DN*Animal* *Screen* Fish

IPC (LR/HR)

Cat. No. 5422211310

Test kit for the qualitative detection of fish DNA, 96 real-time PCR reactions with IPC

16 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 specialized 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 Technical Service Department or local distributors.

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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* Fish IPC Kit

Kit for the qualitative real-time PCR detection of fish DNA

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 such as the EU ban of fish meal for ruminant feeding).

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

- High sensitivity (0.01% w/w in unprocessed samples)
- High specificity (primers and probe)
- Robust test methods
- Fast results
- Low amplicon contamination risk
- Exclusion of false-negative results due to IPC

2 TEST PRINCIPLE AND APPLICATION SCOPE

The DN*Animal Screen* Fish IPC kit was designed for a general detection of fish DNA in DNA extracted i.e. from food and feed matrices.

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 fish detection system in this kit uses FAM® as reporter dye and the IPC (internal positive control) uses Rhodamin6G ® (measured in the HEX® or VIC™ channel). The probes have non-fluorescent quenchers.

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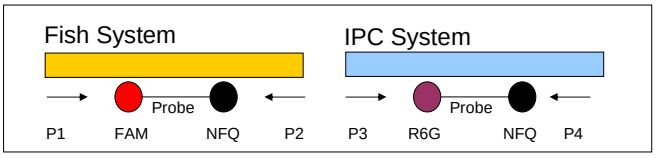

The kit is validated for use on Agilent MX3005P/3000P, Agilent Aria MX, ABI7500/Fast and ABI 7900HT.

Specificity of the kit

The fish screening covers more than 96% of the recent fish species and all commercially relevant ones. Detected are all ray-finned fish species as well as some cartilaginous fish species (e.g. spiny dogfish, but not ray). Details are listed in the table below.

Detection empirically validated

Altlantic wolffish *(Anarhichas lupus)*
American *plaice (Hippoglossoides platessoides*)
Arctic char *(Salvelinus alpinus)*
Atlantic herring *(Clupea harengus)*
Atlantic cod *(Gadus morhua)*
Atlantic halibut *(Hippoglossus hippoglossus)*
Atlantic mackerel *(Scomber scombrus)*
Atlantic salmon *(Salmo salar)*
Bigeye tuna *(Thunnus obesus)*
Blue whiting *(Micromesistius poutassou)*
Brill *(Scophthalmus rhombus)*
Chum salmon *(Oncorhynchus keta)*
Coho salmon *(Oncorhynchus kisutch)*
Comon dab *(Limanda limanda)*
European eel (*Anguilla anguilla*)
European flounder *(Platichthys flesus)*
European perch *(Perca fluviatilis)*
European pilchard *(Sardina pilchardus)*
European Plaice *(Pleuronectes platessa)*
European Pollock *(Pollachius pollachius)*
European seabass *(Dicentrarchus labrax)*
European Sturgeon *(Huso huso)*

Detection empirically validated (continued)

Gilt-head bream (*Sparus aurata*)
Greenland Halibut *(Reinhardtius hippoglossoides)*
Haddock *(Melanogrammus aeglefinus)*
Lemon sole *(Microstomus kitt)*
Megrim *(Lepidorhombus whiffiagonis)*
Nile perch *(Lates niloticus)*
Northern pike *(Esox lucius)*
Pink salmon *(Oncorhynchus gorbuscha)*
Pouting *(Trisopterus luscus)*
Rainbow trout *(Oncorhynchus mykiss)*
Siberian Sturgeon *(Acipenser baerii)*
Skipjack tuna *(Katsuwonus pelamis)*
Talapia *(Oreochromis)*
Tongue sole *(Cynoglossus spp.)*
Turbot *(Scophthalmus maximus)*
Wels catfish *(Silurus glanis)*
Whitefish *(Coregonus)*
Witch *(Glyptocephalus cynoglossus)*
Zander *(Sander lucioperca)*

No Detection

empirically validated

Cuckoo ray (*Leucoraja naevus)*

according to sequence alignment

Australian ghostshark *(Callorhinchus milii)*

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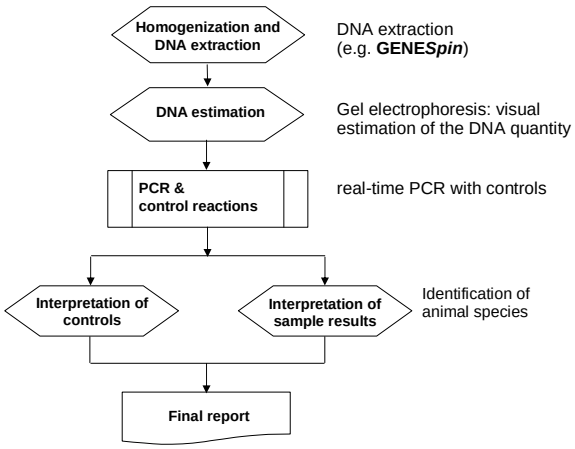
3 TEST PROCEDURE – FLOWCHART



4 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.

5 COMPONENTS OF THE KIT

The kit contains all components to run control and specific reactions for a total of 96 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 post-PCR areas where PCR products (amplicons) may be present.

2x **MasterMix QL RT IPC (LR/HR+) GS-P-08.036, Fish**

1 ml composed of

- **650 µL BasicMix** QL RT • GS-P-26.012 • EFGi TP 2x (NR)
- **390 µL OligoMix** QL RT IPC (LR/HR+) GS-P-08.036 Fish

Mix prior to use!

2x **Positive control DNA**: **Genomic DNA Codfish** (50 µL, 10 copies/µL)

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

2x **ROX™ dye,** 23 µL (to be used only for High ROX cyclers, see instructions below)

6 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.

7 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

8 SAMPLE PREPARATION

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.
If DNA purity is satisfactory, the sample can usually be used undiluted. If inhibition occurs, the DNA can be diluted in order to use 50 - 100 ng of the sample DNA per reaction.
DNA amount can be determined by one of the following methods:
- Real-time PCR monitor run with the respective kit (preferred method)
- Fluorimetric or spectrophotometric or other physical measurements, e.g. gel electrophoresis may be applied.

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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 spiked with DNA, which should subsequently undergo PCR analysis.

9 PCR

9.1 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).

9.2 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 or other cyclers laid out for high ROX (HR) concentration (please enquire in case of doubt), add additional ROX™ dye: ABI 5700, 7000, 7300, 7700, 7900, Opticon 2.
ROX™ is added increasing the volume of the MasterMix to 20.45 µL.
20.45 µL HR MasterMix thus consist of 12.5 µL BasicMix + 0.45 µL ROX (50x) + 7.5 µL OligoMix per reaction.

The following reactions are required for a RT-PCR run with 1, 8, 23 or 46 samples and 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
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*

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.

9.3 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 (fish) 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 = DNA extraction duplicates

9.4 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
Fish	8036a	FAM™/SYBR® Green I
IPC	8036i	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

Agilent AriaMX G8830A:

System	Target Name	Dye
Fish	8036a	FAM™
IPC	8036i	HEX™
Reference	ROX	ROX™

FAM signal is collected using the FAM detector
R6G signal is collected using the HEX detector
Passive reference: ROX

ABI 7500 (7500 Fast) / ABI 7900 HT

System	Detector	Reporter	Quencher
Fish	8036a	FAM™	NONE
IPC	8036i	VIC™	NONE

Passive Reference: ROX

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9.5 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 optimise the PCR parameters.

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

ABI 7900HT
Activate 9600 emulation mode

Save the created file/document.

10 RESULTS

10.1 Evaluation

Refer to your cycler´s manual for details. An evaluation (Excel™) sheet for ABI7500, ABI7900, MX3005P and AriaMX 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 linear portion of the plot. 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.
AriaMX: Auto calculated threshold with default background based threshold settings can be used:
Cycle range: 5 thru 9
Sigma multiplier: 10

Baseline:

ABI 7900: manual, 3-15
ABI 7900: manual, 3-15 or automatic baseline
Mx3005P: adaptive
AriaMX: auto baseline correction can be used

10.2 Interpretation of Results

Export Ct values to the Excel™ sheet provided for ABI7500, ABI7900, MX3005P and AriaMX (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	Fish: IPC:	Mean Ct (C+) +10 Mean CT (NTC) +3
dRn Limits	Fish: IPC:	Mean dRn (C+) x 0.2 Mean dRn (NTC) x 0.33
Outliers	Maximum acceptable outliers (C+): 0 Maximum acceptable outliers (NTC): 0	

Inhibition control, scoring of IPC

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

Test reaction (Fish)

Ct	dRn	Result
Ct sample ≤ Ct Cut-off	dRn sample ≥ dRn Limit	Positive
Ct sample ≤ Ct Cut-off	dRn sample < dRn Limit	Check amplification!
Ct sample > Ct Cut-off	dRn sample ≥ dRn Limit	Positive
Ct sample > Ct Cut-off	dRn sample < dRn Limit	negative
Ct sample < Mean Ct (K+) –5	dR(n) sample ≥ dR(n) Limit	Check amplification!
No Ct	-	negative
***Note*:** In case of "Check amplification!" the amplification plots must be checked visually for presence of a sigmoid PCR amplification signal.		

Final result from combination of inhibition control (IPC) and test reaction

IPC	Fish test	Final result
Test valid	Reaction positive	Positive
Test valid	Reaction negative	Negative
Test invalid	Reaction positive	Positive
Test invalid	Reaction negative	Inhibited

10.3 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 f is 33% of the MEAN dRn.

Evaluation of Fish specific test

Calculate the MEAN Ct Value from positive control. To calculate the Ct cut-off, add 10 Ct. Calculate the MEAN dRn value from positive control. The dRN cut-off is 20% of the MEAN dRn.

Combine results of IPC and the fish test to the final result.

Please remember that the kit cannot be used for a differentiation of several fish species, it detects all food-relevant ray-finned fish as well as Spiny Dogfish (*Squalus acanthias*).

10.4 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.

11 LIMIT OF DETECTION

The absolute detection limit (LOD_{abs}) of the method is ≤ 10 copies per reaction. The relative limit of detection (LOD_{rel}) was validated as 0.01% of fish DNA in other species (at a total DNA amount of 200 ng/rxn).
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.

12 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.

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 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

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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.

14 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.

15 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).