DN*Animal*

DN*Animal* *Screen* Mammal & Bird

IPC (LR/HR)

Cat. No. 5422212110

Test kit for the qualitative real-time PCR detection of mammalian and avian DNA

DN*Animal Screen* Mammal & Bird IPC (LR/HR)_ID2872 V9 09.08.2018

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. For technical assistance and more information please contact the Eurofins GeneScan Technologies Technical Service Department or your local distributor.

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DN*Animal Screen* Mammal & Bird IPC (LR/HR)
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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* Mammal & Bird Kit

Kit for the qualitative real-time PCR detection of mammalian and avian 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 i.e. for vegetarian food, food for religious communities, export and trade, as well as for feed (i.e. in the context of feeding restrictions).

The kits from the Eurofins GeneScan DN*Animal* kit line comprise several major advantages:

- High sensitivity (LOD ≤ 0.01% w/w)
- High specificity (primers and probe)
- Robust test methods
- Fast results
- No amplicon contamination risk

The DN*Animal Screen* Mammal & Bird kit was designed for a general detection of Mammal and Bird in DNA from food and feed matrices.

The kit is validated for use on Agilent MX3005P/3000P/Aria MX, ABI7500/Fast, Roche LC480, ABI 7900HT and BioRad CFXTouch96.

Specificity of the kit: Important notes!

Please be aware that humans are mammals and human DNA will be detected with this kit. For this reason, thorough contamination prevention is necessary in order to avoid contamination and false-positive results!

The detection system of this kit does not detect reptiles and amphibians.

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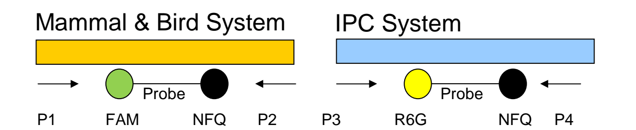
The test comprises the following steps:

1. DNA extraction (not included in this kit)
2. Real-time PCR detection of the target

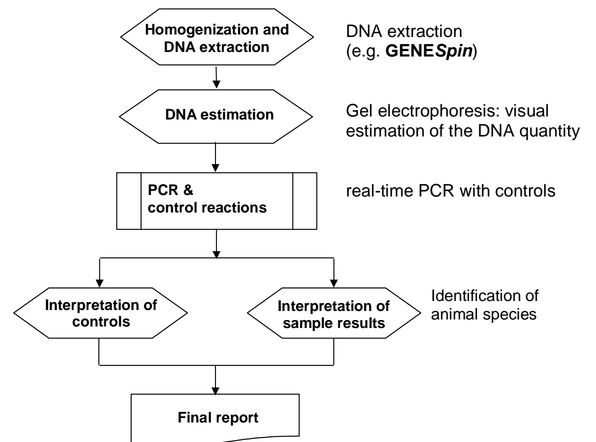
Rigid food or feed processing can degrade DNA in a way that makes PCR amplification impossible.
However, in most matrices, even in gelatin, animal species can be analyzed. Due to processing steps commonly used in food and feed production, the fragment length of the sample DNA is usually reduced, but DNA is not completely degraded and the base sequence of the fragments is not changed.

Specific DNA sequences 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 system in this kit uses FAM® as reporter dye and the IPC uses R6G. The probes use non-fluorescent quenchers.



2 TEST PROCEDURE – FLOWCHART



3 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.

4 COMPONENTS OF THE KIT

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

MasterMix QL RT IPC (LR) GS-P-08.043, Mammal & Bird
1 mL composed of

- 650 µL **BasicMix** QL RT IPC (NR) • 26.012 • EFGi2x TPn (NR) 1.5U
- 390 µL **OligoMix** QL RT IPC (LR/HR+) GS-P-**08.043** myostatine

Mix prior to use!

Positive control DNA: **Genomic DNA Cattle**
(50 µL, 10 copies/µL)

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

ROX™ dye, 23 µL -
(to be used only for High ROX cyclers, see instructions below)

5 PREPARATION AND STORAGE OF REAGENTS

Store the kit light-protected at -20°C. Never store solutions and materials for DNA extraction together with samples or amplicons. Never use them in areas where PCR products may be present.
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 accordingly.

6 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

7 SAMPLE PREPARATION

7.1 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 the sample DNA undiluted for PCR.
In case of inhibitory effects present in the DNA preparation further purification of the DNA or a repeat of the PCR analysis with diluted sample DNA is feasible. The dilution factors depend on the degree of inhibition and the DNA concentration. Excessive dilution compromises the practical LOD (pLOD) and shall be avoided
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.

8 PCR

8.1 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) Be aware that human DNA will be detected with the kit.
d) Wear disposable powder-free gloves.
e) Wear lab coat and cover your hair.
f) Wear a mask in order to avoid contamination of samples and/or PCR with aerosols.
g) Never store kits and materials for DNA extraction together with samples or amplicons.
h) Always perform extraction controls and PCR controls (NTCs).

8.2 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.

If you use one of the following cyclers, add also more ROX™dye: ABI 5700, 7000, 7300, 7700, 7900, Opticon2 or other cyclers laid out for high ROX concentration (please enquire in case of doubt). ROX™ is added increasing the volume of the MasterMix to 20.45 µL. 20.45 µL HR MasterMix consist of 12.5 µL BasicMix + 0.45 µL ROX (50x) + 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
ROX (only for HR!)	*2.7 µL*	*9 µL*	*22.5 µL*	*43.2 µL*

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. If the cycler requires a high ROX™ level, add ROX™.
4. Mix thoroughly.
5. The composed MasterMix can be stored up to 4 h in the refrigerator, but must not be used longer.

8.3 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.

6. Label all PCR reaction tubes.
7. Add 20 µL of the composed MasterMix to the wells.
8. Add 5 µL of stabilisation buffer to NTCs.
9. Add 5 µL of control (cattle) DNA to positive controls.
10. 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

8.4 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.

Program your template with the following settings:

Stratagene Mx3005P (3000P)

System	Assay	Filter
Mammal & Bird	8043a	FAM™/SYBR® Green I
IPC	8043i	HEX™
Reference	ROX	ROX™

The fluorescence signals are scanned in the following order: (1) ROX, (2) FAM, (3) HEX. The filter set gain settings are for ROX, FAM and HEX: 1x

ABI 7500 (7500 Fast)/ ABI 7900 HT

System	Detector	Reporter	Quencher
Mammal & Bird	8043a	FAM™	NONE
IPC	8043i	VIC™	NONE

Passive Reference: ROX

ABI 7500 Fast
PCR is performed in the “Standard 7500” run mode.
ABI 7900HT
Activate 9600 emulation mode.

Agilent AriaMx™

System	Target Name	Dye
Mammal & Bird	8043a	FAM™
IPC	8043i	HEX™
Reference	ROX	ROX™

Passive reference: ROX.

Bio-Rad CFX96 Touch™

System	Targets	Dyes
Mammal & Bird	8043a	FAM™
IPC	8043i	VIC™

A reference dye is not used.

LightCycler480 (System II)
Color Compensation for FAM and R6G is performed according to the manual.

System	Filter
Mammal & Bird	FAM™
IPC	VIC™/Hex™/Yellow555

A reference dye is not used.

8.5 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 than the mentioned ones, it may be necessary to optimize the PCR parameters.

- Save the created file/document.

9 RESULTS

9.1 Evaluation

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

Threshold:

Agilent Mx- Cyclers, ABI Cyclers: manual
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
If appropriate, Auto calculated threshold with default Background based threshold settings can be used: Cycle range: 5 thru 9; Sigma multiplier: 10

Bio-Rad CFX Touch: auto
Check visually

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Baseline:

- **ABI RT-cyclers:** manual, 3-15 or automatic baseline.
- **Agilent MX:** Adaptive.
- **Agilent AriaMX:** Auto baseline correction can be used.
- **Bio-Rad CFX Touch**: Baseline Subtracted Curve fit with fluorescence drift correction.

Roche LC 480

Apply the colour compensation object (created for this analysis/kit) to the experiment.

To obtain CP values: use the "Abs Quant/2nd Derivative Max" analysis mode and High Confidence settings.

To obtain endpoint fluorescence values: use the "Endpoint Genotyping" analysis mode.

9.2 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 1	Mammal & Bird: Mean Ct (C+) +4.5 IPC: Mean CT (NTC) +3
Ct Cut-offs 2	Mammal & Bird: Mean Ct (C+) -10 IPC: Mean CT (NTC) -3
EPF Endpoint Fluorescence Limits	IPC: Mean EPF (NTC) x 0.33
Outliers	Maximum acceptable outliers (C+): 0 Maximum acceptable outliers (NTC): 0

Inhibition control, scoring of IPC

Cq (Ct) and Endpoint fluorescence (EPF)	Results
Cq_{IPC} Cut-off -2 ≤ Cq_{IPC} sample ≤ Cq_{IPC} Cut-off-1 **and** EPF_{IPC} sample ≥ EPF_{IPC} Limit	Sample valid
Ct_{IPC} sample > Ct_{IPC} Cut-off-1 **or** EPF_{IPC} sample < EPF_{IPC} Limit	Sample inhibited
No Ct_{IPC}	Sample inhibited
Ct_{IPC} sample < Ct_{IPC} Cut-off-2	Check amplification!

Note:
For all runs and in particular in case of LightCycler480 use, it is recommended to check the curves for sigmoid amplification.
In case of Cq value of 40 or "Check amplification!" the linear scale amplification plot must be analysed for presence or absence of sigmoid PCR amplification signal.
Only if the amplification curve is sigmoid, the result is valid, if not, the reaction is inhibited and the result not valid.

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Test reaction (Mammal & Bird)

Cq (Ct)	Results
Cq Cut-off 2 ≤ Cq sample ≤ Cq Cut-off 1	Reaction positive
Cq sample > Cq Cut-off 1	Reaction negative
Cq sample < Cq Cut-off 2	Check amplification!
No Cq	Reaction negative

Note:
For all runs and in particular in case of LightCycler480 use, it is recommended to check the curves for sigmoid amplification.
In case of Cq value of 40 or "Check amplification!" the linear scale amplification plots must be checked critically for presence or absence of sigmoid PCR amplification signal.
Only if the amplification curve is sigmoid, the result is positive, if not, the result is negative.

Final result from combination of inhibition control and test reaction

IPC	Mammal & Bird test	Final result
Sample valid	Reaction positive	positive
Sample valid	Reaction negative	negative
inhibited	Reaction positive	positive
inhibited	Reaction negative	Inhibited

Evaluation of the IPC

Calculate the MEAN Cq Value from NTC. Refer to data from IPC-detector. To calculate the Cq cut-off-1, add 3 Ct. To calculate Cq cut-off-2 subtract 3 Cq.
Calculate the MEAN EPF Value from NTC. Refer to data from IPC-detector. The EPF cut-off is 33% of the MEAN EPF.

Evaluation of Mammal & Bird specific test

Calculate the MEAN Ct Value from positive control. To calculate the Ct cut-off-1, add 4.5 Ct. To calculate Ct cut-off-2, subtract 10 Ct.

Combine results of IPC and the Mammal & Bird test to the final result.

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.

10 LIMIT OF DETECTION

The relative limit of detection has been validated at 0.01% of Mammals and Birds DNA in other species (total DNA ratio). LOD95% ≤ 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.

11 PRODUCT USE LIMITATIONS

The GeneScan 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.

12 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 and before 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

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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 provided in our price lists.

13 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.

14 TROUBLESHOOTING

Result	Possible mistakes/reasons	Possible verification and measures
No PCR signals from samples	Inhibition of PCR by inhibitory substances.	Clean DNA further* or dilute DNA solution.
	Inhibition by too much DNA.	Check DNA concentration/dilution.
No PCR signals from positive controls	Wrong PCR program.	Check and correct PCR program.
No amplification, neither from control DNA nor from sample DNA	MasterMix not properly prepared	Prepare fresh MasterMix, repeat PCR.
	Wrong PCR program.	Check program.
Positive PCR result for NTC	Contamination with DNA/amplicons 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 sample material/DNA/ amplicons/ 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).