ID-Check Horse Speciation Kit

User Guide

Test for the real-time PCR detection of horse DNA in food, feed and environmental samples

Catalog #12019423

BIO-RAD

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Section 1
Introduction

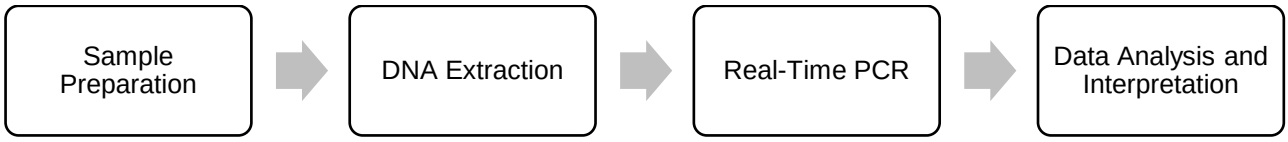
The identification of meat species is an important area in the food industry for reasons of transparency of labeling, religious or cultural restrictions, and economic transactions. Due to international scandal in the food supply chain related to meat adulteration, there is an ever-growing concern to overcome and anticipate potential fraud events in this sector, arising from intentional use of meat species with lower commercial value or adventitious contamination. Both result in non-compliance with the product label (Directive 2002/86/CE), the quality and security requirements and, ultimately jeopardize public health and diminish consumer confidence. Analytical tools such as real-time PCR allow for highly specific and sensitive target detection, suitable in most cases regardless of the processing steps to which the product has been subjected and the composition complexity.

Section 2
ID-Check Horse Speciation Kit Technology

ID-Check Horse Speciation Kit is a premium quality molecular biology solution to provide the general laboratory and research personnel an easy and quick ready-to-use kit for the detection of a selection of specific meat species. Under optimal conditions, these kits provide high sensitivity, able to detect at least 0.001% adulteration. The kit was designed to allow the simultaneous amplification (in multiplex) of the target meat species and an Internal Amplification Control (IAC) to exclude false negatives due to PCR inhibition. The duplex detection system helps maximize amplification accuracy and simplify the reaction set-up. The kit also includes an amplification control to monitor target DNA detection.

ID-Check Horse Speciation Kit provides a real-time PCR method for detecting horse (*Equus caballus*) mitochondrial DNA in food, feed and environmental samples. 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 multiple copies, leading to higher detection sensitivity compared to single copy target genes found in the nuclear genome. Amplification detection is based on Taqman technology, i.e., fluorescence emission is detected upon degradation of the hydrolysis probe during PCR reaction.

This test allows the detection of horse DNA in food, feed, and environmental samples. It includes the following four main steps:



Section 3
Kit Components

The ID-Check Horse Speciation Kit contains sufficient reagents for 100 tests.

Reagent ID	Reagent	Cap Color	Quantity Provided, µl
D01.01	Primer/probe mix	Brown	1 tube, 200
L000437	Master mix (2x)	Clear	1 tube,1250
D01.03	Negative control	Green	1 tube, 1000
D01.04	Positive control	Red	1 tube, 100

Section 4
Shelf Life and Storage

Once received, the kit must be stored at -20 ± 5°C. Reagents stored at this temperature can be used until the expiration date indicated on the package label. To avoid unnecessary freeze/thaw cycles, the PCR mix can be prepared in advance (refer to the mix table provided in the Invisorb Spin Tissue Mini Kit and ID-Check Speciation Kit quick guide (Bulletin 3443) and aliquoted into smaller volumes. Do not exceed 5 freeze/thaw cycles. Protect reagents from light to prevent degradation.

Section 5
Materials Required but Not Supplied

Equipment

- Food processor for homogenizing test samples
- Spectrophotometer to quantify and assess DNA purity (optional)
- Biosafety cabinet to minimize contamination with foreign DNA
- Vortexer
- Microcentrifuge
- Analytical balance with milligram readability
- Heating thermoshaker capable of maintaining 52 ± 1°C, with a mixing speed of at least 1,300 rpm
- 10, 200, and 1,000 µl micropipets
- Bio-Rad real-time PCR system*; for example, the CFX96 Touch Deep Well (catalog #3600037), CFX Opus Deepwell (catalog #17007991) or CFX Opus 96 (catalog #17007992) Real-Time PCR Systems

Note: We recommend using an uninterrupted power supply (UPS) with the thermal cycler.

* Contact Bio-Rad Technical Support for information on recommended instruments.

Supplies

- Invisorb Spin Tissue Mini Kit (DNA extraction kit, catalog #12019412)
- 99.7% isopropanol
- 96-100% ethanol
- Spatula
- 1.5 ml microcentrifuge tubes (for example, catalog #17008941)
- 1.5 ml conical screwcap tubes, sterile (for example, catalog #2240110XTU)
- PCR plates, tubes, sealing tape, and caps
- Sterile filter tips adaptable to 10, 200, and 1,000 µl micropipets
- Powder-free gloves
- Bleach, 5%
- Cleaning agent such as DNA AWAY or RNase AWAY

Section 6
Safety Precautions and Recommendations for Best Results

- This test must be performed by trained personnel
- The quality of results depends on strict compliance with Good Laboratory Practices (for example, the EN ISO 7218 standard), especially concerning PCR:
    - Never circulate laboratory equipment (pipets, tubes, etc.) from one workstation to another
    - Always use a positive control and a negative control for each series of amplification reactions
    - Do not use reagents after their expiration date
    - Vortex reagents from the kit before using them to ensure homogeneity
    - Periodically verify the accuracy and precision of pipets, as well as correct functioning of the instruments
    - Change gloves often, especially if you suspect they are contaminated
    - Clean work spaces periodically with 5% bleach and other decontaminating agents such as DNA AWAY
    - Use powder-free gloves and avoid fingerprints and writing on tube caps. Both will interfere with data acquisition

- Real-Time PCR Systems

    - Improper use of the CFX96 Touch Deep Well, CFX Opus Deepwell or CFX Opus 96 Real-Time PCR Systems may cause personal injury or damage to the instrument. Some components may pose a risk of personal injury due to excessive heat if improperly handled. For safe use, the real-time PCR system must be operated only by qualified laboratory personnel who have been appropriately trained. Servicing of instrument must be performed only by Bio-Rad Field Service Engineers

Section 7
Protocol

It is strongly recommended to read the entire protocol before starting the test.

A. Sample Preparation and DNA Extraction

All kinds of food and feed samples suitable for real-time PCR amplification can be used, as long as the quality parameters for purity, concentration and integrity are fulfilled. It is recommended to carefully homogenize samples that are composed of more than one ingredient. In case of surface sampling, it is advised to perform DNA collection using swabs pre-moistened in distilled water or sterile saline solution and rubbing the test surface (100 cm^2 area). Dry swabs should be added directly to lysis buffer and homogenized by vortexing. In all case, it is necessary to adjust the quantity of sample in accordance with the manufacturer's instructions for the total genomic DNA extraction kit being used.

Sample preparation and DNA extraction protocols for the Invisorb Spin Tissue Mini Kit have been optimized for use with the ID-Check Speciation Kits and are provided in the Invisorb Spin Tissue Mini Kit and ID-Check Speciation Kit quick guide. Following these instructions will improve the overall performance of the ID-Check Speciation kits.

Use the following table when preparing samples in accordance with the Invisorb Spin Tissue Mini Kit. Measuring the DNA concentration and normalizing it prior to qPCR is highly advised to avoid inaccurate results.

Product Type	Sample	Lysis Buffer	Elution Volume	Expected Yield
Surface swabs (buffer)	200 µl	400 µl	50 µl	1-10 ng/µl
Surface swabs (dry)	Direct	400 µl	50 µl	1-10 ng/µl
Plant-based vegetarian products	0.1 g	400 µl	50 µl	10-50 ng/µl
Fresh meat	0.1 g	400 µl	100 µl	200-400 ng/µl
Meat-based products	0.1 g	400 µl	100 µl	100-300 ng/µl

When working with some matrices such as sheep milk/cheese, chili meals, highly seasoned ready-to-eat meals, lard, and fatty foods like sausage and bacon, etc., it is important to consider the following:

- The time it takes to lyse the samples completely may be significantly longer than other matrices and is important to follow the recommendations in the quick guide

- An additional washing step is recommended

- Performing a 1:5 dilution of the lysate is recommended to remove inhibitors

B. PCR Reaction Preparation

Allow all reagents to thaw at room temperature, vortex and spin briefly to avoid drops on the cap.

Prepare a reaction mix for each DNA sample according to the table:

Reagent	Volume
Master mix (2x)	10 µ
Primer/probe mix	2 µl
Negative control	5 µl
Total Volume	**17 µl**

1) Homogenize the reaction mixture and pipet 17 µl into individual wells according to the PCR plate set-up.

2) Add 3 µl of DNA template to each well. The ideal concentration of DNA is 40 ng/µl. Total DNA added to the PCR reaction should not exceed 120 ng.

At least one positive control reaction and one negative control reaction must be included in the PCR run. Replace the sample in these wells with 3 µl of kit positive control and 3 µl of kit negative control (or RNase/DNase free water), 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.

C. Real-Time PCR

Set up the PCR run with the following amplification conditions:

Step	Temperature	Time	Cycles
Enzymatic activation	95°C	5 min	1
Denaturation	95°C	20 sec	30
Hybridization/extension/ plate reading[1]	60°C	1 min	

[1]Fluorescence data must be obtained during this step through the FAM (target DNA) and HEX (internal amplification control) channels.

D. Data Analysis

Results should be interpreted with the CFX Manager Software, Industrial Diagnostic Edition (IDE). The software monitors DNA amplification through the detection of fluorescence emitted by each probe, attributing a Cq value for each reporter dye found in each individual sample. Target DNA amplification is monitored in the FAM channel and the internal amplification control in the HEX channel. After setting the threshold baseline, the analysis outcome should be interpreted according to the scenario described below.

The reaction includes an internal 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 horse DNA detected in 100% of the experiments is at least 12 pg of pure genomic DNA and 0.001% horse DNA (10 ppm) in spiked matrices.

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

Control	Target (FAM)	IAC (HEX)
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 Cq 26 If amplification is observed for the negative control, the reagents have become contaminated while setting up the run, invalidating test results.

A result is considered positive when Cq ≤ 30.

Result	Target (FAM)	IAC (HEX)
Positive	+	+/-
Negative	-	+
Inconclusive[1,2]	-/?	-

[1] 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 PCR reaction. When applicable, the limit of detection (LOD) of the method should be adjusted in accordance with the dilution factor.
[2] The appearance and characteristics of the amplification curves should be thoroughly considered. Incomplete amplification curves often denote a low amount of DNA template. In this case, the positivity of the result is dubious, and the PCR reaction should be repeated using a higher amount of DNA template.

Section 8
Performance Characteristics

Specificity
ID-Check Horse Speciation Kit was designed to specifically detect horse species. The following DNA extracts were tested according to the general assay instructions and no amplification was obtained for

species different from horse.

Meat	
Cow *(Bos Taurus)*	Goat (*Capra hircus*)
Chicken (*Gallus gallus*)	Pig *(Sus scrofa domesticus)*
Deer (*Cervus elaphus*)	Rabbit (*Oryctolagus cuniculus*)
Duck (*Anas platyrhynchos*)	Sheep (*Ovis aires*)
European Wild Boar (*Sus scrofa*)	Turkey (*Meleagris gallopavo*)

Fish and Seafood	
Codfish (*Gadus morhua*)	Monkfish (*Lophius litulon*)
Gilt-head bream (*Sparus aurata*)	Mussel (*Perna cnaliculus*)
Grouper (*Epinephelus aeneus*)	Shrimp (*Peaneus vannamei*)

Fruits and Vegetables	
Almond (*Prunus dulcis*)	Ginger (*Zingiber officinale*)
Apple (*Bravo de esmolfe*)	Hazelnut (*Corylus avellana*)
Apricot (*Prunus armeniaca*)	Linseed (*Linum usitatissimum*)
Beans (*Phaseolus vulgaris*)	Lupine (*Lupinus albus*)
Brazil Nut (*Bertholletia excels*)	Macadamia nut (*Macadamia integrifolia*)
Broccoli (*Brassica oleracea*)	Maize (*Zea Mays*)
Cabbage turnip (*Brassica rapa*)	Mushrooms (*Agaricus bisporus*)
Carrot (*Daucus carota*)	Mustard (*Sinapis alba*)
Cashew (*Anacardium occidentale*)	Nutmeg (*Myristica fragrans*)
Cauliflower (*Brassica oleracea* var. *botrytis*)	Oats (*Avena sativa*)
Celery (*Apium graveolens*)	Onion (*Allium cepa*)
Chives (*Allium schoenoprasum*)	Parsley (*Petroselinum crispum*)
Cinnamon (*Cinnamonium verum*)	Peach (*Prunus persica*)
Coriander (*Coriandrum sativum*)	Peanut (*Arachis hypogaea*)
Cumin (*Cuminum cyminum*)	Pear (*Pyrus communis*)
Garlic (*Allium sativum*)	Pecan nut (*Carya illinoinensis*)
Pine nut (*Pinus pinea*)	Soy (*Glycine max*)
Pistachio (*Pistacia vera*)	Sunflower (*Helianthus annuus*)
Plum (*Prunus domestica*)	Thyme (*Thymus vulgaris*)
Potato (Solanum *tuberosum*)	Tomato (*Solanum lycopersicum*)
Pumpkin (*Cucurbita pepo*)	Walnut (*Juglans regia*)
Rice (*Oryza sativa*)	Wheat (*Triticum aestivum*)
Saffron (*Crocus sativus*)	White pepper (*Piper nigrum*)
Sesame (*Sesamum indicum*)	

Detection Limit and Sensitivity

The LOD is often matrix dependent and the sensitivity of the analysis may be reduced depending on the total DNA extracted from the actual ingredient being tested, as well as its quality. Therefore, the LOD needs to be determined through in-house validation. The method LOD was determined by evaluating the method sensitivity in 20 independent experiments. All validation experiments were performed with internal DNA standards with decreasing amounts of horse DNA. Standards were composed of pure genomic DNA extracted from horse and either serially diluted to 4 x 10^{-5} ng/µl (0.12 pg per reaction) or spiked with test matrices prepared in a concentration of 10% (w/w) and scalar diluted to 1 ppm. Under optimal conditions, the lowest amount of horse DNA detected in 100% of the experiments is at least 12 pg of pure genomic

DNA and 0.001% horse DNA (10 ppm) in spiked matrices.

Repeatability and Reproducibility
ID-Check Horse Speciation Kit has been demonstrated to have an excellent dynamic range, with relative standard deviation of 5%.

Robustness
The method has been found to be highly reliable, and unaffected by deliberately introduced small variations. The results from at least 10 independent experiments, performed in duplicate, concurred and the expected outcome achieved.

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

Performance Characteristics for Validation
The false positive and false negative rates were evaluated using a total of 422 samples (233 positive and 189 negative) in 10 independent experiments, performed in at least duplicate. For the present method, both the % of false positive and % of false negative results equaled 0, and the positive predicted value (PPV) and negative predicted value (NPV) equaled 100%.

Section 9
Revision History

Release date	Document number	Change
January 2023	5127 Ver A	New document

Visit bio-rad.com/IDCheck for more information.

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Life Science Group

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