Instructions for use
InviScreen® Swine Halal Speciation Kit

INVITEK diagnostics

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REF 6013009200 | Σ 100 tests | -25 °C / -15 °C | RUO

1. Intended use

InviScreen® Swine Halal Speciation Kit is specifically designed for the qualitative detection of swine (*Sus scrofa*) DNA in food and feed samples. This kit is intended for use by trained professionals in laboratory settings to assist in ensuring compliance with Halal requirements. The PCR kit enables users to identify potential contamination with swine-derived ingredients that may compromise the Halal status of the products. This kit is suitable for the analysis of various matrices, including raw materials, finished products, and environmental samples within the food and feed industry. It aids in monitoring and controlling the presence of swine DNA, thereby supporting efforts to maintain Halal integrity throughout the production and supply chain. Users should be aware that this kit does not provide information about other aspects of Halal compliance, such as the processing methods, ingredients, or other potential non-Halal contaminants.

2. Product description

InviScreen® Swine Halal Speciation Kit is a real-time PCR method designed to provide a sensitive and qualitative detection of swine (*Sus scrofa*) DNA in food, feed, and environmental samples. With an striking level of sensitivity, the kit enables the detection of swine DNA down to a limit of 0.0001% under optimal conditions. The method relies on the amplification of species-specific mitochondrial DNA. This was found to be an advantageous approach as mitochondrial genes are present in multi-copies, leading to higher detection sensitivity in comparison to single copy target genes found in the nuclear genome. This kit allows for simultaneous amplification of the target species and an internal amplification control (IAC) to mitigate the risk of false negative results resulting from PCR inhibition. This duplex detection system enhances amplification accuracy while simplifying reaction setup. The real-time PCR method provided in this kit can be conducted using commonly available PCR instruments equipped with FAM and VIC/HEX fluorescence channels.

3. Kit contents

REF.	COMPONENT	FUNCTION	CAP COLOR	QUANTITY
D33.01	Primer/Probe Mix[1]	Targeted detection		1 tube, 1000 µL
D33.02	qPCR Master Mix[1]	Amplification		1 tube, 1000 µL
D33.03	Negative Control	Negative Control		1 tube, 100 µL
D33.04	Positive Control	Positive Control		1 tube, 100 µL

[1] Reagents are supplied with a 5% of extra volume

4. Storage

Reagents should be stored sealed at -20 ± 5°C and may be used until the expiration date shown on the package label. Expiry date refers to the product under rightful handling and storage conditions. It is not recommended the use of the kit after the expiry date stated on the box. Avoid unnecessary repeated freeze/thawing cycles. Protect reagents from light exposure to prevent degradation.

5. Equipment and materials required (not provided)

- Food processor [2]
- DNA extraction kit
- Real-Time PCR instrument [3]
- Spectrophotometer/Fluorometer (optional) [4]
- Plates and/or tubes for qPCR
- 1.5 mL microcentrifuge tubes
- PCR cabinet [5]
- Micropipettes (10, 200 and 1000 µL) and filter tips
- Vortex and microcentrifuge

[2] To ensure accurate analysis of samples with multiple ingredients that are not in a powdered or granulated form, it is recommended to homogenize them carefully.

[3] The assay was validated on a Bio-Rad CFX96 instrument.

[4] It is recommended to quantify and assess the purity of the extracted DNA.

[5] To minimize the risk of contamination with foreign DNA, we recommend that the PCR reaction set-up is performed in a controlled environment.

6. Suitable test sample material

All kind of food and feed samples suitable for PCR analysis can be used, as long as the quality parameters for purity, concentration and integrity are fulfilled. It is recommended to carefully homogenize samples that contain more than one ingredient in its composition. In case of surface sampling, it is advised to perform sample collection using pre-moistened swabs in distilled water or sterile saline solution and rub the test surface (100 cm^2 area). In either case, it is necessary to adjust the quantity of sample in accordance to the manufacturer instructions of the total genomic DNA extraction kit in use. The use of InviSorb® Spin Tissue Mini Kit for DNA extraction is highly recommended.

7. Test procedure

a. PCR reaction preparation

Allow all reagents to thaw at room temperature, vortex and spin briefly to avoid drops on the vial cap. For each DNA sample prepare a reaction mix according to the table below:

REAGENT	VOLUME
qPCR Master Mix	10 µL
Primer/Probe Mix	10 µL
Total Volume	**20 µL**

Alternatively, the user may choose to prepare a working mix by mixing the total volume of the Primer/Probe Mix with the qPCR Master Mix in a separate tube (not provided). Vortex the mix gently and spin it down to obtain a working mix enough for 100 reactions. It is recommended to label the vial corresponding to the working mix.

1. Homogenize the reaction mixture and pipette 20 µL into individual wells according to the predicted PCR plate set-up.

2. Add 5 µL of DNA template to each well. The ideal concentration of DNA is 30 ng/µL. Total DNA added to the PCR reaction should never exceed 150 ng.

At least one positive control reaction and one negative control reaction must be included in the PCR run, replacing the sample in these wells with 5 µL of Positive Control and 5 µL of Negative Control respectively.

It is recommended to prepare the reaction mixture carefully in a controlled environment, preferably in a nucleic acid-free zone. The addition of the positive control and sample DNA should preferably be carried out in a separate room.

b. Amplification protocol

The amplification conditions are as follows:

	STEPS	TEMPERATURE	TIME	CYCLES
1	Enzymatic Activation	95 °C	5 min	1
2	Denaturation	95 °C	20 seg	40
3	Hybridization/extension plate reading *	60 °C	1 min	

* Fluorescence data must be obtained during this step through FAM (target DNA) and HEX/VIC (internal amplification control) channels.

c. Results interpretation

The results should be interpreted in accordance to the analysis software recommended by the Real-Time PCR instrument manufacturer. The software monitors DNA amplification through the detection of fluorescence emitted by each probe, attributing a Ct value for each reporter dye found in each individual sample. Target DNA amplification is monitored in FAM channel and the amplification control in HEX/VIC channel. After setting the threshold baseline, the analysis outcome should be interpreted according to the scenarios referred bellow.

The reaction includes an endogenous amplification control to monitor the reaction and to exclude the occurrence of PCR inhibition. A poor signal indicates the presence of PCR inhibitors in the reaction which compromises an effective and accurate speciation test. Under optimal conditions, the lowest amount of Swine DNA detected in 100% of the experiments was at least 0.0001% of Swine DNA (≤ 1 ppm).

A result is considered positive when Ct ≤ 35.

RESULT	Target DNA (FAM)	IAC (HEX/VIC)
Positive	+	+/-
Negative	-	+
Inconclusive [6,7]	-/?	-

[6] PCR inhibition may be due to the presence of excessive DNA and/or PCR inhibitors. It is recommended to dilute the DNA extracted from the sample 1:10 or 1:100 in DNAse/RNAse free water and repeat the Real-Time PCR reaction. When applicable, the LOD of the method should be adjusted in accordance to the dilution factor.
[7] The appearance and characteristics of the amplification curves should be thoroughly considered. Incomplete amplification curves often indicate low amount of DNA template. In this case, the positivity of the result is dubious, and the Real-Time PCR reaction should be repeated using a superior amount of DNA template.

8. Quality control

The test can only be considered valid under the following control conditions:

CONTROLS	Target DNA (FAM)	IAC (HEX/VIC)
Positive Control	+	+
Negative Control	-	+/-

If no amplification is observed for the positive control, the test results are invalid and must be repeated. The positive control template is expected to amplify before Ct 30.

If amplification is observed for the negative control it indicates that the reagents have become contaminated while setting up the run, invalidating test results.

9. Performance characteristics

Specificity: InviScreen® Swine Halal Speciation Kit was designed to specifically detect swine species. The following DNA extracts (Table below) were tested according to the general assay instructions and no amplification was obtained for species other than swine (highlighted in **Bold**).

MEAT

Chicken (*Gallus gallus*)
Cow (*Bos Taurus*)
Deer (*Cervus elaphus*)
Duck (*Anas platyrhynchos*)
Goat (*Capra Hircus*)
Pig (*Sus scrofa*)
Rabbit (*Oryctolagus cuniculus*)
Sheep (*Ovis aires*)
Turkey (*Meleagris gallopavo*)
Wild Boar (*Sus scrofa* breed
European wild boar)
Horse (*Equus caballus*)

FISH AND SEAFOOD

Codfish (*Gadus morhua*)
Grouper (*Epinephelus aeneus*)
Mussel (*Perna canaliculus*)
Gilt-head bream (*Sparus aurata*)
Monkfish (*Lophius litulon*)
Shrimp (*Peaneus Vannamei*)

FRUITS AND VEGETABLES

Almond (*Prunus dulcis*)
Apricot (*Prunus armeniaca*)
Brazil Nut (*Bertholletia excelsa*)
Broccoli (*Brassica oleracea*)
Cabbage turnip (*Brassica rapa*)
Cashew (*Anacardium occidentale*)
Celery (*Apium graveolens*)
Chives (*Allium schoenoprasum*)
Hazelnut (*Corylus avellana*)
Linseed (*Linum usitatissimum*)
Lupine (*Lupinus albus*)
Macadamia nut (*Macadamia integrifolia*) White pepper (*Piper nigrum*)
Maize (*Zea mays*)
Mustard (*Sinapis alba*)
Nutmeg (*Myristica fragrans*)
Oats (*Avena sativa*)
Parsley (*Petroselinum crispum*)
Peanut (*Arachis hypogaea*)
Pecan nut (*Carya illinoinensis*)
Pine nut (*Pinus pinea*)
Pistachio (*Pistacia vera*)
Potato (*Solanum tuberosum*)
Pumpkin (*Cucurbita pepo*)
Rice (*Oryza sativa*)
Rye (*Secale cereal)*
Sesame (*Sesamum indicum*)
Soy (*Glycine max*)
Sunflower (*Helianthus annuus*)
Thyme (*Thymus vulgaris*)
Tomato (*Solanum lycopersicum*)
Walnut (*Juglans regia*)
Wheat (*Triticum aestivum*)
White pepper (*Piper nigrum*)

Detection limit and sensitivity: The limit of detection (LOD) for a given analysis is often influenced by the sample matrix and the quantity and quality of the DNA extracted from the target ingredient. To determine the LOD for a specific method, in-house validation experiments are necessary. In our study, the LOD was determined through ten independent experiments that evaluated the sensitivity of the method. Internal DNA standards with decreasing amounts of swine DNA were used in the validation, either as pure genomic DNA or spiked matrices. Under optimal conditions, the method was able to detect at least 0.0001% of swine DNA (≤ 1 ppm).

Repeatability and reproducibility: InviScreen® kits demonstrated to have an excellent dynamic range, with relative standard deviation of Ct values less than 5% for all tested concentrations.

Robustness: The method revealed to be highly reliable, and unaffected by small variations deliberately introduced. The obtained results from at least 10 independent experiments, performed in duplicate, were concurring with the expected outcome.

Trueness: Trueness of the method proved to be of 100%, as the obtained results from at least 10 independent experiments, performed in duplicate, were concurring with the expected outcome.

Performance characteristics for validation: To evaluate the false positive and false negative rates, a total of 232 samples were tested in independent experiments, with 167 samples known to be positive and 65 samples known to be negative. Each experiment was performed at least twice. Our method showed no false positive or false negative results, with both rates at 0%, resulting in 100% positive predicted value (PPV) and negative predicted value (NPV).