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Kylt® SGP & 9R DIVA

PCR Detection Kit for *Salmonella* Gallinarum, Pullorum (separate detection) & DIVA 9R

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100 / 25 reactions
in vitro Diagnosticum for birds

A. Introduction

- Kylt® SGP & 9R DIVA PCR Detection Kit is for detection and differentiation of *Salmonella* Gallinarum, *Salmonella* Pullorum (separate detection) and *Salmonella* Gallinarum vaccine strain 9R in isolates derived from cultural processes with suitable sample material originating from birds.

- Kylt® SGP & 9R DIVA comprises all reagents and controls for detection and differentiation of bacterial DNA of *Salmonella* Gallinarum (SG), *Salmonella* Pullorum (SP) and *Salmonella* Gallinarum vaccine strain 9R (9R) in a multiplex PCR. The qualitative testing of Kylt® SGP & 9R DIVA is based on the Polymerase Chain Reaction (PCR) in one reaction vessel. By using specific oligonucleotides, the target genes of interest in a sample are specifically amplified. Following amplification the PCR reaction is analyzed by agarose gel electrophoresis for qualitative test result. By analyzing the detection of the target genes in the sample, the Negative Control and Positive Control the SG, SP or 9R-specific status of the 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® SGP & 9R DIVA contains the following reagents:

Reagent	Colour code of lid	Quantity in kit with 25 / 100 reactions	Storage conditions
2x PCR-Mix	○ white	1 x / 4 x 280 µl	-18 °C to -20 °C
10x Loading Dye	○ transparent	1 x / 4 x 60 µl	-18 °C to -20 °C
Primer-Mix	○ white	1 x / 4 x lyophilisate (final 160 µl each)	-18 °C to -20 °C
Positive Control (PC)	● red	2 x / 4 x lyophilisate (final 20 µl each)	-18 °C to -20 °C
Negative Control (NC)	● blue	1 x 1 ml	-18 °C to -20 °C

- After receipt, the kit and its components are immediately stored at -18 °C to -20 °C. Avoid repeated freezing and thawing of the kit or its components and keep them thawed as short as possible. If needed, generate appropriate aliquots upon its first thawing. The kit and its components are to be used within the indicated shelf life (see label on the outer packing), if stored properly. The components of different batches may not be mixed.

- The Primer-Mix needs to be stored at -18 °C to -20 °C. Before first use the lyophilized Primer-Mix is rehydrated: add 160 µl of Negative Control per vial of Primer-Mix, briefly incubate at room temperature and mix by repeated vortexing. Generate aliquots of suitable volumes and store them at -18 °C to -20 °C.

- The kit can be used on all commercially available PCR thermal cyclers.

- 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):

  - DNA preparation kit / protocol (e.g. Kylt® DNA Extraction-Mix II or Kylt® RNA/DNA Purification)
  - Table top microcentrifuge
  - Vortex
  - Micropipettes covering volumes of 1 µl to 1000 µl
  - Centrifuge for PCR tubes or plates
  - PCR thermal cycler
  - Dry heating block (if applicable for DNA preparation)
  - Magnetic stirrer (if applicable for DNA preparation)
  - Equipment, media and disposables for agarose gel electrophoresis

- Accessory Kylt® products (not included in all products):
  - Kylt® DNA Extraction-Mix II (article no 31398 for 100 preparations) for simplified and economic DNA extraction from swabs, pure bacterial isolates or bacterial enrichments)
  - Kylt® RNA/DNA Purification (articles no 31314 / 31315 for 250 / 50 preparations) for purification of high quality RNA and DNA from vast variety of veterinary samples or from pure bacterial or viral culture isolates or enrichments.

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 provides the ability to monitor the specificity and efficiency of the reagents used, i.e. the performance of PCR itself and of the PCR thermal cycler, respectively. The sample testing is only valid if both the Negative Control and the Positive Control reactions are included in every PCR run and used to verify the validity of such run.

D. Protocol

- The overall protocol of the SGP & 9R analysis consists of the following workflow:

1. sample processing
2. DNA preparation
3. reaction setup and amplification (PCR)
4. agarose gel electrophoresis
5. 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 to -20 °C or -70 °C to -80 °C until further processing of the SGP & 9R detection. Avoid repeated freezing and thawing of the DNA preparations.

1. Sample Processing

- The kit is intended for use on material derived from cultural processes. Pure or mixed colony material is directly transferred into respective tube for Kylt® DNA Extraction (see next chapter), such as conical screw cap tube; therefore a little amount of a single colony is picked with a sterile loop wire or sterile pipette tip and transferred into the tube.

2.DNA Preparation

a) Kylt® DNA Extraction (requires Kylt® DNA Extraction-Mix II)

  - Kylt® DNA Extraction is applied for simplified and economic extraction of bacterial DNA from pure bacterial isolates or bacterial enrichments:
  - 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 DNA Extraction-Mix II is stirred on a magnet stirrer, it must be used as a homogenous suspension. In case of pure or mixed colony material massive load with specific bacterial DNA is expected, thus use 500 µl of DNA Extraction-Mix II and thoroughly resuspend the transferred material. 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 final 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.

b) DNA Preparation by other methods

- All kind of sample matrices may be processed with appropriate DNA preparation kits, such as Kylt® RNA/DNA Purification (please refer to chapter C. "Equipment and Reagents not Included") or appropriate in-house methods.
- For detailed information on the DNA preparation process, please refer to the Direction For Use or Standard Operating Procedure of the respective kit or in-house method, respectively.

3. Reaction Setup, Reverse Transcription and Amplification

- Before each use, briefly vortex and spin down the 2x PCR-Mix and the rehydrated Primer-Mix (see also chapter B "Reagents and Materials").
- 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.
- Prepare a Master-Mix containing the 2x PCR-Mix, 10x Loading Dye and the SGP & 9R Primer-Mix for the appropriate number of reactions. Then add 18 µl of the Master-Mix to each of the PCR tubes. The PCR is set up in the given order:

Reagent	Volume (µl)	
	Per reaction	e.g. n=7
2x PCR-Mix	10.0	70.0
10x Loading Dye	2.0	14.0
SGP & 9R Primer-Mix	6.0	42.0
Total Master-Mix	**18.0**	**126 µl, dispense 18 µl per reaction**
DNA (Negative Control / sample / Positive Control)	2.0	
Total Reaction	**20.0**	

- Return the 2x PCR-Mix and the Primer-Mix back to -18 °C to -20 °C right after application. Avoid the formation of bubbles when pipetting the Master-Mix, samples and controls.
- Add 2 µl of the Negative Control to the corresponding tube and seal it.
- Add 2 µl of the sample DNA (final DNA preparation) to the corresponding tube and seal it.

- To minimize risk of potential cross-contaminations, 2 µl of the Positive Control are added to the corresponding tube after all previous samples and control reactions are set up. Before each use, briefly vortex and spin down the rehydrated Positive Control (see also chapter B "Reagents and Materials").
- It is recommended to briefly spin the tubes before start of the PCR run.
- Place cavities in the PCR thermal cycler and run the test using the following parameters:

Step	Temperature	Duration	
Activation of Polymerase	94 °C	3 min	
Denaturation	94 °C	30 sec	35 cycles
Annealing	64 °C	30 sec	
Extension	72 °C	60 sec	
Post-PCR cooling (optionally)	7 °C	hold	

- Please follow the specified instructions of your PCR thermal cycler as recommended by the manufacturer.

4.Agarose Gel Electrophoresis

- The expected SGP and 9R product sizes are:
  - *Salmonella* Pullorum: **only** 255 bp
  - *Salmonella* Gallinarum: 175 bp **and** 255 bp
  - *Salmonella* Gallinarum vaccine strain 9R: 120 bp **and** 175 bp **and** 255 bp
- You may run any standard agarose gel electrophoresis method appropriate for this product size. An appropriate method is described briefly below:
  - Prepare a 2% agarose gel for separation of the DNA sample after PCR amplification.
  - Load the wells of the agarose gel with 5 µl of the PCR reactions from the DNA sample(s), Positive Control and Negative Control, respectively. Load at least an additional well of agarose gel with sufficient volume of e.g. an 100 bp reference DNA ladder. Make notes of the position of sample(s), controls and ladder.
  - Run the electrophoresis at a voltage of approximately 15 V/cm (the distance in cm refers to the distance between electrodes) for 45 min to 60 min.
  - Following electrophoresis, stain the gel with appropriate amount of nucleotide / intercalating dye (e.g. Ethidium bromide, GelRed Nucleic Acid stain or SYBR green) and visualize by using the corresponding technique. For more details, please refer to the respective Direction For Use of the dye.

5.Data Analysis – Validity and Qualitative Result

- The readily stained agarose gel must give discrete bands of expected sizes for control reactions and the reference DNA ladder. The actual PCR-test analysis starts with the validity check of the entire PCR run. Therefore, check the results of Positive Control and Negative Control for presence / absence of expected product sizes of appr. 120 bp, 175 bp and 255 bp. Afterwards, the SGP and 9R -specific status of each sample is analyzed by looking for presence / absence of the expected product size as described above.

Test Evaluation

- The **PCR test run** is only **valid** if the Negative Control is negative and the Positive Control is positive with regard to the expected SGP and 9R specific product sizes of appr. 120 bp, 175 bp and 255 bp.
- A **sample** is SGP and 9R **negative**, if absence of all three specific products with sizes of 120 bp, 175 bp and 255 bp is observed.
- A **sample** is *Salmonella* Pullorum **positive**, if presence of only one PCR product with a size of 255 bp is observed.
- A **sample** is *Salmonella* Gallinarum **positive**, if presence of two PCR products with a size of 175 bp **and** 255 bp is observed.
- A **sample** is *Salmonella* Gallinarum vaccine strain 9R **positive**, if presence of three PCR products with sizes of 120 bp **and** 175 bp **and** 255 bp is observed.

Production:

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