GENERON
Advanced Transfer Technologies

VERYfinder DETECTION ASSAY TOTAL MEAT

Cat. N. PMA99A

User Guide

1 - Introduction

The horse meat scandal dating 2012 demonstrated the need for food industry and consumers to have reliable and sensitive analytical tools facilitating routine control tests of meat species in different foods and feedstuffs. These analysis would avoid unfair market competition and protect consumers from false labeled meat products for economical, religious and health reasons.

Although Real-time PCR is widely accepted as a robust method for the identification of undesired contaminating ingredient species due to its high sensitivity and specificity, there are few published reports on its application for quantitation of species in complex DNA samples. Some of the drawbacks found are inherent in any procedure based on DNA because DNA yields may depend on the source material, method of extraction, or fragmentation of DNA that takes place in highly processed food.

When several species are present in the sample, ascertaining the relative amounts of each species in the mix requires a unique reference target sequence identical in all of the possible species.

2 - VERYfinder Total Meat Detection Assay

This assay can serve three different purposes:

- it provides a positive control on the total PCR-amplifiable animal DNA present in the sample;
- it validates the quantitation of different species DNA, allowing the calculation of the percentages in the mixed sample;
- third, it traces the presence of DNA that has not been detected by the species-specific detectors.

The second and third applications require the use of quantitative standards dispersed in water (Quantiscale) or in a customizable dispersive matrix (Quanticust) provided separately by Generon (e.g.: according to the animal target users would like to quantify, Quantiscale and Quanticust products are Cat. N. PMBxxW or PMBxxX).

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 animals meat presence 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	1	300	1	300
Negative Control	1	1000	1	1000

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

We suggest to use VERYfinder Total Meat Detection Assay 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	Lab Suppliers
DNAse/RNAse Free Water	Lab Suppliers
Vortexer	Lab Suppliers
Benchtop Centrifuge for 50 ml Tubes	Lab Suppliers
Thermal Water Bath or Block	Lab Suppliers
Pipette sets	Lab Suppliers
Pipette tips (Barrier)	Lab Suppliers
Tube rack for 1.5 ml tubes	Lab Suppliers
2.0 and 1.5 ml micro-tubes	Lab Suppliers
Micro centrifuge for 1.5-2.0 ml micro-tubes	Lab Suppliers
DNA Extraction VACUUM BOX + Vacuum pump or Venturi meter	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]	Lab Suppliers
VERYfinder Total Meat Detection Assay	Generon (Cat. N. PMA99A)
GENERase ULTRA PLUS Mastermix	Generon (Cat. N. ENG009)
VERYfinder Quantiscale or Quanticust	Generon (Cat. N. PMAxxW or X)
Optical Adhesive Seal and Optical reaction plate or Optical Caps and Strips	Lab Suppliers
Micropipette sets	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. If VERYfinder Total Meat Assay is used along with specific animal target Quanticast or Quantiscale products, proceed adding 12 µl of each standard points in order to carry a regression curve for a specific animal target quantification.

VII. 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).

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 vegan(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	35
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 animal 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:

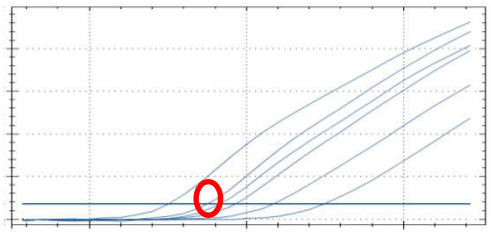
TEST	Total Meat (FAM)	Internal Amplification Control (HEX)
Positive Control	+	+
Negative Control	-	+

Then the possible results for any sample are:

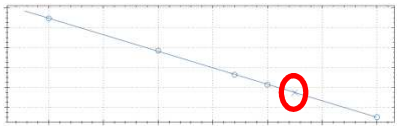
TEST	Total Meat (FAM)	Internal Amplification Control (HEX)
Positive Sample	+	+/-
Negative Sample	-	+
Invalid Sample (inhibited)	-	-

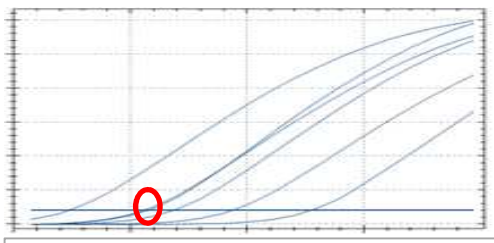
In case of inhibition DNA isolation and purification for the sample need to be improved or you may need to dilute your sample before performing a new test. Refer to the Troubleshooting paragraph (section 8) for further suggestions.

In case of VERYfinder animal target Quanticast or Quantiscale maching, see below an example of quantification example

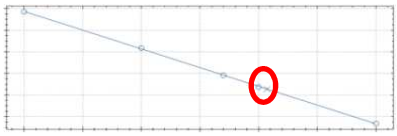


Veryfinder Equine Assay + Quantiscale Horse
Std. Points: 0,1% - 1% - 5% - 10% - 100% (100% = 24 ng Horse DNA)
Eff_{PCR}=108% ; R^2=0.988
Unknown sample = 17%±30%





Veryfinder Total meat Assay + Quantiscale Bovine
Std. Points: 0,1% - 1% - 10% - 100% (100% = 24 ng Ox DNA)
Eff_{PCR}=94% ; R^2=1.00
Unknown sample: 12%±30%



Example of a quantification of contamination in a complex matrix (tomato meat sauce) using the double standard curve approach. The product was claimed to contain 15% w/w bovine meat. 24 ng/reaction of the DNA extracted from the sample underwent analysis. Tests were performed using the VERYfinder equine and VERYfinder total meat assays, referencing results to a standard curve prepared using VERYfinder Quantiscale (horse and bovine respectively). Graphs show the calculated amount of equine and total meat DNA in the sample and through an easy proportioning - $Equine_{PCR}$:$Real_{Equine}$=$Total\ Meat_{PCR}$:100 - it becomes evident all of the meat DNA contained in the sample is equine.

6 – Inclusivity Panel

Meat (Raw and Heat treated matrices)		
Beef *(Bos taurus)*	Goat (*Capra hircus*)	Rabbit (*Oryctolagus cuniculus*)
Buffalo (*Bubalus bubalis*)	Horse (*Equus caballus*)	Swine *(Sus scrofa domesticus*)
Donkey (*Equus asinus*)	Poultry (*Gallus gallus domesticus*)	Sheep (*Ovis aries*)
Duck *(Anas* spp.)	Quail (*Coturnix coturnix*)	Wild boar (*Sus crofa*)
Fish (Raw matrices)		
Anchovy (*Engraulis encrasicolus)*	Mackerel *(Scombrus scombru*)	Trout (*Salmo trutta*)
Cod (*Merluccius merluccius*	Salmon (*Onchorthynchus kisutch)*	Tuna (Thunnus albacares)
Sardine (*Sardina pilchardus*)		

7 – Exclusivity Panel

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

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*)	

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 if analysis aimed for a animal target quantification and semi-quantification. In case of a qualitative analysis , If other reactions look normal, there may be no need to repeat the run.

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.

Quick Reference Guide

Product Line:	VERYfinder
Type:	Qualitative
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	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
TOTAL MEAT 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	35
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	Total Meat (FAM)	Internal Amplification Control (HEX)
Positive Control	+	+
Negative Control	-	+

Then the possible results for any sample are:

TEST	Total Meat (FAM)	Internal Amplification Control (HEX)
Positive Sample	+	+/-
Negative Sample	-	+
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.