GENERON
Advanced Transfer Technologies

VERYfinder DETECTION ASSAY EQUINE SEMI-QUANTITATIVE

Cat. N. PMA08S

User Guide

1 - Introduction

The recent scandal related to horse meat sold as bovine put under a spotlight the fact that food industry and consumers, even though the scope is different, share a common interest in the possibility to have a fast and accurate method determining the authenticity of the ingredient used for food preparation.

The food industry often makes use of products, where it is difficult to verify the real content in raw material stated by the manufacturer. This problem translates into economic and commercial risks for the company. On the other side consumers want to be sure the products they eat: deserve the price; don't contain risks for health; are not infringing religious/ethical rules.

DNA testing allows an efficient and sensitive identification of plant and animal derivatives, easily detecting accidental contaminations or potential fraud related to false declaration on the label of the species constituting the food.

Real-Time PCR is the most sensitive method for the detection and quantification of specific DNA sequences of different species. The method combined with an appropriate nucleic acid extraction system allows the analysis of raw materials, semi-finished and finished products as well.

This assay provides the user with a simple and reliable procedure for detecting the presence of the DNA of a specific organisms in food matrices. The assay utilizes the Polymerase Chain Reaction (PCR) to amplify a genetic target typical of the equine (species of interest).

The validation performed at Generon exploited ION Force DNA Extractor FAST (Cat. N. EXD001) as DNA extraction method from raw and heat treated matrices (20' at 121°C). The Limit of detection (LOD 0.01%) and the Limit of Quantification (LOQ 0.05%) have been calculated based upon a solution concentrated at 2 ng/µl (DNA/DNA) containing equine DNA on a background of DNA from a simulating matrix. These representing the minimum detectable amount of sought species after spiking a simulating matrix. This approach has been used in both raw matrices and heat treated matrices due to the impossibility of having a real standard as each matrix undergoes a different industrial process. Species can moreover be unevenly distributed in the matrix or separated in the homogenization process.

Due to the high sensibility of the test some matrices might cause a background signal, we therefore suggest to operate a DNA quantification after the extraction and to normalize its concentration accordingly to our validation. The plot should then be evaluated using the positive controls provided in the assay. Cut off is strictly dependent from the positive control provided.

2 - VERYfinder Equine Semi-Quantitative Detection Assay

When used along with GENERase ULTRA PLUS Mastermix (Cat. N. ENG009) this Real-Time PCR assay detects a specific DNA sequence in the DNA of equine in less than 1.5 hours. The amplification of the target sequence is measured by the use of a specific fluorescence-labeled probe (FAM).

2.1 - Assay Content

	Box 50 reactions		Box 100 reactions	
	N. vials	Volume (µl)	N. vials	Volume (µl)
VERYfinder OLIGO Mix * (OLIGOS and Probe pre-blended mix)	1	150	2	150
Positive Control R (0,1 %)	1	300	1	300
Positive Control HT (1 %)	1	300	1	300
Negative Control	1	1000	1	1000

** reagents are supplied with a 5% of extra volume.*

We suggest to use VERYfinder Equine Semi-Quantitative Detection Assay (VERYfinder Equine Semi-QT) along with the following Polymerase Enzyme Ready-to-use mastermix: GENERase ULTRA PLUS Mastermix (Cat. N. ENG009). When using this GENERase ULTRA PLUS an additional detection channel (HEX) becomes available to detect the Internal Amplification Control (IAC) to excluding false negative results due to a PCR inhibition.

2.2 - Storage & Expiry information

Expiry date: see date on the packaging, product validity refers to the product kept intact in its original packaging. Protect reagents from light exposure as far as OLIGO Mix reagents are photosensitive. Store frozen.

3 – Materials and equipments needed

3.1 – Extraction[1]

Material/Equipment	Source
Extraction Kit	Generon ION Force DNA extractor FAST (Cat. N. EXD001)
Chemicals: n-esane	Lab Suppliers
Tubes, 50 ml and 15 ml	Generon or other Lab Suppliers
DNAse/RNAse Free Water	Generon or other Lab Suppliers
Vortexer	Generon or other Lab Suppliers
Benchtop Centrifuge for 50 ml Tubes	Generon or other Lab Suppliers
Thermal Water Bath or Block	Generon or other Lab Suppliers
Pipette sets	Generon or other Lab Suppliers
Pipette tips (Barrier)	Generon or other Lab Suppliers
Tube rack for 1.5 ml tubes	Generon or other Lab Suppliers
2.0 and 1.5 ml micro-tubes	Generon or other Lab Suppliers
Micro centrifuge for 1.5-2.0 ml micro-tubes	Generon or other Lab Suppliers
DNA Extraction VACUUM BOX + Vacuum pump or Venturi meter	Generon or other Lab Suppliers

Each step of sample preparation (grinding, transferring, weighing, etc.) must be done according to GLP so that chance of cross-contamination between samples is minimized. It is recommended to use disposable equipment when possible.
If the food samples are not in a powdered or granular form, they should be processed (grinded or blended) before DNA extraction. The majority of DNA extraction methods supports from 20 to 50 mg of starting material. Generon ION Force DNA Extractor FAST (Cat. N. EXD001) allows processing up to 20 grams of starting material in order to maximize sample's lot representation.
Once the sample has been pulverized/homogenized, it can be weighed and the appropriate amount extracted according to DNA extraction method selected. Refer to manufacturer user manual for extraction procedure details.

3.3 – Detection via Real-Time PCR

Material/Equipment	Source
Real-Time PCR System [2]	Generon or other Lab Suppliers
VERYfinder EQUINE Semi-QT Detection Assay	Generon (Cat. N. PMA08S)
GENERase ULTRA PLUS Mastermix	Generon (Cat. N. ENG009)
Optical Adhesive Seal and Optical reaction plate or Optical Caps and Strips	Generon or other Lab Suppliers
Micropipette sets	Generon or other Lab Suppliers
DNA Quantification System	Generon or other Lab Suppliers

(1) Equipment necessary only when ION Force DNA Extractor FAST (Cat. N. EXD001) is used.
(2) The assay can be used with Biorad CFX and MiniOpticon, Stratagene MxSeries, ABI 7300-7500-7900-Step ONE-StepONE Plus, Light Cycler 480, Eppendorf realplex, Rotor-Gene Q etc. The assay is not compatible with Roche Light Cycler I and II.

4 – Real-Time PCR detection

4.1 – Reaction setup

I. Allow the reagents to thaw (GENERase ULTRA PLUS Mastermix, VERYfinder OLIGO MIX, Positive Controls and Negative Control). Vortex tubes when thawed and spin to collect contents at the bottom of the vial.

II. Mix 150 µl of VERYfinder OLIGO Mix with 750 µl of GENERase ULTRA PLUS Mastermix to prepare VERYfinder Working Mastermix (WMX).

III. Vortex briefly and spin down in order to homogenize the mix.

IV. Transfer 18 µl of WMX into each well.

V. Add 12 µl of Negative Control into wells acting as negative control.

VI. Add 12 µl of each sample into wells testing the unknown samples: in order to perform a proper semi-quantification all the unknown samples should be normalized at the concentration of 2 ng/µl as the positives controls supplied within the assay. Quantification should be executed using a suitable DNA quantification system (we suggest Quantus™ Fluorometer Promega – Cat. N. E6150).

VII. Add 12 µl of Positive Controls into wells acting as Semi-QT controls; we strongly recommend to use the Positive Control R (Raw) when testing raw matrices and Positive Control HT (Heat Treated) when testing cooked / high processed food matrices.

VIII. Close wells and ensure no bubbles are present at the bottom of the wells.

4.2 – Instrument setup

With GENERase ULTRA PLUS Mastermix set the following parameters on your thermocycler:

I. Total Reaction volume: 30 µl

II. Fluorophores/Quenchers: Target Equine (FAM/BHQ1-NFQ); Target IAC (HEX/BHQ1-NFQ);

III. Thermal profile:

Step	T (°C)	Duration	Loops
Taq Activation	95	3 min	1
DNA Denaturation	95	10 sec	45
Annealing/Extension + Plate Reading	60	45 sec	

5 – Data Interpretation

Results evaluation must be done according to the analysis software recommended by the Real-Time PCR instrument manufacturer. After performing PCR, each individual sample is analyzed through the instrument software to produce a Cq value (quantification cycle) for each reporter dye. These values are then used to determine the presence and, afterwards, semi-quantify the amount of equine DNA material in each sample.
Set the Baseline to Auto. The analysis outcome should be evaluated following this table:
If the following conditions are met:

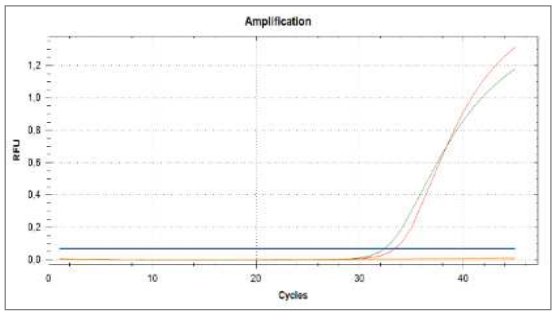
TEST	Equine (FAM)	Internal Amplification Control (HEX)
Positive Control	+	+
Negative Control	-	+

Then the possible results for any sample are:

TEST	Equine (FAM)	Internal Amplification Control (HEX)
Cq Unknown sample < Cq Semi-QT Control	Target species % > Semi-QT Control %	+/-
Cq Unknown sample > Cq Semi-QT Control	Target species % < Semi-QT Control %	+
Invalid Sample (inhibited)	-	-

RED LINE= Semi-QT Control for EQUINE- target

GREEN LINE = Unknown sample



In case of inhibition DNA isolation and purification for the sample need to be improved or you may need to dilute your sample before performing a new test. Refer to the Troubleshooting paragraph (section 8) for further suggestions.

6 – Inclusivity Panel

Species tested for inclusivity	
Horse (*Equus caballus*)	Donkey (*Equus asinus*)

7 – Exclusivity Panel

The following DNA extracts showed no amplification curve when tested according to the general assay instruction.

Meat (Raw and Heat Treated matrices)		
Beef *(Bos taurus*)	Poultry *(Gallus gallus domesticus)*	Swine *(Sus scrofa domesticus*)
Buffalo (*Bubalus bubalis*)	Quail (*Coturnix coturnix*)	Turkey *(Meleagris gallopavo*)
Duck *(Anas* spp.)	Rabbit (*Oryctolagus cuniculus)*	Wild boar (*Sus crofa*)
Goat (*Capra hircus*)	Sheep *(Ovis aries)*	
Vegetables		
Barley (*Hordeum vulgare*)	Mushroom (*Agaricus campestris*)	Sesame (*Sesamum indicum*)
Basil (*Ocinum Basilicum*)	Mustard (*Brassica nigra*)	Soybean (*Glycine max*)
Beans (*Phaseolus vulgaris*)	Oat (*Avena sativa*)	Spelt (*Triticum monococcum*)
Carrot (*Daucus carota*)	Olive (*Olea europaea*)	Garlic (*Allium sativum*)
Corn (*Zea mays*)	Onion (*Allium cepa*)	Spinach (*Spinacia oleracea*)
Cucumber (*Cucumis sativus*)	Parsley (*Petroselinum crispum*)	Tomato (*Solanum lycopersicon*)
Eggplant (*Solanum melongena*)	Pepper (*Capsicum annuum*)	Wheat (*Triticum aestivum*)
Garlic (*Allium sativum*)	Rice (*Oryza sativa*)	Zucchini (*Cucurbita pepo*)
Lupine (*Lupinus albus*)	Rye (*Secale cereale*)	
Fish (Raw matrices)		
Anchovy (*Engraulis encrasicolus)*	Mackerel *(Scombrus scombru*)	Trout (*Salmo trutta*)
Cod (*Merluccius merluccius*	Salmon (*Onchorthynchus kisutch)*	Tuna (Thunnus albacares)
Sardine (*Sardina pilchardus*)		

8 – Troubleshooting

I. Concomitant no target or IAC amplification or amplification plots grossly abnormal. Possible causes and corrective actions:

- An excess of DNA in the target might inhibit the reaction may be affected due to an excess of DNA and/or PCR inhibitors. Test samples diluted 1:10 and 1:100. Please, use DNase/RNase Free Water to prepare dilutions.
- Inadequate sealing of optical caps/film caused sample evaporation. Redo the analysis using proper tools and proper optical caps/film to secure perfect sealing.
- Did not use the proper consumables. Redo the analysis and use only optical grade 96-well plates and optical adhesive seal or optical 8-well strips and caps.
- Samples were not properly prepared. Remake the sample DNA preps. Ensure that the DNA extraction method is properly performed.

II. Positive Control reactions failed to amplify, but other reactions appear correct (e.g. the IAC is amplified):

- Positive Controls DNA were not added to the reaction wells. PCR run should be repeated.

III. Negative Control reactions are positive:

- Contamination of the negative control vial or the VERYfinder PCR mix with VERYfinder-positive DNA. Use more care to prevent contamination while handling assay reagents and setting up assays.

In case support is needed contact Generon at: support@generon.it

9 – Disclaimers

The product is intended for research use only. Generon makes no warranty of any kind, either expressed or implied, except that the materials from which its products are made of standard quality. If any materials are defective, Generon will provide a replacement product. Generon shall not be liable for any damages, including special or consequential damage, or expense arising directly or indirectly from the use of this product. Please do not interchange components between assays of different lot numbers. This assay is designed to be used by laboratory personnel following the common molecular biology precautions.

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Product Line:	VERYfinder
Type:	Semi-Quantitative
Storage:	Frozen
Execution time:	about 120 minutes
Expiry date:	see date on the packaging, product validity refers to the product kept intact in its original packaging and constantly under suitable temperature conditions as mentioned above.

Assay Box Content

	Box 50 reactions		Box 100 reactions	
	N. vials	Volume (µl)	N. vials	Volume (µl)
VERYfinder OLIGO Mix (OLIGOS and Probe pre-blended mix)	1	150	2	150
Positive Control R (0,1%)	1	300	1	300
Positive Control HT (1%)	1	300	1	300
Negative Control	1	1000	1	1000

All reagents are supplied with a 5% of extra volume.

Not Provided Article: GENERase ULTRA PLUS Mastermix (Cat. N. ENG009) or equivalent.

Reaction Set-Up

Protect reagents from light exposure as far as OLIGO Mix reagents are photosensitive.

Before setting the analysis, we strongly advise to leave the reagents to warm up at room temperature. Vortex briefly OLIGO mix, afterwards spin to collect contents at the bottom of the vials. Spin GENERase ULTRA PLUS Mastermix (Cat. N. ENG009) before opening it.

Prepare VERYfinder WORKING Mastermix by adding 150 µl of VERYfinder OLIGO Mix into each tube prefilled with 750 µl of GENERase ULTRA PLUS Mastermix (Cat. N. ENG009) in order to obtain a single volume of 900 µl of VERYfinder WORKING Mastermix. Vortex briefly VERYfinder WORKING Mastermix with the aim of homogenizing the mix and excluding $MgCl_2$ gradient that could impair the results. Spin to collect contents at the bottom of the vial *(Note: label GENERase vials with target name after OLIGO Mix addition)*. Vortex briefly Positive Control and samples before proceeding further, spin to collect contents at the bottom of the vial.

Transfer VERYfinder WORKING Mastermix and samples into the plate as follows:

Reagents per well	Volume
Unknown Sample	12 µl
Positive Control Negative Control	
VERYfinder WORKING Mastermix	18 µl
Final Volume	30 µl

Detector Setup

Target	Reporter Dye	Quencher Dye
EQUINE Target	FAM	BHQ1-NFQ
IAC (Internal Amplification Control)	HEX (*)	BHQ1-NFQ

(*)According to your thermocycler you can replace HEX detector in the plate setting with VIC or JOE in case your own Real Time Platform does not possess the HEX reading channel.

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Thermal cycling

Step	T (°C)	Duration	Loops
Taq Activation	95	3 min	1
DNA Denaturation	95	10 sec	45
Annealing/Extension + Plate Reading	60	45 sec	

The thermal profile presented above was optimized for GENERase ULTRA PLUS Mastermix (Cat. N. ENG009).

Results analysis

If the following conditions are met:

TEST	Equine (FAM)	Internal Amplification Control (HEX)
Positive Control	+	+
Negative Control	-	+

Then the possible results for any sample are:

TEST	Equine (FAM)	Internal Amplification Control (HEX)
Cq Unknown sample < Cq Semi-QT Control	Target species % > Semi-QT Control %	+/-
Cq Unknown sample > Cq Semi-QT Control	Target species % < Semi-QT Control %	+
Invalid Sample (inhibited)	-	-

In case of inhibition DNA isolation and purification for the sample need to be improved or you may need to dilute your sample before performing a new test. Refer to the Troubleshooting paragraph , section 8 in the User Guide, for further suggestions.

Warning and Precaution

Please do not interchange components of assays with different lot numbers. This assay is designed to be used by laboratory personnel following the common molecular biology precautions (GLP).

Disclaimer

Generon s.r.l. guarantees the buyer exclusively concerning the quality of reagents and of the components used to produce the Assay. Generon S.r.l. is not responsible and cannot anyway be considered responsible or jointly responsible for possible damages resulting from the utilization of the product by the user. The user consciously and under his own responsibilities decides for the utilization purposes of the product and uses it the way he considers most suitable in order to reach his goals and/or objectives.

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The product was internally tested by our quality control. Any responsibility is waivered if the warranty of quality control does not refer to the specific product. The user is personally responsible for data that he will obtained and/or he will supply to third parties using this assay. Once the sealed package is open the user accepts all the conditions without fail; if the package is still sealed the product can be returned and the user can be refunded.