for 320 to 480 samples (depending on device and protocol).

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 and catalogue number
- Quantity and size of products
- Indicate if your account is VAT exempt

Production:

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PROTOCOL AT A GLANCE
Real-Time PCR Setup

1

Pulse-vortex and spin down

Vortex

2

Dispense Reaction-Mix and add 4 µl NC, DNA preparation, PC

+ 16 µl Mix

+ 4 µl NC,
DNA preparation, PC

3

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

4

Analysis

normalized fluorescence
positive sample
CT
exponential phase
threshold
baseline
negative sample
cycle