vetproof® STM Vaccine Detection 1 Kit
- 5'Nuclease - Order No. V 900 03

Instruction for Use
Version 1, August 2019 - EN

For veterinary diagnostics only (swine)

▽48 rxn

Real-time PCR-kit for the identification of the live vaccine strain Salmoporc (*Salmonella* Typhimurium 421/125, distributed by Ceva Santé Animale, Dessau, Germany). In addition the test kit detects *Salmonella* spp. and *S.* Typhimurium (STM) field strains in a second channel, so field strains can be differentiated from the vaccination strain.

The intended use of **vet**proof® STM Vaccine Detection 1 Kit exclusively is the detection of the vaccine strain, detection in other channels serve for control purposes only. In cases of positive STM vaccine detection (FAM channel), the field strain assay (HEX channel) indicates whether the sample also contains a *Salmonella* field strain. A positive result for *S.* Typhimurium in melting curve analysis (ROX Channel) in combination with a positive result in the field strain channel indicates a presumed occurrence of this serotype as a field strain in the sample. This has to be confirmed by classical microbiological methods.

The Internal Control (IC, Cy5 channel) is included in the assay to rule out PCR inhibition, preventing false-negative results. A negative result in all channels with a positive internal control shows that the samples are free from all respective parameters.

A. Kit contents

Component	Format	Function
Microplate, prefilled with 48 reactions, (lyophilized)	Aluminum bag containing 8-tube PCR-strips • V 900 03-1 (LP) with „low profile" 8-tube PCR-strips* • V 900 03-2 (RP) with „regular profile" 8-tube PCR-strips*	• Ready-to-use PCR-reaction-mix containing primer and hydrolysis probes specific for STM vaccine strain 421/125, *Salmonella* field strains and the Internal Control (IC) as well as Taq-DNA-Polymerase and Uracil-DNA Glycosylase (UNG, heat labile)**. • Protect from light and moisture.
Control Template	Vial 1 (purple cap)	• 1 x 300 µl • Contains a stabilized solution of DNA. • For use as a PCR run positive control.
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Component	Format	Function
H_2O PCR-grade	Vial 2 (colorless cap)	• 2 x 1 ml • Nuclease-free, "PCR-grade" H_2O. • PCR run negative control.
8-Cap Strips	Plastic bag containing 8-cap strips	• 12 x 8-cap strips to seal the reaction tubes. • To close 8-tube PCR-strips after adding sample.

* Compatibility of PCR-tubes with real-time PCR-instruments is provided online by BIOTECON Diagnostics: www.bc-diagnostics.com/compatibility-chart

** The PCR reaction mix contains Taq polymerase for PCR and Uracil-DNA N-Glycosylase (UNG) for efficient degradation of previously amplified DNA. The real-time PCR-kit contains dUTP instead of dTTP: This technique relies on the incorporation of deoxyuridine triphosphate (dUTP) during all amplification reactions. The UNG cleaves DNA at any site where a deoxyuridine residue has been incorporated. The resulting abasic sites are hydrolyzed due to the high temperatures during the initial denaturation step, and can no longer serve as PCR templates.

Store the kit at 2 °C to 8 °C until the expiration date printed on the label. Store the 8-tube PCR-strips with the lyophilized reagents in the aluminum bags and protect them from light and moisture. Close the bag carefully after use.

Additional equipment required

- Real-time PCR-cycler suitable for detection of FAM-, HEX-, ROX and Cy5 signals, and generation of melting curves
- Nuclease-free pipette tips
- Pipettes
- Centrifuge for 8-strip PCR-tubes (150 x g)

B. Sample preparation

Sample material and enrichment

Relevant sample material such as boot socks, dust, tissue or fecal samples has to be enriched in the recommended volume ratio in buffered peptone water (BPW) for 18 ± 2 hours at 37 ± 2 °C according to ISO 6579-1:2017.

If PCR inhibition occurs (check Internal Control, see chapter E) BIOTECON Diagnostics suggest a selective sub cultivation with pre-warmed Mossel Broth (1 ml of first enrichment in 9 ml Mossel Broth Medium for at least 5 ± 0.5 h (at the most 24 h) at 37 ± 1°C and 150 revolutions per minute). This sub cultivation is recommended, if the analysis of *Salmonella* spp. with PCR-kits has generated Cq values > 30.

DNA extraction

For DNA extraction, the **food**proof® StarPrep Three Kit (order number S 400 18) is recommended (for details see ordering information from BIOTECON Diagnostics).

C. Real-time PCR time-temperature protocol

The following procedure is optimized for a real-time PCR-instrument with a FAM (STM Vaccine 421/125), HEX (field strains of *Salmonella* and other vaccines with the exception of the STM Vaccine strain 421/125), ROX (*S.* spp.), and Cy5 (Internal Control) detection channel, capable of performing a melting curve analysis. Melting curve analysis is required for the differentiation of field strain *S.* Typhimurium and closely related serotypes.

Program the real-time PCR-instrument before preparing the samples. Use the real-time PCR-protocol below for the **vet**proof® STM Vaccine Detection 1 Kit (for details on how to program the experimental protocol, see the operator's manual of your real-time PCR-cycler):

Pre-incubation	**1** cycle
Step 1:	37 °C for 4 minutes
Step 2:	95 °C for 5 minutes
Amplification	**50** cycles
Step 1:	95 °C for 5 seconds
Step 2*:	60 °C for 60 seconds
* Fluorescence detection in step 2	
Melting Curve	**1** cycle
Step 1:	95 °C for 50 seconds
Step 2:	60°C for 50 seconds
Step 3*:	ramp up to 80 °C
* Fluorescence detection during 60 - 80 °C ramp with approx. 1 measurement/°C	

Note:

- For the Mx3005P real-time PCR-Cycler (Bio-Rad), step 1 of amplification needs to be changed:
  Step 1: 95 °C for **15 seconds**.
  Filter Set Gain Settings need to be set to: CY5 x1, ROX x1, HEX-JOE x4, FAM x4.
- For the CFX96™ real-time PCR (Bio-Rad), step 2 of the melting curve needs to be changed to: Step 2: 50 °C for 50 seconds.
- For Applied Biosystems® 7500 Fast (Thermo Fisher Scientific), the Fast System Software must be used for melting curve analysis. Step 2 of the melting curve needs to be changed to: 45 °C for 60 seconds
- For PCR-Cyclers without HEX channel, VIC channel can be used instead. For the PikoReal® Yakima Yellow (YY) needs to be used instead of HEX.
- For some real-time PCR-instruments the type of the probe quencher as well as the usage of a passive reference dye has to be specified. The **vet**proof® STM Vaccine Detection 1 Kit contains probes with a non-fluorescent ("dark") quencher and no passive reference dye.
- For LightCycler® 480 Systems I and II, color compensation is required and will be supplied by BIOTECON Diagnostics (Color Compensation Set 3; Order No. A 500 10). Please contact BIOTECON Diagnostics for further information.

D. Preparation of the PCR-Mix

Proceed as described below to prepare a 25 µl standard reaction.

Always wear gloves when handling the PCR-tubes. Sample material should be suitable for PCR concerning purity, concentration and absence of inhibiting substances (see chapter B).

Note: The lyophilized reaction mix is stable only if the PCR-strips are stored in the provided aluminum bag with the silica gel pads to avoid liquid absorption.

1. Take the needed number of 8-strip PCR tubes out of the aluminum bag. Use scissors to cut the required amount of reaction wells. Tightly seal the bag and make sure the silica gel is included.
2. Place the 8-strip PCR tubes containing the lyophilized reagents in a suitable PCR tube rack. Check that the reagent pellets are at the bottom of the tubes. If not, briefly centrifuge or flick the pellets to the bottom before proceeding.
3. Uncap the tube strips cautiously and discard the clear cap strips.

   Note: Do not leave strips open for extended periods of time. To avoid unwanted liquid absorption, only open strips briefly before filling.

4. Pipet 25 µl sample extract into each PCR tube and resuspend the pellet by cautiously pipetting up and down.
   - If less sample volume is available, dissolve the pellet in H_2O (PCR-grade) and add samples to a total volume of 25 µl.
   - For the negative control, add 25 µl H_2O PCR-grade (vial 2, colorless cap).
   - For the positive control, add 25 µl of Control Template (vial 1, purple cap).
5. Seal the vessels tightly with a new set of colorless cap strips.

   Note: Alternatively, resuspend the pellet after closing the vessels by mixing thoroughly. To reduce the risk of cross-contamination, only one PCR-strip should be processed at a time.

6. Briefly (5 seconds) spin the PCR-strips in a suitable centrifuge (150 x g).
7. Put the samples in the real-time PCR-cycler and start the program (see chapter F).

   Note: For the LightCycler 480, a special adapter is required (Oder No. Z 100 24). For some PCR-instruments the PCR-strips should be placed evenly distributed into the cycler block (e.g. place two strips in column 1 and 12).

E. Data interpretation

Amplification

The amplification of vaccine strain *Salmonella* Typhimurium 421/125 DNA is analyzed in the FAM detection channel. DNA-amplification of *Salmonella* Typhimurium field strain (and closely related serotypes) is detected in channel HEX. DNA of *Salmonella* spp. is detected in ROX, Internal Control in the Cy5 detection channel.

- Positive amplification signals in the FAM and ROX detection channels show the presence of vaccine strain *Salmonella* Typhimurium 421/125 in the tested sample.
- Positive amplification signals in the HEX and ROX detection channels show the presence of *Salmonella* Typhimurium (or closely related serotypes) in the tested sample.
- A positive amplification signal in ROX detection channel indicates the presence of *Salmonella* spp.
- A melting curve peak (T_m 67-73 °C) in ROX detection channel indicates the presence of *Salmonella* Typhimurium
- To exclude matrix-based PCR inhibition if FAM, HEX and ROX channels are negative, check the IC in CY5 channel. A negative result is valid, if the IC is detected. Should all channels show a negative result, please refer to chapter G for troubleshooting.

STM Vaccine [FAM]	*Salmonella* field strains [HEX]	*Salmonella* spp. [ROX]	Tm 67-73 °C [ROX]	IC [Cy5]	Interpretation of the result
+	-	+	+	+/-	Positive for STM vaccine 421/125
-	+	+	+	+/-	Negative for STM vaccine 421/125 Positive for *S.* Typhimurium field strains
+	+	+	+	+/-	Positive for STM vaccine 421/125 and *S.* Typhimurium field strains
-	+/-	+	-	+/-	Negative for STM vaccine 421/125 Positive for *Salmonella* field strains other than Typhimurium
-	-	-	-	+	Negative for all *Salmonella* spp.
-	-	-	-	-	Invalid result

Melting Curves

Samples that are positive in HEX and ROX detection channels can be further differentiated using a melting curve analysis in the ROX channel. A positive signal in melting curve analysis is used for the identification of *S*. Typhimurium.

A requirement for the clear assignment of the serotype *S.* Typhimurium is an adequate calibration of the PCR-cycler for the channels FAM, HEX, ROX and Cy5. Please refer to the operating manual of your real-time PCR-cycler for further information.

Result Interpretation of the melting curves

Melting peak range temperature (Tm) for *S*. Typhimurium.

	T_m 67 – 73 °C
ROX	***Salmonella* Typhimurium**

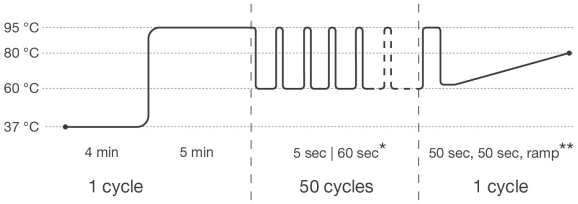
For some real-time PCR-cycler the peak range can differ from the melting peak range temperature of the table. For this case the control template contains a mixture of all target sequences and can be used as an approximate reference for the melting peak ranges in the particular PCR run. Furthermore some sample preparation matrices can slightly shift melting peak temperatures (not more than ± 1 °C variance from the peak of the control template).

The peak height and Tm of positive samples may vary depending on matrices, number of DNA-copies and cycler used.
Note that the presence or absence of specific melting peaks should be checked manually for all positive samples, as the peak finding algorithms of the respective PCR-instrument software may not detect all relevant peaks of the melting curve. A guarantee for the identification by melting curves cannot be given.

F. Protocol and workflow

Set up the PCR-instrument program before preparing the samples. Assign these channels:

- FAM (STM Vaccine 421/125), HEX (*Salmonella* field strain and vaccine strains other than 421/125), ROX (*Salmonella* spp.), Cy5 (Internal Control)



Pre-incubation: 1 Cycle
Step 1: 37 °C for 4 minutes
Step 2: 95 °C for 5 minutes
Amplification: 50 Cycles
Step 1: 95 °C for 5 seconds
Step 2*: 60 °C for 60 seconds
Melting curve: 1 Cycle
Step 1: 95 °C for 50 seconds
Step 2: 60 °C for 50 seconds
Step 3**: increase to 80 °C

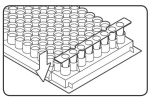
* Measurements in step 2 during amplification ** and in step 3 during melting curve analysis

Implementation: real-time PCR

Take appropriate precautions to prevent contamination, e.g. by using filter tips and wearing gloves.

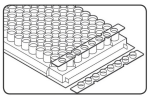
1. PLACE STRIPS IN RACK

Take needed number of PCR tubes out of the aluminum bag. Important: close the bag tightly afterwards. Place strips in a suitable PCR tube rack. If needed, gently tab the tubes to move the lyophilized pellets to the bottom of all tubes.



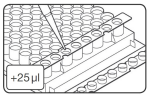
2. OPEN PCR-STRIPS

Open strips carefully just before filling and discard caps.
Do not leave open longer than necessary.



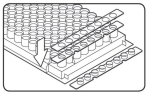
3. ADD SAMPLES AND CONTROLS

Pipet 25 µl of samples, negative control (colorless caps) or Control Template (purple cap) into respective wells.
If using a lower sample volume, add PCR-grade H_2O to a total volume of 25 µl.



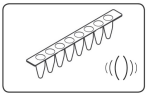
4. SEAL

Seal the tubes with new 8-cap strips accurately.



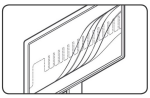
5. MIX AND CENTRIFUGE

Resuspend the pellet by mixing thoroughly after sealing. Alternatively resuspend the pellet by repeatedly pipetting up and down in step 3.
Spin strips for 5 seconds at approx.150 x g in a suitable centrifuge.



6. START REAL-TIME PCR RUN

Cycle samples as described above. Evenly spread the strips in the cycler block (e.g. two strips can be placed in column 1 and 12).



G. Troubleshooting

Observation	Possible Reason	Recommendation
No signal increase, even with positive controls.	Incorrect detection channel has been chosen.	• Set channel settings to FAM, VIC/HEX, ROX and Cy5.
	Pipetting errors.	• Check for correct reaction setup. Repeat the PCR run. • Always run a positive control along with your samples.
	No data acquisition programmed.	• Check the cycle program.
No signal increase in channel CY5.	Inhibitory effects of the sample material (e.g., caused by insufficient purification).	• Dilute samples or pipet a lower amount of sample DNA (e.g., 20 µl PCR-grade H_2O and 5 µl sample DNA instead of 25 µl sample DNA).
Fluorescence intensity is too low.	Inappropriate storage of kit components.	• Store the lyophilized PCR-mix at 2 °C to 8 °C, protected from light and moisture.
	Low initial amount of target DNA.	• Increase the amount of sample DNA. Depending on the chosen DNA isolation method, inhibitory effects may occur.
Strong decrease of fluorescence baseline.	Resuspension of lyophilized PCR-mix not complete.	• Always resuspend lyophilized PCR-mix thoroughly.
Negative control samples are positive.	Cross contamination.	• Exchange all critical solutions. • Repeat the complete experiment with fresh aliquots of all reagents. • Always handle samples, kit components and consumables in accordance with commonly accepted practices to prevent carry-over contamination. • Add positive control after all sample and negative control reaction tubes have been sealed.
Fluorescence intensity varies.	Insufficient centrifugation of the PCR-strips. Resuspension of the PCR-mix only in the upper part of the reaction tube.	• Always centrifuge PCR-strips.
	Outer surface of the vessel or the seal is dirty (*e.g.,* by direct skin contact).	• Always wear gloves when handling the vessels and seal.
Pellets are hard to dissolve.	The lyophilized PCR-mix started to rehydrate.	• Store the lyophilized PCR-mix tightly sealed in the aluminum bag with silica gel pad. • Open strips just before filling.

H. Symbols

REF	Product reference number		Expiry
	Kitsize / reactions		Protect from moisture
	Store at		Protect from heat and direct sunlight
	Batch		Manufacturer

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