DN*Animal Screen* Halal IPC (LR)

Cat. No. 5422221210

Test kit with 96 real-time PCR reactions for the qualitative real-time PCR detection of pork and horse/donkey DNA with IPC for ABI7500/Fast, Agilent MX3005P/MX3000P, AriaMx, Bio-Rad CFX96 and Roche LC480

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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 Screen* Halal Kit

Kit for the qualitative real-time PCR detection of pork and horse/donkey DNA in DNA from 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 food for religious communities, vegetarian or vegan food, export and trade.

This kit can be used to test food for haram species in muslim food, but can also be used to check i.e. adulteration of other food with pork or horse/donkey meat.

Our DN*Animal* kits comprise several major advantages:
- High sensitivity (down to 0.01%)
- High specificity (primers and probe)
- Robust test methods
- Fast results

1.1Test Principle

The test comprises the following steps:

1. DNA extraction
2. Real-time PCR

Rigid food or feed processing can degrade DNA in a way that makes PCR amplification impossible.
However, in most matrices, even in gelatin, animal species can be analyzed. 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 and the base sequence of the fragments is not changed.

Specific DNA sequences for *Equidae* (horse, donkey, mule, hinny and zebra) and porcine DNA (both, domestic pig and wild boar) are amplified by RT-PCR (Real-time Polymerase Chain Reaction) and detected with high specificity due to the Taqman™ probes.

The probe of the detection systems in this kit use FAM™ and R6G as reporter dyes and the IPC uses Cy5™ as reporter dye. All probes use non-fluorescent quenchers.

The kit is validated for use on ABI7500/Fast, Agilent MX3005P/MX3000P, AriaMX, CFX96 and Roche LC480.

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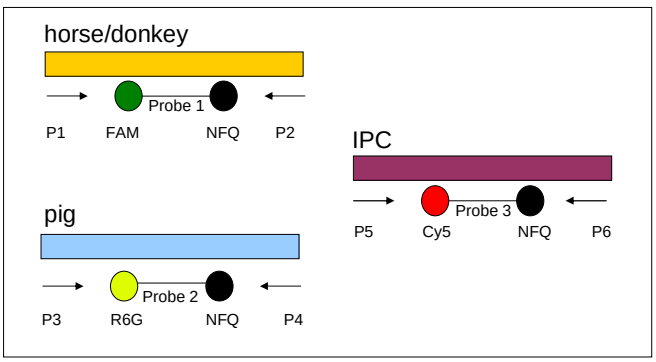


FAM™, R6G and Cy5™ are the fluorescent reporter dyes attached to the 5' ends of the probes for species screening and for the IPC (internal positive control). Non-fluorescent quenchers (NFQ) are used for all probes.

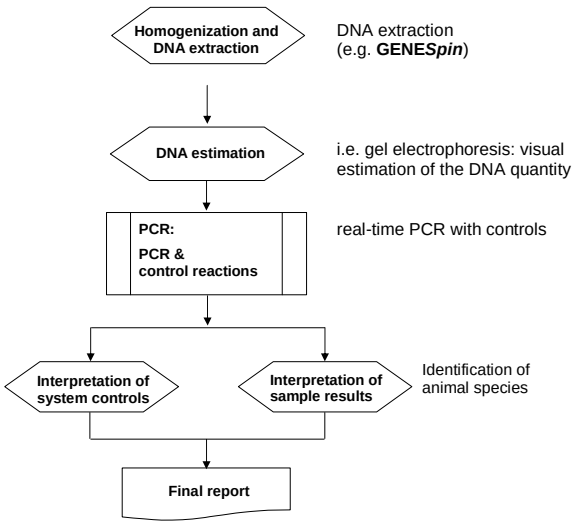
Caution:
This kit is not suitable for samples with known high content of one of the both species (please consider chapter 8.2: Asymmetric target situation).

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.

Test Procedure – Flowchart



3 COMPONENTS OF THE KIT

The kit contains all components to run control and specific reactions for a total of 96 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-08.042, Halal**

1 ml composed of

- **650 µL BasicMix** (BM) QL RT • GS-P-26.012 • EFGi TP 2x (NR) 1.5U, pH 8.0
- **390 µL OligoMix** (OM) QL RT IPC (LR) GS-P-08.042 • Halal

Mix prior to use!

2 x **Positive control DNA** (50 µL, 10 copies/µL): **Genomic DNA gGS-P-08.042**

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

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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/plates 0.2 ml and optical caps/seals
– Applied Biosystems® 7500/Fast, Agilent MX3005P/MX3000P, Agilent AriaMx™, Bio-Rad CFX96 Touch™ or Roche LightCycler® 480

– **On LC480, Color Compensation must be performed before the first test is run. Please use our color compensation kit with cat. no. 5427200302.**

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 affect overall sensitivity of the analysis. Low DNA amount 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 the sample DNA undiluted for PCR. In case inhibitors are present in the DNA, dilution of the sample DNA is feasible.
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 kits and materials for DNA extraction together with samples or amplicons.
e) Always perform extraction controls and PCR controls (NTCs).

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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.

The following reactions are required for a RT-PCR run:

Samples (and extraction controls)	**1**	**8**	**23**	**46**
NTCs	2	2	2	2
Pos. controls	2	2	2	2
Samples/extraction controls (duplicates)	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
OligoMix	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 (incl. extraction controls). Please add approx. 5% of the respective volumes to account for pipetting errors.

1. Thaw 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. Add 20 µL of the composed MasterMix to the wells.
2. Add 5 µL of stabilization buffer to NTCs.
3. Add 5 µL of control DNA gGSE-P-08.042 to positive controls (C+).
4. Add 5 µL of sample DNA each to duplicates of 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	ECa	ECb

Plate layout for 45 samples + extraction control (EC);

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

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7.4 Programming of Plate Documents

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

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

Program your template with the following settings:

ABI 7500/Fast

System	Detector	Reporter	Quencher
Horse/Donkey	8042a	FAM™	NONE
Pig	8042b	VIC™	NONE
IPC	8042i	CY5™	NONE

Passive Reference: ROX

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

Agilent MX3005P/MX3000P

System	Assay	Filter	pmt
Horse/Donkey	8042a	FAM™/SYBR® Green I	1
Pig	8042b	HEX™	1
IPC	8042i	Cy5	1
Reference	ROX	ROX™	1

The fluorescence signals are scanned in the following order: (1) ROX, (2) FAM, (3) HEX (4) Cy5.
Set filter set gain settings for all filters to pmt = 1.

AriaMX

System	Targets	Dyes
Horse/Donkey	8042a	FAM™
Pig	8042b	HEX™
IPC	8042i	Cy5™

Bio-Rad CFX96 Touch

System	Targets	Dyes
Horse/Donkey	8042a	FAM™
Pig	8042b	VIC™
IPC	8042i	Cy5™

Roche LightCycler®480 Instrument I

Detection Format: Multi Color Hydrolyses Probe

System	Filter
Horse/Donkey	FAM (483-533)
Pig	VIC/HEX/Yellow555 (523-568)
IPC	Cy5 (615-670)

During Cycling the Analysis Mode “Quantification” is selected. Ramp rate is set to 4.4 for heating and 2.2 for cooling.

On LC480, Color Compensation must be performed before the first test is run. Please use our color compensation kit with cat. no. 5427200302.

7.5Cycling Conditions

The PCR temperature profile for ABI7500/Fast MX3005P/MX3000P, AriaMX, CFX96 and LC480 is:

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

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

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8 RESULTS

8.1 Evaluation

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

Threshold:

ABI 7500/Fast and Agilent MX 3005/MX3000P:
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 portion of the plot, which is linear. The threshold line should neither be placed in the plateau phase nor in the initial linear phase of amplification.

Agilent AriaMx™:
Auto calculated threshold with default background based threshold settings can be used:
Cycle range: 5 thru 9
Sigma multiplier: 10

Bio-Rad CFX96 Touch™:
Auto calculated threshold can be used but should be checked visually

Baseline:

ABI 7500/Fast: Set manually, 3-15 or alternatively automatic

Agilent MX3005/MX3000P: Adaptive

Agilent AriaMx™: Adaptive

Bio-Rad CFX96 Touch™: Baseline Subtracted Curve Fit, Apply fluorescence drift correction.

8.2 Interpretation of Results

Export CT values to the Excel™ sheet provided (please request to kits@eurofins.com), or do the evaluation following the parameters below.

Definitions

Ct/Cp Cut-offs	Horse/Donkey Cut-off -1: Pig Cut-off -1: IPC Cut-off -1: Horse/Donkey Cut-off -2: Pig Cut-off -2: IPC Cut-off -2:	Mean CT (C+) -10 Mean CT (C+) -10 Mean CT (NTC) -3 Mean CT (C+) +7 Mean CT (C+) +7 Mean CT (NTC) +3
dRn/dR/ Endpoint fluorescence (EF) Limits	Horse/Donkey: Pig: IPC:	Mean dRn (C+) x 0.2 Mean dRn (C+) x 0.2 Mean dRn (NTC) x 0.33
Outliers	Maximum acceptable outliers (C+): Maximum acceptable outliers (NTC):	0 0

Inhibition control, scoring of IPC

Ct/Cp and dRn/dR/EF (Endpoint fluorescence)	**Results**
Ct/Cp_{IPC} Cut-off-1 ≤ Ct/Cp_{IPC} sample ≤ Ct/Cp_{IPC} Cut-off-2 **and** $dRn/dR/EF_{IPC}$ sample ≥ $dRn/dR/EF_{IPC}$ Limit	Sample valid
Ct/Cp_{IPC} sample < Ct/Cp_{IPC} Cut-off-1 **and** $dRn/dR/EF_{IPC}$ sample ≥ $dRn/dR/EF_{IPC}$ Limit	Check amplification!
Ct/Cp_{IPC} sample > Ct/Cp_{IPC} Cut-off-2 **or** $dRn/dR/EF_{IPC}$ sample < $dRn/dR/EF_{IPC}$ Limit	Sample inhibited
No Ct/Cp_{IPC}	Sample inhibited

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Test reaction horse/donkey or pig

Ct/Cp	**dRn/dR/Endpoint fluorescence (EF)**	**Result**
Ct/Cp Cut-off-1 ≤ Ct/Cp sample ≤ Ct/Cp Cut-off-2	dRn/dR/EF sample ≥ dRn/dR/EF Limit	Reaction positive
Ct/Cp sample < Ct/Cp Cut-off-1	dRn/dR/EF sample < dRn/dR/EF Limit	Check amplification!
Ct/Cp sample < Ct/CpCut-off-1	dRn/dR/EFsample ≥ dRn/dR/EF Limit	Check amplification!
Ct/Cp sample > Ct/Cp Cut-off-2	dRn/dR/EF sample ≥ dRn/dR/EF Limit	Reaction positive
Ct/Cp sample > Ct/Cp Cut-off-2	dRn/dR/EF sample < dRn/dR/EF Limit	Reaction negative
No Ct/Cp sample	-	Reaction negative
Note: In case of "Check amplification!" the linear scale amplification plots must be carefully checked for presence or absence of a sigmoid PCR amplification signal. If a sigmoid curve can be observed, the sample is positive; if not, the sample is negative.		

Final result from combination of inhibition control and test reaction

IPC	**Test reaction**	**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/Cp value from NTC. Refer to data from IPC-detector. To calculate the Ct/Cp cut-off-1, subtract 3 Ct/Cp, to calculate the Cut-off-2 add 3 Ct/Cp. Calculate the MEAN dRn/dR/Endpoint fluorescence (EF) value from NTC. Refer to data from IPC-detector. The dRn/dR/Endpoint fluorescence cut-off is 33% of the MEAN dRn/dR/Endpoint fluorescence.

Evaluation of horse/donkey and pig test
Calculate the MEAN Ct/Cp value from positive Control. Refer to data from horse/donkey or pig detector, respectively. To calculate the Ct/Cp cut-off-1, subtract 10 Ct/Cp, to calculate the Cut-off-2, add 7 Ct/Cp. Calculate the MEAN dRn/dR/Endpoint fluorescence value from positive control. Refer to data from horse/donkey or pig detector, respectively. The dRn/dR/Endpoint fluorescence cut-off is 20% of the MEAN dRn/dR/Endpoint fluorescence.

Please keep in mind that the specificity of the tests is "*Equidae*" (horse, mule, hinny, donkey, zebra) for one test and pig and wild boar for the other test.

Asymmetric target situation:
Negative results in one test reactions must be regarded invalid if the other PCR system is positive and shows CT values for the sample < mean Ct(C+) - 5 Ct. In this case the validity of the negative result cannot be guaranteed and the negative test result needs to be confirmed with separate analysis!

8.3 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% (w/w) of horse or porcine DNA in other animal species (total DNA amount of 200ng/rxn).
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. Thus, the LOD is strongly dependent on the type of sample and the DNA extraction procedure.
Quantification is not possible with this kit.

10 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.

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.

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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.
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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.