DN*Animal Ident*
RT IPC (LR)
Horse

Cat. No. 5422220110

Test kit for the qualitative detection of horse DNA with IPC, 96 real-time PCR reactions

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Test kits, their components and instructions for use are subject to alterations. They are intended for research purposes only.

DN*Animal Ident* Horse Kit

Kit for the qualitative real-time PCR detection of horse DNA in food and feed

1 INTRODUCTION

National regulations in most countries of the world require declaration of components of food and feed and a corresponding labeling.

Declaration of components of animal origin can be crucial e.g. for vegetarian food, food for religious communities, export and trade.

Our DN*Animal Ident* kits comprise several major advantages:

- High sensitivity (down to 0.01% admixture)
- High specificity (primers and probe)
- Robust test methods
- Fast results
- No amplicon contamination risk

1.1 Test Principle

The test comprises the following steps:

1. DNA extraction
2. Real-time PCR

Due to processing steps commonly used in food production, the fragment length of the sample DNA is usually reduced, but DNA is not completely degraded. The base sequence of the fragments is not changed and identification of animal species is mostly still possible even in highly processed food and feed samples. Specific DNA sequences are amplified by RT-PCR (Real-time Polymerase Chain Reaction) and detected with high specificity due to the Taqman™ probes.

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Points to consider:

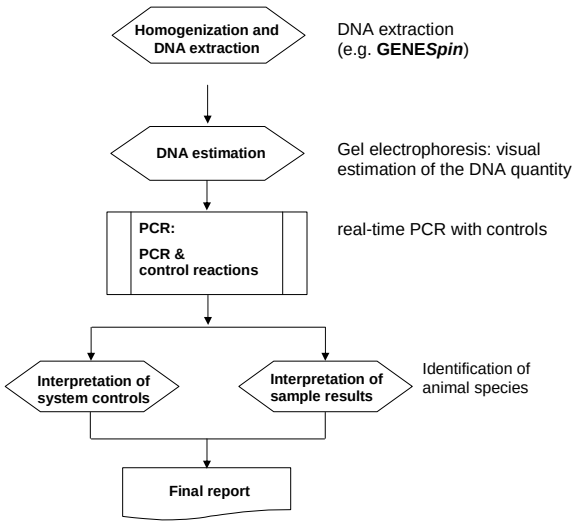
- Animal species can be analyzed in most matrices, even in gelatin. However, rigid food or feed processing can degrade DNA in a way that amplification is impossible.
- The detection system of this kit may cross-react with DNA from some donkey breeds.

2 TIME SCHEDULE

Step	Hands-on time	Time involved
DNA-extraction	0.5 h	1.5 h
PCR-reaction	0.5 h	3.5 h
Total time	1.0 h	5.0 h

The hands-on time has been calculated for a single sample. When several samples are handled simultaneously, the time increases.

2.1 Test Procedure – Flowchart



3 COMPONENTS OF THE KIT

The kit contains all components to run control and specific reactions for a total of 100 PCR reactions.

Important Note: Store all following components at -20°C. Never store solutions and materials for DNA extraction together with samples or amplicons. Never use them in areas where PCR products (amplicons) may be present.

2x **MasterMix QL RT IPC (LR) GS-P-08.020, Horse cytB**

1 ml composed of

- 650 µL BasicMix QL RT (NR) GSE-P-26.001 • EFGi 2x (NR) 1.5 U

- 390 µL OligoMix QL RT IPC (LR) • GS-P-08.020 • Horse cytB

Mix prior to use!

2 x **Positive control DNA**: **Genomic DNA Horse (cytB)** (50 µL, 10 copies/µL)

1x **DNA stabilisation buffer**, 150 µL (for NTCs)

4 PREPARATION OF THE KIT'S REAGENTS

Store the kit at –20°C until opened for the first time. Thaw reagents just before use, mix them by vortexing and centrifuge briefly before use.
Just thaw as many BasicMix and OligoMix vials as necessary. Frequent freezing and thawing might cause inactivation of the reagents. If smaller volumes are needed, aliquot reagents at first use.
Refer to the reagent label for specific instructions regarding the correct storage.

5 MATERIAL AND EQUIPMENT NOT INCLUDED IN THE KIT

– water, DNase-free
– Vortex
– Micropipettes (variable 1-1000 µL) and filter tips
– PCR optical tubes or plates 0.2 ml and optical caps or seals
– RT-PCR Thermocycler

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6 SAMPLE PREPARATION

6.1 DNA extraction

The extraction of DNA from the sample is a crucial step, and in order to guarantee optimal quantity and purity, an appropriate method has to be chosen for each sample matrix. For most products, the DNA can be extracted with a kit from our DNA*Extractor* kit line or with our GENE*Spin* kit.
Absolute DNA amount and purity (meaning: free of PCR inhibiting substances) affect overall sensitivity of the analysis. Low DNA amount subjected to analysis results in poor LOD with regard to the specific test sample. Insufficiently pure DNA displaying inhibitory effects may even make testing impossible.
It is recommended to use 50 - 100 ng of the sample DNA per reaction. The concentration of DNA should be adjusted accordingly prior to the set up of the reactions. DNA amount can be determined prior to analysis by one of the following methods:
- Real-time PCR monitor run (preferred method)
- Fluorimetric or spectrophotometric or other physical measurements, e.g. gel electrophoresis may be applied.

Especially the spectrophotometric methods can suffer significant errors due to interfering DNA impurities or partial DNA denaturation/degradation.
For samples known with regard to extraction yield and purity, DNA measurement may be omitted.

Each sample should be treated in duplicate.
It is recommended to perform an extraction control for each set of samples extracted simultaneously, i.e. a complete DNA extraction without sample material, which should subsequently undergo PCR analysis.

7 PCR

7.1 Special precautions during PCR analysis

PCR is an exponential reaction. Detection of single target DNA copies is possible. The extreme sensitivity requires special precautions for handling and equipment. A successful amplification leads to several billion amplicons in the reaction tube. Each of them might lead to a false positive result when contaminating sample material or PCR, e.g. by spreading in aerosols.
Most important rules to avoid false-positive results are:

a) Separate the different procedures spatially. Use separate rooms for sample preparation, amplification and analysis of amplicons and dedicate separate equipment and materials for each procedure. Mixing of PCR components should be done in a separate room, best on a clean bench. Keep all working steps separate from rooms where gel electrophoresis of amplicons is performed.
b) Use filter tips for micropipettes.
c) Wear disposable powder-free gloves.
d) Never store DN*Animal Ident* Kits and materials for DNA extraction together with samples or amplicons.
e) Always perform extraction controls and PCR controls (NTCs).

7.2 Preparation of MasterMix

Calculate the number of reactions and amount of equivalent MasterMix volume before thawing and mixing the reagents and starting the practical work.
The MasterMix is made of pre-made reagents ready for direct use after mixing of the two components.

Please be aware that the NTCs and positive controls (C+) are indispensable for the evaluation of the test and must be included in every run.

PCR is performed in a volume of 25 µL in 0.2 mL reaction tubes/plates according to the RT-PCR cycler instructions.

20 µL MasterMix for low ROX (LR) cyclers consist of 12.5 µL BasicMix + 7.5 µL OligoMix per reaction.

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The following reactions are required for a RT PCR run:

Samples	1	8	23	46
NTCs	2	2	2	2
Pos. controls	2	2	2	2
Samples (duplicate)	2	16	46	92
Total no. reactions	**6**	**20**	**50**	**96**
Total MM volume	**120 µL**	**400 µL**	**1 mL**	**2 mL**
BasicMix	75 µL	250 µL	625 µL or 1 tube	1250 µL or 2 tubes
Oligo Mix	45 µL	150 µL	375 µL or 1 tube	750 µL or 2 tubes

Number of reactions and volumes of reagents needed for 1, 8, 23 or 46 samples).

1. Thaw only the volumes of BasicMix and OligoMix needed for analysis. Shake thoroughly.
2. Remove the required volumes and mix in a fresh tube. Freeze the rest.
3. Mix thoroughly.
4. The composed MasterMix can be stored up to 4 h in the refrigerator, but must not be used longer.

7.3 PCR Setup

Every run requires two no template controls (NTC) and two positive controls (C+). It is highly recommended to test the samples in duplicate.

1. Label all PCR reaction tubes.
2. Add 20 µL of the composed MasterMix to the wells.
3. Add 5 µL stabilisation buffer to NTCs.
4. Add 5 µL of pos. control DNA to positive controls.
5. Add 5 µL of sample DNA to test reactions.

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	NTC	C+	C+	1a	1b	2a	2b	3a	3b	4a	4b
B	5a	5b	6a	6b	7a	7b	8a	8b	9a	9b	10a	10b
C	11a	11b	12a	12b	13a	13b	14a	14b	15a	15b	16a	16b
D	17a	17b	18a	18b	19a	19b	20a	20b	21a	21b	22a	22b
E	23a	23b	24a	24b	25a	25b	26a	26b	27a	27b	28a	28b
F	29a	29b	30a	30b	31a	31b	32a	32b	33a	33b	34a	34b
G	35a	35b	36a	36b	37a	37b	38a	38b	39a	39b	40a	40b
H	41a	41b	42a	42b	43a	43b	44a	44b	45a	45b	46a	46b

Plate layout for 46 samples.

NTC = no template control; C+ = positive control; a and b = DNA extract duplicates

7.4 Programming of Plate Documents

Before starting the practical work, program the plate document und the cycling conditions.

For description of the instrument programming please refer to the user manual of the respective instrument and software version.

For setup information for your individual cycler, please send an email to kits@eurofins.com or see our website www.eurofins.com/kits.

Program your template with the following settings:

ABI 7500 (7500 Fast)

System	Detector	Reporter	Quencher
Horse	20HOR	FAM™	NONE
IPC	20IPH	VIC™	NONE

Passive reference: ROX

ABI 7500 Fast
PCR is performed in the “Standard 7500” run mode.

Agilent Mx3005P (3000P)™

System	Assay	Filter
Horse	20HOR	FAM™/SYBR® Green I
IPC	20IPH	HEX™

Passive reference: ROX
The fluorescence signals are scanned in the following order: (1) ROX™, (2) FAM™, (3) HEX™. (PMT Gain =1)

Agilent AriaMx™

System	Detector	Reporter	Quencher
Horse	20HOR	FAM	NONE
IPC	20IPH	HEX	NONE

Passive reference: ROX

Bio-Rad CFX96 Touch™

System	Detector	Reporter	Quencher
Horse	20HOR	FAM	NONE
IPC	20IPH	VIC	NONE

No passive reference used

Cycling Conditions

The run parameters are to be programmed as follows:

Thermal Cycler Times and Temperatures		
***1** HOLD*	*CYCLE* **(45 repeats)**	
enzyme act.	*denaturation*	*ann. & extension*
10 min at 95°C	**15 sec at 95°C**	**90 sec at 60°C**
no data collection	no data coll.	data collection

For other thermocyclers, it may be necessary to optimize the PCR parameters.

8 RESULTS

8.1 Evaluation

Refer to your cycler´s manual for details or see our website www.eurofins.com/kits. An evaluation (Excel™) sheet can be requested at kits@eurofins.com.

Threshold:

- **ABI 7500 (Fast), Agilent Mx3005P (3000P)™** The threshold should be placed in the region of exponential amplification across all of the amplification plots. This region is depicted in the log view of the amplification plots as the linear portion of the plot. The threshold line should neither be placed in the plateau phase nor in the initial linear phase of amplification. Thresholds are typically in the range 0.03 – 0.1.
- **Bio-Rad CFX96 Touch™:** Auto calculated threshold can be used.
- **Agilent AriaMx™:** Auto calculated threshold with default Background based threshold settings can be used: Cycle range: 5 thru 9 Sigma multiplier: 10

Baseline:

- **ABI 7500 (Fast):** manual, 3-15 or automatic baseline
- **Agilent Mx3005P (3000P)™:** Adaptive
- **Bio-Rad CFX96 Touch™:** Baseline Subtracted Curve Fit, Auto calculated
- **Agilent AriaMx™:** Auto baseline correction can be used

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8.2 Interpretation of Results

Export CT values to the Excel™ sheet provided (please request to kits@eurofins.com), or create an own evaluation sheet following the parameters below, where you will find an overview of the algorithms applied for scoring of the results in our Excel™ Evaluation Sheet.

Definitions

CT Cut-offs (CT = Ct/Cp/Cq)	Horse:	Mean CT (C+)	+6
	IPC:	Mean CT (NTC)	+3
EF* Limits	Horse:	Mean EF (C+)	x 0.2
	IPC:	Mean EF (NTC)	x 0.33
Outliers	Maximum acceptable outliers (C+):		0
	Maximum acceptable outliers (NTC):		0

*Depending on your cycler the final intensity value, called Endpoint fluorescence (EF) is termed as: ΔRn, dRn, Rn, RFU, or EF

Inhibition control, scoring of IPC

CT and EF	Results
CT_{IPC} sample ≤ CT_{IPC} Cut-off **and** dRn_{IPC} sample ≥ EF_{IPC} Limit	Sample valid
CT_{IPC} sample > CT_{IPC} Cut-off **or** dRn_{IPC} sample < EF_{IPC} Limit	Sample inhibited
No CT_{IPC}	Sample inhibited
CT_{IPC} sample < Mean CT_{IPC} NTC -3	Sample inhibited

Test reaction horse-specific

CT = Ct/Cp/Cq	EF	Results
CT sample ≤ CT Cut-off	EF sample ≥ EF Limit	Reaction positive
CT sample ≤ CT Cut-off	EF sample < EF Limit	Check amplification!
CT sample > CT Cut-off	EF sample ≥ EF Limit	Reaction positive
CT sample > CT Cut-off	EF sample < EF Limit	Reaction negative
No CT	-	Reaction negative
***Note*:** In case of "Check amplification!" the amplification plots must be checked visually for presence of a PCR amplification signal.		

Final result from combination of inhibition control and test reaction

IPC	Horse-specific	Final result
Sample valid	Reaction positive	Positive
Sample valid	Reaction negative	Negative
Sample inhibited	Reaction positive	Positive
Sample inhibited	Reaction negative	Inhibited

Evaluation of the IPC
Calculate the MEAN CT Value from NTC. Refer to data from IPC-detector. To calculate the CT cut-off, add 3 CT. Calculate the MEAN EF Value from NTC. Refer to data from IPC-detector. The EF cut-off f is 33% of the MEAN EF.

Evaluation of horse-specific test
Calculate the MEAN CT Value from positive control. To calculate the CT cut-off, add 6 CT. Calculate the MEAN EF value from positive control. The EF cut-off is 20% of the MEAN EF.

Ambiguous results
If independently extracted DNAs show deviations in the results for "A" and "B", this may be due to sample material non-homogeneity or to non-uniformities in the DNA-extraction efficiency. Repeat DNA extraction and homogenize sample more thoroughly. If the ambiguous result persists, it is most likely due to a concentration close to the LOD.

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9 LIMIT OF DETECTION

The relative limit of detection has been validated at 0.01% of horse DNA in other animal species (total DNA ratio).
The absolute detection limit of the method is 10 copies per reaction.
The limit of detection for the analysis of individual samples depends on several factors: the presence of PCR-inhibiting substances, the capability of the DNA extraction method to remove these inhibitors and the degree of DNA damage, and thus strongly on the type of sample and the DNA extraction procedure.

Quantification is not possible with this kit.

10 PRODUCT USE LIMITATIONS

The GeneScan Technologies DN*Animal* Kit is developed, designed, and sold for research purposes only. It is not to be used for diagnostic purposes or analysis of food and feed unless expressly cleared for that purpose by the appropriate regulatory authorities in the country of use. All due care and attention should be exercised in the handling of many of the materials described in this text.

11 PRODUCT WARRANTIES AND SATISFACTION GUARANTEE

Eurofins GeneScan Technologies GmbH ("GeneScan") warrants the products manufactured by it will be free of defects in materials and workmanship when used in accordance with the applicable instructions for a period equal to or shorter of one year from the date of shipment of the product(s) or the expiration date marked on the product packaging under the storage conditions, recommended in the instructions and/or on the package. Application protocols published by GeneScan are intended to be only guidelines for the buyers of the products. Each buyer is expected to validate the

applicability of each application protocol to his individual application. GeneScan makes no other warranty, expressed or implied. There is no warranty of merchantability or fitness for a particular purpose. GeneScan's sole obligation with the respect to the foregoing warranties shall be, at its option, to either replace or to refund the purchase price of the product(s) or part thereof that proves defective in materials or workmanship within the warranty period, provided the customer notifies GeneScan promptly of any such defect. GeneScan shall not be liable for any direct, indirect or consequential damages resulting from economic loss or property damages sustained by buyer or any customer from the use of the product(s). A copy of Eurofins GeneScan GmbH terms and conditions can be obtained on request, and is also provided in our price lists.

12 IMPORTANT NOTES

- The TaqMan processes are covered by patents issued to Hoffmann-La Roche AG and Applied Biosystems Inc., which are applicable in certain countries.
- Eurofins GeneScan Technologies does not encourage or support the unauthorized or unlicensed use of these processes. Use of this kit is recommended for persons that either have a license or are not required to obtain a license.
- Registered names, trademarks, etc. used in this document, even when not specifically marked as such, are not to be considered unprotected by law.

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13 TROUBLESHOOTING

Result	Possible mistakes/reasons	Possible verification and measures
No PCR signals from samples	Inhibition of PCR by inhibitory substances. Inhibition by too much DNA.	Clean DNA further* or dilute DNA solution. Check DNA concentration/dilution.
No PCR signals from positive controls	Wrong PCR program.	Check and correct PCR program.
No amplification, neither from reference DNA nor from sample DNA	MasterMix not properly prepared Wrong PCR program.	Prepare fresh MasterMix, repeat PCR. Check program.
Positive PCR result for NTC	Contamination with DNA when mixing the PCR components.	Optimize your precautions. Check your solutions. Decontaminate your equipment. Repeat the PCR.
Positive PCR result for extraction control	Contamination with DNA or sample material during DNA extraction or PCR setup.	Check your solutions. Repeat extraction and PCR.

* Repeat extraction of DNA from the sample. Repeat washing with 75 % ethanol three times. If necessary, clean DNA further e.g. with our DNA Cleaning Columns (cat. no. 5224700310).

14 TECHNICAL SERVICE

If you have any questions or experience any difficulties regarding this kit or GeneScan Technologies products in general, please do not hesitate to contact us. GeneScan Technologies customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at GeneScan Technologies. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques. For technical assistance and more information please call the GeneScan Technologies Technical Service Department or local distributors.