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DN*Animal*

DN*Animal Ident* Meat

IPC (LR/HR)

Cat. No. 5422212010

Test kit for the qualitative detection of bovine and porcine DNA with 2 x 48 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* Meat IPC Kit

Kit for the qualitative real-time PCR detection of bovine and porcine 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 became particularly important in times of BSE (bovine spongiform encephalopathy), and can be crucial e.g. 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 Technologies DN*Animal* kit line 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

The DN*Animal Ident* Meat IPC kit was designed for a general detection of bovine and porcine DNA in extracted DNA from i.e. food and feed matrices.

The tests comprise the following steps:

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

Animal species can be analyzed 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 is validated for use on Agilent MX3005P/3000P, Agilent AriaMX, ABI7500/Fast, Bio-Rad CFX Touch,Roche LC480 and ABI 7900HT.

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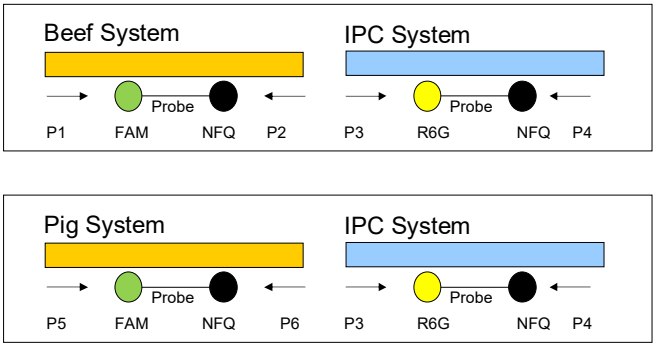
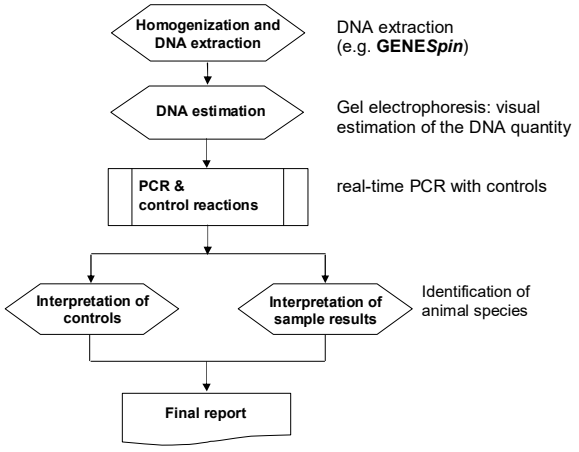


Specificity of the kit: Important note!

- The kit cannot be used for a differentiation of different bovine species, it detects besides cattle *(Bos taurus)* also zebu *(Bos indicus)*, yak *(Bos mutus)*, bison *(Bison sp.)* and gaur *(Bos frontalis)*. Water buffalo (*Bubalus bubalis*), however, is not detected with this kit.
- 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.

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 2 x 48 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. (1x) **OligoMix:** OM QL RT IPC (LR/HR+)
   Module GS-P-08.030 **Cattle**
   390 µL primer/probe mix for bovine DNA detection
2. (1x) **OligoMix:** OM QL RT IPC (LR/HR+)
   Module GS-P-08.041 **Pig**
   390 µL primer/probe mix for porcine DNA detection
3. (2x) **BasicMix:** BM (NR) GS-P-26.012
   EFGi TP 2x (NR) 1.5U
   650 µL reaction mix without primers and probes

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

4. (2x) **ROX™ dye,** 23 µL (to be used only for High Rox cyclers, see chapter 8)
5. (1x) **Genomic DNA Cattle [*Bos taurus*]**
   50 µL (10 copies/µL)
6. (1x) **Genomic DNA Pig [*Sus scrofa domestica*]**
   50 µL (10 copies/µL)
7. (2x) **DNA stabilization buffer** (for NTCs)
   150 µL

5 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, prepare aliquots of reagents at first use.
Refer to the reagent label for specific instructions regarding the correct storage.

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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 50 - 100 ng of the sample DNA per reaction. The concentration of DNA should be adjusted accordingly prior to the setup 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
- 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.

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) 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).

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.
- 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.
- 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, Opticon 2 or other cyclers laid out for high ROX (HR) concentration (please enquire in case of doubt).

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- 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.

The following numbers of reactions are required for a RT PCR run for each species test (cattle and pig):

Samples	1	8	23
NTCs	2	2	2
Pos. controls	2	2	2
Samples (duplicate)	2	16	46
Total no. reactions	**6**	**20**	**50**
Total MM volume	**120 µL**	**400 µL**	**1 mL**
BasicMix	75 µL	250 µL	625 µL or 1 tube
Oligo Mix	45 µL	150 µL	375 µL or 1 tube
ROX (only for HR!)	*2.7 µL*	*9 µL*	*22.5 µL*

Number of reactions and volumes of reagents needed for 1, 8, 23 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.

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 (cattle or pork) DNA to positive controls.
5. Add 5 µL of sample DNA to test reactions.

8.4 Plate layout example

	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	NTC	NTC	C+	C+	1a	1b	2a	2b	3a	3b	4a	4b
F	5a	5b	6a	6b	7a	7b	8a	8b	9a	9b	10a	10b
G	11a	11b	12a	12b	13a	13b	14a	14b	15a	15b	16a	16b
H	17a	17b	18a	18b	19a	19b	20a	20b	21a	21b	22a	22b

Table 4: Plate layout example
A – D: Test for bovine DNA; E – H: Test for porcine DNA;
a and b = DNA extract duplicates; NTC = No Template Control/negative control

8.5 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:

Agilent Mx3005P (3000P), Agilent AriaMX

System	Assay	Filter
Beef	8030a	FAM™/SYBR® Green I
IPC	8030i	HEX™
Reference	ROX	ROX™

System	Assay	Filter
Pork	8041a	FAM™/SYBR® Green I
IPC	8041i	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: pmt = 1x
ABI 7500 (7500 Fast) / ABI 7900 HT

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System	Detector	Reporter	Quencher
Beef	8030a	FAM™	NONE
IPC	8030i	VIC™	NONE

System	Detector	Reporter	Quencher
Pork	8041a	FAM™	NONE
IPC	8041i	VIC™	NONE
Passive Reference:		ROX	

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

Bio-Rad CFX96 Touch

System	Detector	Reporter	Quencher
Beef	8030a	FAM™	NONE
IPC	8030i	VIC™	NONE

System	Detector	Reporter	Quencher
Pork	8041a	FAM™	NONE
IPC	8041i	VIC™	NONE

Default maximum Ramp rate is used.
A reference dye is not used.

LightCycler480

- Color Compensation for FAM and R6G is performed according to the manual.
- FAM signal is collected using the FAM detector.
- R6G signal is collected using the VIC/HEX/Yellow555 detector.
- Ramp rate is set to 4.4 for heating and 2.2 for cooling.
- A reference dye is not used.

8.6 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. **The evaluation is done separately for both detection systems (pig and cattle).**

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, which may be too low and into the background fluorescence or where splitting between replicates may be observed.
Roche LC 480: No threshold settings.

Baseline:

- **ABI RT-cyclers:** manual, 3-15 or automatic
- **Stratagene Mx:** Adaptive
- **Bio-Rad CFX96 Touch:** Baseline Subtracted Curve Fit Apply fluorescence drift correction
- **Agilent AriaMX:** Auto baseline correction can be used.

Analysis mode:

Roche LC 480:

1. Apply the colour compensation object (created for this analysis/kit) to the experiment.
2. To obtain CP values: use the "Abs Quant/2nd Derivative Max" analysis mode and High Confidence settings.
3. To obtain endpoint fluorescence values: use the "Endpoint Genotyping" analysis mode.

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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	Beef/ Pork Cut-off-1	Mean Ct (C+) -5
	IPC Cut-off-1	Mean Ct (C+) -3
	Beef/ Pork Cut-off-2	Mean Ct (C+) +8
	IPC Cut-off-2	Mean Ct (NTC) +3
dRn Limits	Beef/ Pork:	Mean dRn (C+) x 0.2
	IPC:	Mean dRn (NTC) x 0.33
Outliers	Maximum acceptable outliers (C+): 0 Maximum acceptable outliers (NTC): 0	

Inhibition control, scoring of IPC

Ct and dR(n)	Results
Ct Cut-off 1 < Ct sample ≤ Ct Cut-off 2 **and** dR(n) sample ≥ dR(n) Limit	Sample valid
Ct sample > Ct Cut-off 2 **or** dR(n) sample < dR (n) Limit	Sample invalid
No Ct	Sample invalid
Ct sample < Ct Cut-off 1	Sample invalid

Test reaction (beef / pork)

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

Note: In case of “Check amplification!” the amplification plots must be checked critically visually for presence or absence of sigmoid PCR amplification signal.
The LC480 displays Cq values >40 as 40, therefore it is recommended to check all amplification curves with Ct 40 when the cut-off values 1 is >40.

Final result from combination of inhibition control and test reaction

IPC	bovine/porcine test	Final result
Sample valid	Reaction positive	Positive
Sample valid	Reaction negative	Negative
Sample invalid	Reaction positive	Positive
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 1, subtract 3 Ct.
  - To calculate Ct cut-off 2 add 3 Ct.
- Calculate the MEAN dRn value from NTC. Refer to data from IPC-detector.
  - The dRn cut-off f is 33% of the MEAN dRn.

Evaluation of beef / pork specific test

- Calculate the MEAN Ct Value from positive control.
  - To calculate the Ct cut-off-1, subtract 5 Ct.
  - To calculate Ct cut-off-2, add 8 Ct.
- Calculate the MEAN dRn value from positive control.
  - The dRn cut-off is 20% of the MEAN dRn.

Please remember that the kit cannot be used for a differentiation of several bovine or porcine species.

It is not possible to differentiate between domestic pig and wild boar *(sus scrofa domestica* and *sus scrofa scrofa) and* it detects besides cattle *(Bos taurus) also* zebu *(Bos indicus)*, yak *(Bos mutus)*, bison *(Bison sp.)* and gaur *(Bos frontalis)*.

Water buffalo (*Bubalus bubalis*), however, is not detected with this kit.

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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10 LIMIT OF DETECTION

The relative limit of detection has been validated for the bovine specific reaction 0.01% cattle DNA in pig DNA (w/w) with a total DNA content of 200 ng/reaction and for the pig specific reaction 0.01% pig DNA in cattle DNA (w/w) with a total DNA content of 250 ng/reaction.

The absolute detection limit of the method is ≤10 copies per reaction for both test systems.

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 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.

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 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 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.

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14 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.	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).

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. Eurofins 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 Eurofins 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 Eurofins GeneScan Technologies Technical Service Department or your local distributor.