eurofins | GeneScan

DN*Animal*

DN*Animal Ident* Pig HS

IPC (LR)

for use with low-DNA samples

Cat. No. 5422211810, -05 (S-Kit)

Test kit for qualitative real-time PCR detection of porcine DNA with IPC

eurofins | GeneScan

Table of Contents:

1 INTRODUCTION 4

2 TIME SCHEDULE 7

3 COMPONENTS OF THE KIT 8

4 PREPARATION AND STORAGE OF REAGENTS 9

5 MATERIAL AND EQUIPMENT NOT INCLUDED IN THE KIT 9

6 SAMPLE PREPARATION 10

6.1 DNA extraction 10

7 PCR 11

7.1 Special precautions during PCR analysis 11

7.2 Preparation of MasterMix 12

7.3 PCR Setup 14

7.4 Programming of Plate Documents 16

8 RESULTS 19

8.1 Evaluation 19

8.2 Interpretation of Results 20

9 LIMIT OF DETECTION 24

10 PRODUCT USE LIMITATIONS 24

11 PRODUCT WARRANTIES AND SATISFACTION GUARANTEE 25

12 IMPORTANT NOTES 26

13 TROUBLESHOOTING 27

TECHNICAL SERVICE 28

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

DN*Animal Ident* Pig HS IPC Kit

Kit for the qualitative real-time PCR detection of porcine DNA *(Sus scrofa)* in food and feed, for use with low-DNA samples.

1 INTRODUCTION

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

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 DN*Animal Ident* Pig IPC kit was designed for a general detection of pig DNA in DNA extracted i.e. from food and feed matrices and depends to the Eurofins GeneScan DN*Animal* kit line.
The kit assay targets a mitochondrial gene of pig. Depending on the tissue type cells contain several thousand mitochondrial DNA copies which lead to an extremely high sensitivity of the assay.

Due to this very high sensitivity of the assay, the risk of contamination is very high. Therefore this assay is only recommended for low DNA samples (e.g. gelatine) or in cases where very high sensitivity is required.

In labs where pork samples are analysed on a regular basis and where the risk of contamination is very high, we recommend our DN*Animal* Ident Pork IPC kit (Cat. No. 5422211910).

Important remark:
With this kit, it is not possible to differentiate between domestic pig and wild boar *(sus scrofa domestica* and *sus scrofa scrofa)*. Both will be detected equally.

The test comprises the following steps:

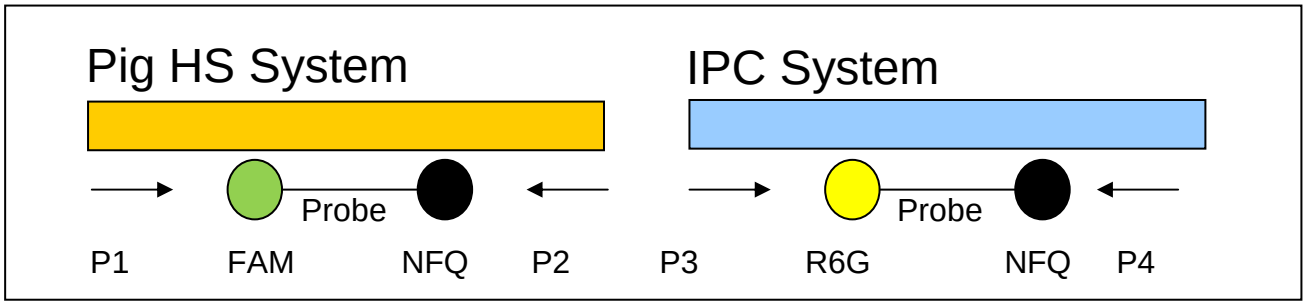
1. DNA extraction (not included in this kit)
2. Real-time PCR detection of the target

Animal species can be analysed in most matrices, even in gelatin. The processing steps commonly used in food and feed production usually reduce the fragment length of the sample DNA, but DNA is not completely degraded and the base sequence of the fragments is not changed.

However, extremely rigid (acid/heat) processing can degrade DNA in a way that amplification is impossible.

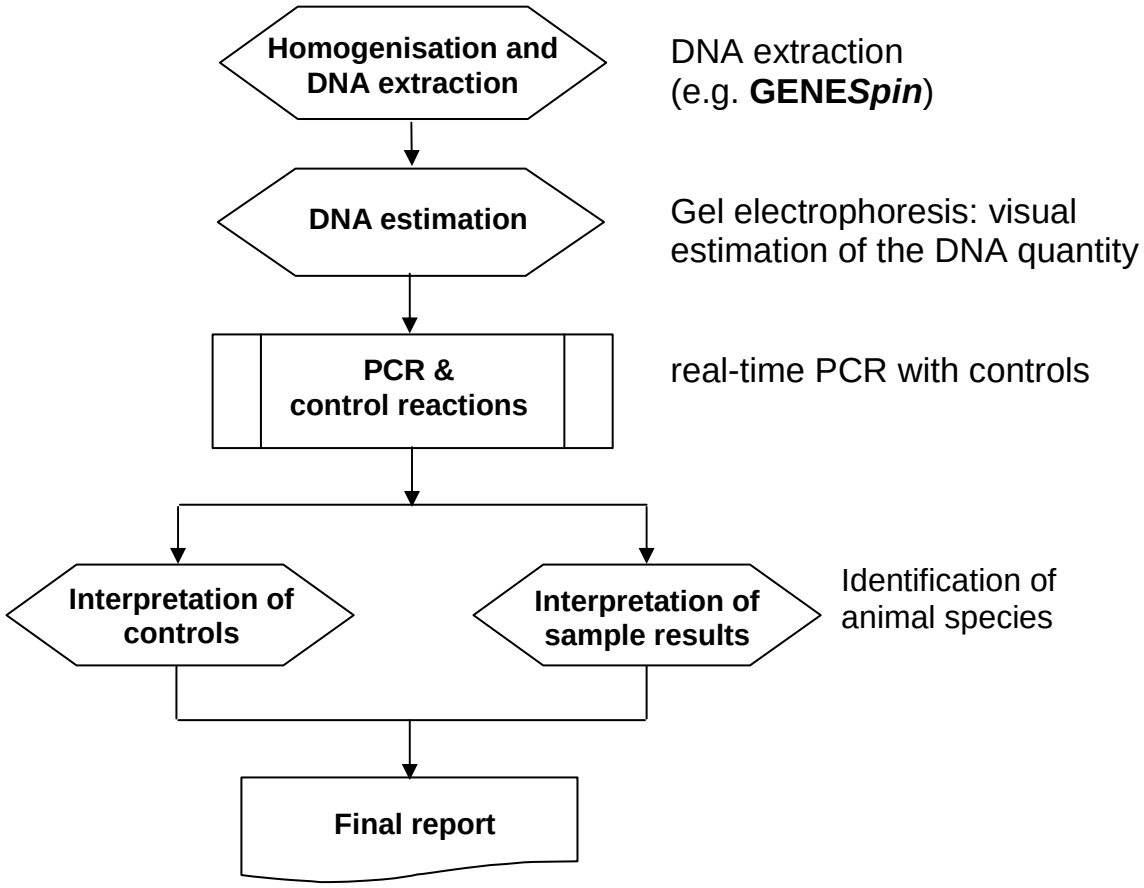
Specific DNA sequences are amplified by RT-PCR (Real-time Polymerase Chain Reaction) and detected with high specificity due to the Taqman™ probes.

The probe of the detection system in this kit uses FAM® as reporter dye and the IPC (internal positive control) uses R6G®. The probes use non-fluorescent quenchers.



The kit was validated for use on Agilent MX3005P/ MX3000P, ABI7500 (Fast) and Roche LC480.

Test Procedure – Flowchart



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.

3 COMPONENTS OF THE KIT

The kit contains all components to run control and specific reactions for a total of 96 (cat# 5422211810) or 48 reactions (S-kit with cat# 5422211805):
PCR reactions.

MasterMix QL RT IPC (LR) GS-P-08.034 pig HS

(cat# -10: 2x, cat# -05 (S):1x)

1 ml composed of

- 650 µL **BasicMix** QL RT (NR) • GS-P-26.001 • EFGi 2x (NR)
- 390 µL **OligoMix** QL RT IPC (LR) • GS-P-08.034 Pork mitochondrial

Mix prior to use!

Positive control DNA, 150 µL • **Genomic DNA** • 100 copies/µL Pork (mito.Targets) (cat# -10: 2x, cat# -05 (S):1x)

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

4 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.

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 thermo cycler

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.
The samples are analysed undiluted. In case of inhibitory effects present in the DNA preparation a repeat of the analysis with diluted samples is feasible.

Each sample should be treated in duplicates.

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* 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 consists 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 with 1, 8, 23 or 46 samples (incl. extraction controls). Add approx. 5% of volume as pipetting error compensation or use complete tubes.

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
OligoMix	45 µL	150 µL	375 µL or 1 tube	750 µL or 2 tubes

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 of stabilisation buffer to NTCs.
4. Add 5 µL of control (pig) 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 = sample duplicates

7.4 Programming of Plate Documents

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

For setup and evaluation information and the evaluation sheet send an email to kits@eurofins.com and refer to the user manual of the respective instrument and software version.

Program your template with the following settings (the use of the assay/detector name is mandatory if the Eurofins GeneScan evaluation sheet is used):

Stratagene Mx3005P/MX3000P

System	Assay	Filter
Pig	8034a	FAM™/SYBR® Green I
IPC	8034i	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 pmt

ABI 7500 (7500 Fast)

System	Detector	Reporter	Quencher
Pig	8034a	FAM™	NONE
IPC	8034i	VIC™	NONE

Passive Reference: ROX

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

Roche LightCycler®480 Instrument I

System	Filter
Pig	FAM (483-533)
IPC	VIC/HEX/Yellow555 (523-568)

Colour Compensation for FAM and HEX is performed according to the manual. Ramp rate is set to 4.4 for heating and 2.2 for cooling. A reference dye is not 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 than the mentioned ones, it may be necessary to optimise the PCR parameters.

8 RESULTS

8.1 Evaluation

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

Threshold: 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. Thresholds are typically in the range 0.03 - 0.1.

Baseline:

ABI 7500/Fast:	manual, 3-15 or automatic baseline
Mx3005P/MX3000P:	Adaptive

Analysis mode Roche LC 480:
Apply the colour compensation object (created for this analysis) 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.

For specific evaluation information for your cycler send an email referring to your cycler to kits@eurofins.com.

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	Pig:	Mean Ct (C+) +6
	IPC:	Mean CT (NTC) +3
dRn Limits	IPC:	Mean dRn (NTC) x 0.33
Outliers	Maximum acceptable outliers (C+): 0 Maximum acceptable outliers (NTC): 0	

Due to the very high copy number of mitochondria in a cell (typically 1000-2000 per cell), the positive control in this system contains 500 mitochondrial DNA target copies per reaction in contrast to 50 copies used in other DNAnimal kits targeting a genomic gene. To limit the risk of contamination the Ct cut-off value was chosen to reliably detect 100 mitochondrial DNA copies per reaction, therefore it is necessary to use only the Ct (Cp) value of the species system for evaluation. The evaluation of the internal positive control remains unchanged and combines the evaluation of the dRn (Endpoint fluorescence) and Ct (Cp) values.

Inhibition control, scoring of IPC

Ct and dRn	**Results**
Ct_{IPC} sample ≤ Ct_{IPC} Cut-off **and** dRn_{IPC} sample ≥ dRn_{IPC} Limit	Sample valid
Ct_{IPC} sample > Ct_{IPC} Cut-off **or** dRn_{IPC} sample < dRn_{IPC} Limit	Sample invalid
No Ct_{IPC}	Sample invalid
Ct_{IPC} sample < Mean Ct_{IPC} NTC -3	Sample invalid

Test reaction (pig)

Ct	Result	Remarks
Ct sample ≤ Ct Cut-off	positive	
Ct sample > Ct Cut-off	negative	*
Ct sample < Mean Ct (K+) – 10	Check amplification!	**
No Ct	negative	

***** *In some cases a sigmoid amplification curve with Ct value > Ct Cut-off can occur. In these cases it is recommended to clean the workplace.*

Especially in laboratories where pork samples get analysed on a routine basis and in countries where pork belongs to the regular diet, contaminations are very likely to occur due to the extremely high sensitivity of the assay. Therefore we do not recommend classifying such samples positive.

****** *Check the amplification curve visually, in case sigmoid PCR amplification cannot be observed, the PCR is negative.*

Final result from combination of inhibition control and test reaction

IPC	Pig test	Final result
Sample valid	Reaction positive	Positive
Sample valid	Reaction negative	Negative
Sample invalid	Reaction positive	Check amplification
Sample invalid	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 dRn Value from NTC. Refer to data from IPC-detector. The dRn cut-off is 33% of the MEAN dRn.

Evaluation of pig specific test

Calculate the MEAN Ct Value from positive control.
To calculate the Ct cut-off, add 6 Ct.

Combine results of IPC and the porcine test to the final result. Please remember that the kit detects both, domestic pig (*Sus scrofa domestica*) and wild boar (*Sus scrofa scrofa*).

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.

9 LIMIT OF DETECTION

The relative limit of detection has been validated as 0.0001 % of porcine DNA in other species (total DNA content of 25 ng/rxn, pig DNA in cattle DNA (w/w)).

10 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.

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 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 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.

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 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.

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 control 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/amplicons when mixing the PCR components.	Optimise 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).

TECHNICAL SERVICE

If you have any questions or experience any difficulties regarding this kit or GeneScan products in general, please do not hesitate to contact us. GeneScan customers are 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 GeneScan. 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.

Eurofins GeneScan Technologies GmbH

Engesser Str. 4
79108 Freiburg, Germany
Phone: + 49-(0)761-5038-100
Fax: + 49-(0)761-5038-111
kits@eurofins.com
www.eurofins.com/kits