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DN*Animal*

DN*Animal Ident* Donkey (LR)

Cat. No. 5422220210

Test kit for the qualitative detection of donkey DNA
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* Donkey Kit

Kit for the qualitative real-time PCR detection of donkey 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* RT kits comprise several major advantages:

- High sensitivity
- High specificity through primers and TaqMan® probe
- Robust test methods
- Fast results

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. The DN*Animal Ident* Donkey kit detects a mitochondrial DNA sequence specific for donkey *(Equus asinus)*.

Important notes:

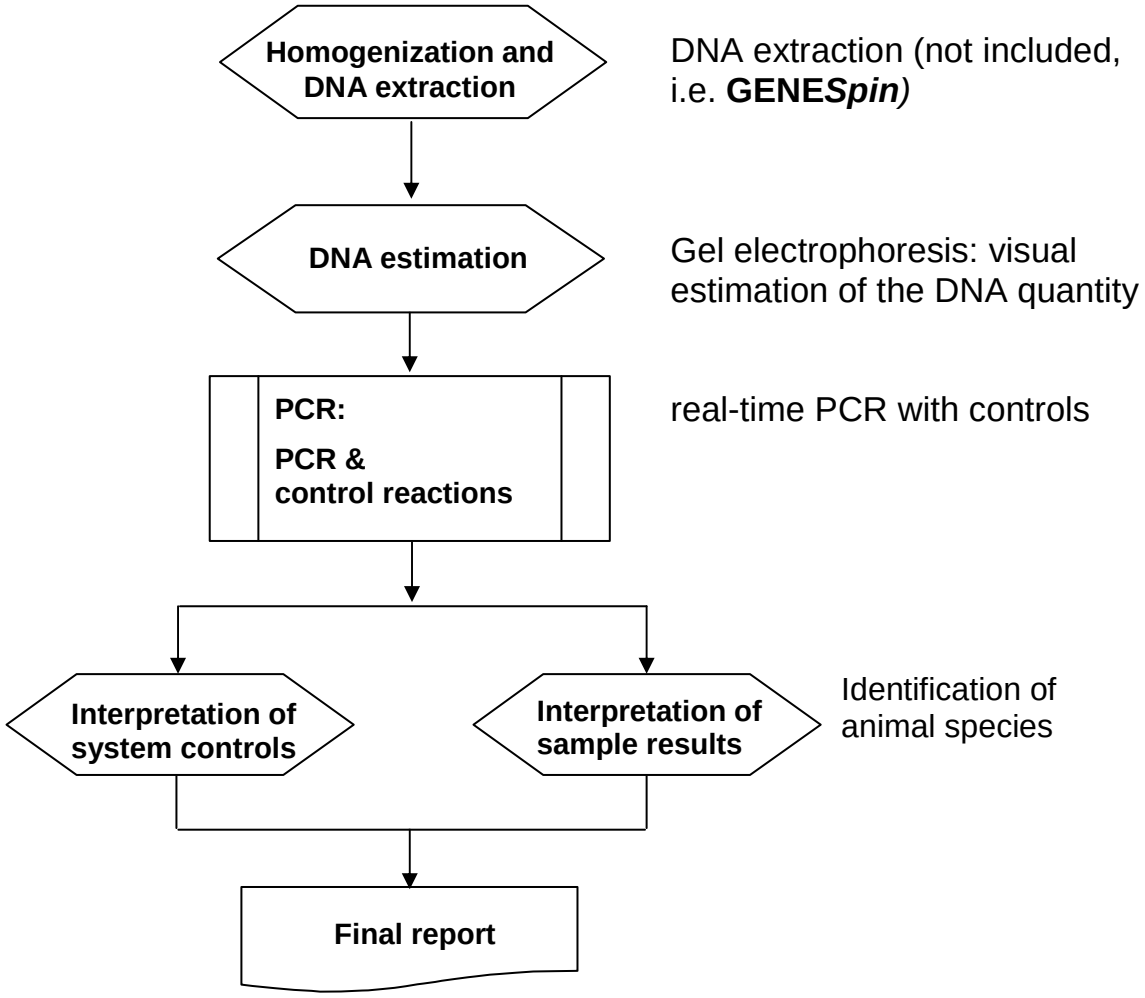
- Animal species can be analysed in most matrices, even in gelatin. However, rigid food or feed processing can degrade DNA in a way that amplification is impossible.
- This system does not detect horse DNA, however, DNA from mule and hinny will be detected.

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

***Table 1**: The hands-on time has been calculated for a single sample. When several samples are handled simultaneously, the time increases.*

3 TEST PROCEDURE – FLOWCHART



4 COMPONENTS OF THE KIT

The kit contains all components to run controls and test reactions for a total of 96 real-time 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.

1. (2x) **OligoMix, Module GS-P-08.019**
   390 µL primer/probe mix for donkey DNA detection

2. (2x) **PREMaster Mix**
   650 µL reaction mix without primers and probes

Mix 1+2 prior to use in empty vial (labels included)!

3. (1x) **Positive Control DNA: Plasmid pGSE641 Donkey** 150 µL (100 copies/µL)

4. (1x) **DNA stabilisation buffer** (for NTCs)
   150 µL

5 PREPARATION OF THE REAGENTS

Store the kit at –20°C until opened for the first time.
Calculate the number of reactions and amount of equivalent MasterMix volume before thawing and mixing the reagents and starting the practical work.
Thaw reagents just before use, mix them by vortexing and centrifuge briefly before use.
The MasterMix is made of pre-made reagents ready for direct use after mixing of the two components.
Mix BasicMix and OligoMix prior to use in empty vial (labels included)! For 50 reactions, mix 1 tube of BasicMix with 1 tube of OligoMix.

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.

6 EQUIPMENT AND MATERIALS NOT SUPPLIED

- Optical tubes or plates and optical caps or seals
- Precision pipette to deliver 5 µL and 20 µL
- Vortex mixer
- Centrifuge (1,500 x g, preferably refrigerated)
- Centrifuge for micro titer plates (pref. refrigerated)
- RT-PCR instrument

7 DNA EXTRACTION

We recommend our GENE*Spin* kit, cat. # 5224400605 for DNA extraction.
GENE*Spin* guarantees good recovery rates also for small genomic DNA fragments (< 1 kb) and even from processed, complex food matrices with a very low DNA content. GENE*Spin* allows processing of up to 200 mg of material. Depending on the type of sample, typical yields with GENE*Spin* are in the range of 0.1-10 µg DNA.

Each sample should be extracted in duplicate.

7.1 Extraction Controls

It is recommended to perform an extraction control for each set of samples extracted simultaneously, i.e. a complete DNA extraction without sample material, and subsequent PCR analysis and gel electrophoresis, to demonstrate the absence of contamination.

7.2 Estimation of DNA Amount

The amount of DNA can easily be estimated e.g. by PicoGreen measurement or by comparing 5 µL of the sample DNA with 5 µL of a DNA amount standard on an agarose gel. (For a general description of gel electrophoresis see e.g. “Molecular Cloning: A Laboratory Manual” (Sambrook & Russel, 2001). Adjust the sample DNA to a concentration of approx. 20 ng/µL, adding H_2O or 0.2 x TE. If no DNA is visible on the gel, use DNA solution undiluted.

8 PCR

8.1 Special Precautions during PCR Analysis

PCR is an exponential reaction. Theoretically, the detection of a single DNA molecule is possible. The extreme sensitivity requires special precautions for handling and equipment. After a successful amplification several billion amplicons are present in the reaction tube. Each of them might lead to a false positive result when contaminating sample material, e.g. by spreading in aerosols.
Observe the following rules to avoid false-positive results:

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

8.2 Procedure

PCR is performed in a volume of 25 µL in 0.2 mL reaction tubes/plates according to the RT-PCR cycler instructions. The reaction conditions have been tested for the Applied Biosystems ABI Prism® 7500 Fast and the Agilent MX3005P/MX3000P.

8.3 Preparation of the PCR Reaction Mix

Mix BasicMix and OligoMix prior to use in empty vial.
For 50 reactions, mix 1 tube of BasicMix with 1 tube of OligoMix.
Please calculate with approximately 5% reagent excess in order to compensate for the pipetting error (1-2 additional reactions/test).

1. Thaw only the volumes of BasicMix and OligoMix needed for analysis. Shake thoroughly.
2. Remove the required volumes and transfer into a fresh tube. MasterMix for one reaction (20 µL) is composed of 12.5 µL BasicMix and 7.5 µL OligoMix. 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.

The following volumes are required for tests with inhibition control (as recommended):

Number of Samples	1	2	11	23
NTCs	2	2	2	2
Pos. controls/NTC+	2	2	2	2
Test reactions and inhibition controls	4	8	44	92
Total no. reactions	**8**	**12**	**48**	**96**
Total MM volume	**160 µL**	**240 µL**	**960 µL**	**1820 µL**
PREMasterMix	100 µL	150 µL	600 µL	1,2 mL or 2 tubes
OligoMix	60 µL	90 µL	360 µL	720 µL or 2 tubes

Number of reactions and volumes of reagents needed for 1, 2, 11 or 23 samples (incl. extraction controls) with inhibition control.

8.4 Control Reactions

For each set of samples:

- **2 NTC:** stabilisation buffer instead of sample DNA
- **2 K+: positive controls/spiked NTCs:** NTCs spiked with 20 copies of positive control DNA
- **1-2 extraction controls:** also submitted to PCR

For each sample:

Inhibition control: PCR with sample DNA and additional 20 copies of positive control DNA.

8.4.1 Preparation of the Inhibition Controls

Separate half of the total volume of MasterMix and add 0.2 µL (= 20 copies) of the undiluted control DNA per reaction. For a 96 well plate (of which half of the reactions are spiked) use 1 tube each of OligoMix and BasicMix, mix both and add 9.6 µL of control DNA. Use 20 µL of spiked MasterMix per well.

8.5 Plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	**NTC**	**NTC**	1a	1b	2a	2b	**K+**	**K+**	1a+	1b+	2a+	2b+
B	3a	3b	4a	4b	5a	5b	3a+	3b*	4a+	4b+	5a+	5b+
C	6a	6b	7a	7b	8a	8b	6a+	6b+	7a+	7b+	8a+	8b+
D	9a	9b	10a	10b	11a	11b	9a+	9b+	10a+	10b+	11a+	11b+
E	12a	12b	13a	13b	14a	14b	12a+	12b+	13a+	13b+	14a+	14b+
F	15a	15b	16a	16b	17a	17b	15a+	15b+	16a+	16b+	17a+	17b+
G	18a	18b	19a	19b	20a	20b	18a+	18b+	19a+	19b+	20a+	20b+
H	21a	21b	22a	22b	ECa	ECb	21a+	21b+	22a+	22b+	ECa+	ECb+

Table 4: Plate layout for 22 samples in duplicates plus extraction control: NTC = No Template Control; NTC+: spiked NTC/positive control, EC: extraction control; a and b = Duplicates of Samples; + = spiked sample/inhibition control

8.6 PCR Setup and Conditions

1. Label all PCR reaction tubes.
2. Add 20 µL of the composed MasterMix to half the number of wells.
3. Add 20 µL of the composed MasterMix with added positive control DNA to the second half of the wells (inhibition controls and positive controls/spiked NTCs).

4. Add 5 µL stabilisation buffer to NTCs and spiked NTCs/positive controls.
5. Add 5 µL of sample DNA to test reactions and spiked test reactions (inhibition controls).
6. If you run extraction controls, treat them like samples (5 µL of extract to test and to spiked reactions).

8.7 Setup and Programming of Plate Documents

Before starting the practical work, program the plate document for your cycler.
For description of the instrument programming please refer to the user manual of your instrument and respective software version. For the plate template, you may use our templates for ABI7500 and MX3005P (request by e-mail for your cycler to kits@eurofins.de) or use the following settings for your own template:

The PCR temperature profile for ABI Prism® 7500 (use Fast model in 7500 Standard Mode) and Agilent MX3005P/MX3000P is shown below. For other thermocyclers, it may be necessary to optimise the PCR parameters.

Temperature	Time	
95°C	10 min	
95°C	15 sec	45 cycles
60°C	90 sec	

ABI 7500 (Fast) in the standard run mode

System	Detector	Reporter	Quencher
Donkey	8019	FAM™	TAMRA™

Passive Reference : ROX™

Agilent (Stratagene) MX3005P/MX3000P

The fluorescence signals are scanned in following order (1) ROX™, (2) FAM™. The filter set gain settings for ROX™ and FAM™ are pmt = 1.

System	Assay Name	Filter
Donkey	8019	FAM™ /SYBR® green (pmt=1)
	ROX	Texas Red®/ROX™ (pmt=1)

Passive Reference: ROX™

8.8 Evaluation of PCR results

8.8.1 Data Analysis

An evaluation sheet for ABI7500 and MX3005P is available on request (kits@eurofins.de) and requires the use of the detector/assay names given above.

ABI 7500 (FAST)

1. Use the default manual baseline range from cycles 3 to 15.
2. Set the threshold: display the deltaRn-axe in the logarithmic mode and locate the threshold line in the exponential phase. Press the update button.
3. Evaluate the Ct-Values and deltaRn values or export them for evaluation (see 8.8.1, step 7 to 9).

Agilent (Stratagene) MX30005P/MX3000P

1. Click Analysis and then Results.
2. Select the area to analyse → Amplification plot.
3. Set in Fluorescence → dRn.

4. Set threshold for the detector: display the deltaRn-axe in the logarithmic mode and locate the threshold line in the exponential phase.
5. Make sure that the baseline settings is adaptive Baseline (Options-analysis term settings Baseline Correction).
6. Select in area to analyse - Text report.
7. Deselect ROX/Reference dye in Assay shown.
8. Add Well; Well Name; Dye; Assay; Well Type; dRN Last; Threshold; Ct (dRN) column to report (8 columns in total).
9. Sort results by assay.
10. Click right mouse click → Export Text Report → Export Text Report to a txt file.
11. Evaluate the Ct-Values and deltaRn values or export them to a spreadsheet, respectively.

8.8.2 Extraction controls and NTCs

The extraction controls indicate that during the course of DNA extraction and further handling no contamination has occurred. If a DNA extraction control reveals a positive result, the extraction procedure and the PCR analysis must be repeated for all samples with positive results.
The NTCs (No Template Controls) indicate that no contamination has occurred during pipetting of PCR reactions and the PCR reagents worked properly, not giving false-positive results. It is crucial that these reactions show no amplification (Ct 45). If a NTC is positive, at least all PCR steps must be repeated for all samples with positive results.

8.8.3 Positive and Inhibition (spiked) controls

Positive and Inhibition controls verify the amplification: approx. 20 copies of the positive control DNA per PCR added to the PCR reaction mix should yield a Ct below the cut-off and a dRn above the dRn limit.

Ct Cut-off:	Mean Ct of the spiked NTCs (K+) + 5
dRn limit:	Mean dRn of the spiked NTCs (K+) x 0.4
Outliers:	No outliers acceptable for (K+) and NTC

$Ct_{SpikedSample}$	$dRN_{Spiked\ Sample}$	Scoring Spike
≤ Ct CutOff	≥ dRN Limit	valid
≤ Ct CutOff	< dRN Limit	Check Amplification
> Ct CutOff	≥ dRN Limit	inhibited
> Ct CutOff	< dRN Limit	inhibited
No Ct	-	inhibited

If inhibitory substances are present, the control PCR result is negative (inhibited). Sometimes further cleaning steps can remove inhibitory substances, e.g. our DNA Cleaning Columns (cat. no. 5224700310).

Dilution of the sample DNA also might solve the problem, but reduces the sensitivity of the test.

8.8.4 Specific reactions

PCR indicates the presence of donkey DNA. The result is positive under the conditions given below and if all control reactions reveal the expected results.

Ct_{Sample}	dRN_{Sample}	Result
≤ Ct CutOff	≥ dRN Limit	positive
≤ Ct CutOff	< dRN Limit	Check Amplification
> Ct CutOff	≥ dRN Limit	positive
> Ct CutOff	< dRN Limit	negative
No Ct	-	negative

		Animal Species PCR		
		positive	**negative**	**Check Amplification**
Spike	**valid**	positive	negative	Check Amplification
	Inhibited	positive	Invalid	inhibited

In case of a valid double negative result for the sample duplicates, no donkey DNA is detectable. The amount of donkey DNA is zero or below the detection limit of this method.

In case of an unclear result (+/-) a very small amount of extracted DNA or a low concentration of donkey DNA might be the explanation. PCR should be repeated.
If the +/- result persists, a new DNA extraction from the sample is recommended.

9 LIMIT OF DETECTION

The absolute detection limit of the method is LOD_{abs} ≤ 10 copies per reaction. The relative limit of detection has been validated with 0.001% donkey DNA and a total DNA content of 100 ng (donkey DNA in cattle w/w).
The total detection limit of the method is at least 60 copies per reaction.

10 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 does not encourage or support the unauthorized or unlicensed use of these processes. Use of these products 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.

11 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 precautions. Check solutions. Decontaminate 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).

12 LITERATURE

Chisholm J, Conyers C, Booth C, Lawley W, Hird H.: The detection of horse and donkey using real-time PCR., Meat Sci.2005 Aug;70(4):727-32

13 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 Technologies GmbH terms and conditions can be obtained on request, and is also provided in our price lists.

14 PRODUCT USE LIMITATIONS

The Eurofins 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.

15 TECHNICAL SERVICE

If you have any questions or experience any difficulties regarding this kit or Eurofins GeneScan Technologies products in general, please do not hesitate to contact us. Our customers are also a major source of information regarding advanced or specialised uses of our products. This information is helpful to other scientists as well as to the researchers at Eurofins 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 Eurofins GeneScan Technologies Technical Service Department or your local distributor.

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