DN*Animal* Screen Fish IPC

REAL-TIME PCR KIT FOR THE QUALITATIVE DETECTION OF FISH DNA WITH INTERNAL POSITIVE CONTROL (IPC)

5422211310	DNAnimal Screen Fish IPC	for use on for use on for use on ABI 7500 (ThermoFisher Scientific), Mx3005P™/Mx3000P™ /AriaMx™ (Agilent), ABI 7900 (ThermoFisher Scientific)

For 96 real-time PCR reactions

GOLD STANDARD DIAGNOSTICS

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1 INTRODUCTION

The DNAnimal Screen Fish IPC real-time PCR detection kit provides materials for the qualitative detection of fish DNA. The kit detects the target sequence in food and feed sample types from which DNA in sufficient quality and quantity can be extracted.

1.1 Test Principle

The procedure of the current kit follows the basic principles of the real-time PCR amplification method. DNA segments are amplified with specific primers. PCR products are measured during each cycle (real-time) with target-specific oligonucleotide probes labelled with fluorescent reporter dyes, which permits detection only after hybridization of the probe with its complementary sequence of the amplified PCR product.

An internal positive control (IPC) contained in the Master Mix is amplified in parallel and indicates inhibition, if it occurs, thus avoiding false-negative/inhibited results for every individual sample.

The DNAnimal Screen Fish IPC kit is considered as fit for purpose for the following real-time PCR cyclers:

- Agilent AriaMx™
- Agilent Mx3005P™, Mx3000P™
- ThermoFisher Scientific Applied Biosystems™ 7500, ABI 7500 Fast (in standard mode)
- ThermoFisher Scientific Applied Biosystems™ 7900 (ABI7900)

2 PRODUCT INFORMATION

2.1 Components of the Kit

Component	Target	No of tubes	Volume per tube
Master Mix MM QL RT IPC (LR/HR+) • 08.036	Fish	2	1040 µL
Genomic positive control DNA, codfish (10 copies /µL)		2	50 µL
DNA stabilisation buffer (for NTCs)		1	150 µL

Table 1: DNAnimal Screen Fish IPC kit components, excess volume included. The additional ROX dye included in the kit is to be used exclusively for High ROX cycler (addition of 0.45 µL ROX dye per 20µL Master Mix).

Store all reagents at –20°C and protect from light and away from PCR samples or post PCR area.

2.2 Additional Equipment, Consumables and Reagents Required

- Appropriate real-time PCR cycler
- Optical tubes or plates and optical caps or seals
  Please note: for assays on Bio-Rad CFX96 Touch™ we recommend to use original Bio-Rad PCR plates (Hard-Shell® 96-Well PCR Plates, low profile, thin wall, skirted, white/clear)
- 1.5 mL micro-tubes and tube rack
- Vortex mixer
- Centrifuge (1500 × g, preferably refrigerated)
- Centrifuge for micro titre plates (preferably refrigerated)
- Pipette sets and appropriate filter tips
- Powder-free disposable gloves

3 RECOMMENDATIONS AND WORKING PRECAUTIONS

PCR is an exponential reaction. In theory, the detection of a single DNA target should be possible. This extreme sensitivity requires special precautions for handling and equipment. After a successful amplification several billion amplicons are present in the reaction tube. Each of which may lead to a false positive result when contaminating sample material, e.g. when spread as aerosols.

- For DNA extraction use only molecular-biology grade reagents and sterile-filtrated, deionised water (DNA-free) and 0.1x TE buffer for DNA dilutions (Tris-HCl pH=8.0 c=1 mmol/L; EDTA pH=8.0 c=0.1mmol/L).
- Always perform appropriate extraction and PCR controls.
- Maintain separate working areas for DNA preparation, reaction setup, amplifications and reaction product analyses.
- Never transfer amplified products into the reaction setup or extraction area.
- Use filter-plugged pipette tips in order to avoid possible cross-contamination.
- Use only powder-free gloves and change them frequently during the process.
- Clean lab-benches and equipment periodically with DNA decontamination fluid. We recommend Roti Nucleic Acid free.
- To obtain reliable quantification results all pipettes have to be calibrated regularly.

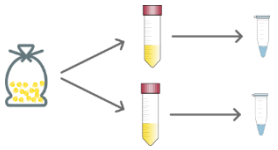
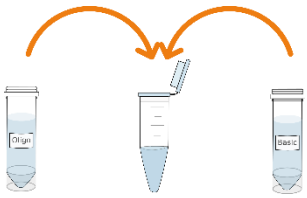
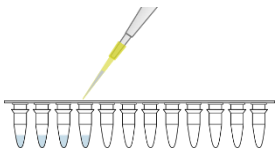
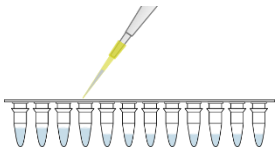
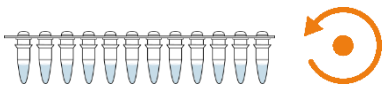
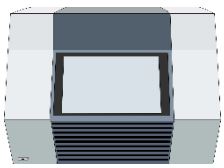
4 PROCEDURAL NOTES

A thorough understanding of this document is necessary for successful use of the product. Reliable results will only be obtained by precise laboratory techniques and accurately following the instructions.

- Store the kit at –20°C until opened for the first time.
- Thaw reagents just before use.
- Do not mix kit components of various kit lots.
- Do not use any component beyond the expiration date of the kit.
- After removing reagents from the refrigerator, allow them to thaw slowly and mix them by vortexing and briefly centrifuge before use.
- Thaw and prepare only as much master mix from the components as needed for PCR.
- Keep the master mix cooled during the working steps. The composed master mix can be stored up to 4 hours in the refrigerator, but must not be used after this period.
- Frequent freezing and thawing might cause inactivation of the reagents. If smaller volumes are needed: aliquot reagents at first use.
- To avoid degradation/inactivation of components immediately start PCR reaction after adding sample DNA to the composed master mix. Do not store the mix with sample DNA.
- Unused reagents should be stored at –20°C. Master mix and positive controls should be mixed by vortexing and briefly centrifuged before use.

5 TEST PROCEDURE

Different approaches and assay formats are possible. Based on a long-term experience, we recommend the workflow outlined below.

1		• Prepare two independent DNA extractions from the unknown sample
2		• Calculate the required Master Mix quantity
3		• Allow the Master Mix reagents to thaw • If applicable: prepare Master Mix by mixing the Basic Mix and the Oligo Mix as calculated below
4		• Vortex and centrifuge carefully
5		• Prealiquot Master Mix: - No template controls (NTC) - Positive control (C+) - Extraction control (EC) - DNA extracts from unknown sample
6		• Add corresponding DNA solution according plate setup
7		• Carefully close all tubes/plates with optical caps/seals • Centrifuge tubes or plate carefully at low speed
8		• Setup PCR cycler • Place tubes or plate into the thermal block of the instrument, close lid and start the run

5.1 Sample DNA for PCR Application of the Replicate

The choice of the appropriate DNA isolation procedure is crucial and depends on the sample type. We recommend the use of either one of our DNA*Extractor* kits or our GENE*Spin* kit for DNA extraction.

We recommend the workflow for the preparation of the DNA extraction replicates for qualitative PCR shown in figure 1. According to DIN EN ISO 21571 (chapter. 5.1.1.) DNA extractions shall be carried out at least on two test portions.

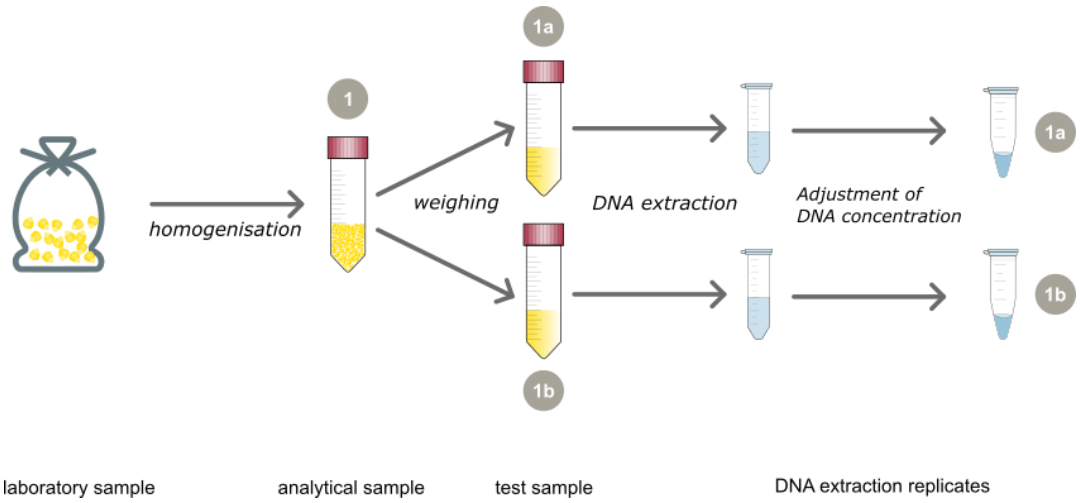


Figure 1: Workflow and terms/definitions for sample preparation and DNA extraction for qualitative analysis.

Absolute DNA amount and purity (meaning: free of PCR inhibiting substances) affect overall sensitivity of the analysis. The use of an insufficient quantity of DNA results in poor LOD with regard to the specific test sample. Insufficiently pure DNA displaying inhibitory effects may even make testing impossible.

Sample DNA (DNA of the replicate) is in general analysed undiluted.

We recommend the DNA quantity listed in the chapter “Recommended DNA Amounts” per undiluted DNA extraction replicate, which would under ideal conditions allow to achieve the limit of detection mentioned in the corresponding validation sheet. In case of the observation of inhibitory effects, we recommend 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 should be avoided. The concentration of the diluted DNA extraction replicates used in the PCR should be measured before the setup of the PCR and further adjusted if necessary.

5.2 Calculation of the Number of Reactions and the Required Volume

PCR is performed in a volume of 25 µL in reaction tubes/plates according to the real-time PCR cyclers' instructions.

Please calculate with approximately 5% reagent excess in order to compensate for the pipetting error.

Reaction Type	**Abbreviations (s. Chapter "Plate Layout")**	**Reaction No per Run**			
		1 sample	**8 samples**	**22 samples**	**45 samples**
DNA extraction replicates (duplicates)	1a / b - 45 a / b	2	16	44	90
NTCs	NTC	2	2	2	2
Extraction control	EC	2	2	2	2
Pos. controls	C+	2	2	2	2
Total number of rxns for calculation of volume		**8**	**22**	**50**	**96**
Calculation of volume	Master Mix volume (total)	160 µL	440 µL	1 mL	1920 mL
	Basic Mix (12.5 µL/rxn)	100 µL	275 µL	625 µL or 1 tube	1200 µL or 2 tubes
	Oligo Mix (7.5 µL/rxn)	60 µL	165 µL	375 µL or 1 tube	720 µL or 2 tubes

Table 3: Number of reactions and volumes of reagents needed for 1, 8, 22 or 45 analytical samples (examples)

5.3 Setup of Reactions

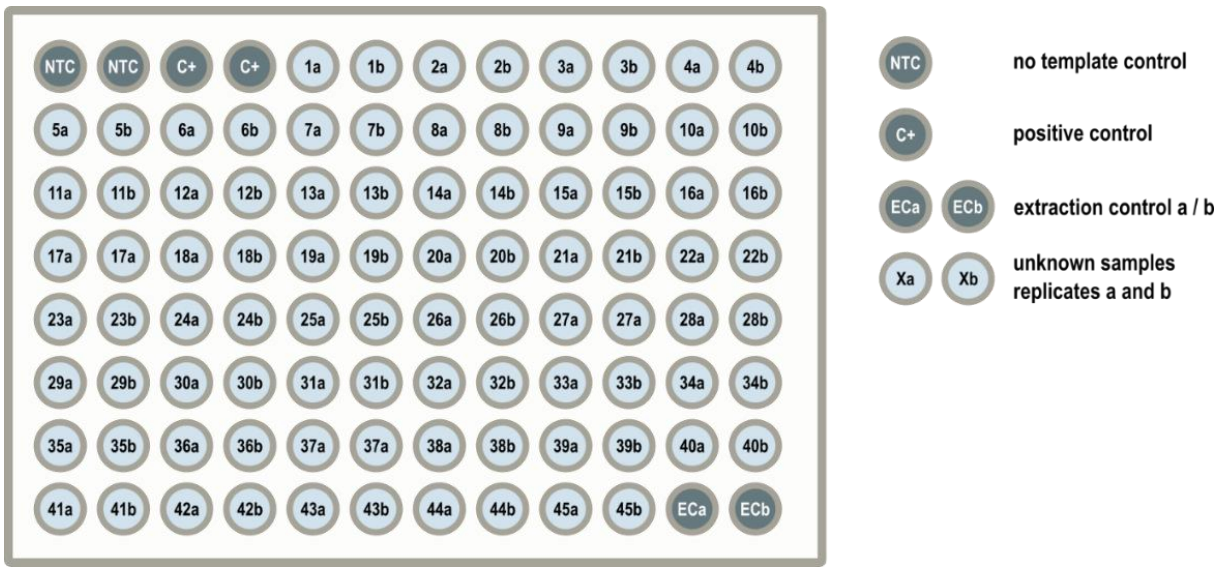
1. Calculate the number of reactions and amount of equivalent Master Mix reagents before thawing the reagents.
2. Vortex and centrifuge the Master Mixes.
   The Master Mixes without template DNA can be stored up to 4 hours in the refrigerator.
3. Add 20 µL of the Master Mix into appropriate tubes or wells of the plate according to the programmed plate document.
4. Add 5 µL of corresponding DNA solution (controls or DNA extraction replicates) and 5 µL of stabilisation buffer for NTCs to the inner side of the tube or well.

We recommend performing an extraction control for each set of samples extracted simultaneously, i.e. a complete DNA extraction without sample material (a blank or empty control), which should subsequently undergo PCR analysis. An extraction control can help as well to examine the reason more closely (e.g., in case of contaminated chemicals) in doubt about the origin of a contamination.

5.3.1 Plate Layout

The following plate layout shows the distribution of reactions for a run. Gold Standard Diagnostics provides a free evaluation (Excel™) sheet that can be requested from your local sales support. If you intend to use this evaluation sheet, please use the setup (adapted to the number of samples required) below.

DNA	Abbreviation	No rxn
No template control	NTC	2
Positive control	C+	2
Extraction control	EC	2
DNA extraction replicate “a”	1a – 45a	1 - 45
DNA extraction replicate “b”	1b – 45b	1 - 45



5.4 Cycler Programming

Cycler	Fish	IPC	Passive reference
Mx3000P™/ Mx3005P™	FAM™ pmt: 1	HEX™ pmt: 1	ROX™ pmt: 1
AriaMx™	FAM™	HEX™	ROX™
ABI 7500 (7500 Fast)*	FAM™ quencher: none	VIC® quencher: none	ROX™
ABI 7900**	FAM™	HEX™	ROX™

Detection channels of targets on different PCR devices
For all target probes: Non-fluorescent quenchers are used.
** ABI 7500: ramping default rate 100%, 7500 Fast: PCR is performed in Standard 7500 run mode*
***ABI 7900: Activate 9600 emulation mode.*

PCR Temperature Profile

	Temperature	Time	No of Cycles
Initial enzyme activation	95°C	10 min	1
Denaturation	95°C	15 sec	45
Annealing & extension	60°C	90 sec	

Additional Information for Species Testing Evaluation Sheet

If you wish to use the species testing evaluation sheet with automated data import, please use the assay detector/ target identifier names given below and follow the instructions on the instruction page of the evaluation sheet:

System	Identifier
Fish	8036a
IPC	8036i

6 EVALUATION

6.1 Cycling Parameters / Instrument Settings

After run completion, data should be evaluated using either the Gold Standard Diagnostics evaluation sheet or by using the appropriate software provided by the cycler manufacturer.
The evaluation (Excel™) sheet can be requested from your local sales support.

Baseline and Threshold Settings

Set baseline and threshold values according to cycler manufacturers' instructions.

We recommend the following settings:

Cycler	Baseline Setting	Threshold Setting
ABI 7500 **ABI 7500 Fast**	Manually cycles 3-15 or automatically	Manually*
Mx3000P™ **Mx3005P™**	Adaptive	Manually*
AriaMx™	Adaptive	Auto**
ABI 7900	Manually cycles 3-15 or automatically	Manually*

Table 4: Threshold and baseline settings, refer to your cycler´s manual for details.

** The threshold should be placed at the beginning of the 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.*
Please note: the threshold should not be placed in the region of background fluorescence or where splitting between replicates may be observed.
*** Check visually, if the threshold is set incorrectly in automatic mode, adjust it manually.*

For raw data export, please follow the instruction in the corresponding cycler analysis software or refer to the Gold Standard Diagnostics evaluation sheet instructions.

7 RESULTS

7.1 Interpretation of Results

Export Cq (=Ct) values to the Gold Standard Diagnostics evaluation sheet, or create an own evaluation sheet following the parameters below. The evaluation (Excel™) sheet can be requested from your local sales support.

Definitions

PCR System	Cq Cut-Offs	Endpoint Fluorescence (EPF)- Limits	Outliers for target and IPC
Fish	cut-off-1: mean Cq (C+) -5 cut-off-2: mean Cq (C+) +10	mean EPF (C+) x 0.20	maximum acceptable outliers (C+): 0 maximum acceptable invalid negative control reactions (NTC): 0
IPC	cut-off-1: mean Cq (NTC*) -3 cut-off-2: mean Cq (NTC*) +3	mean EPF (NTC*) x 0.33	maximum acceptable outliers (NTC*): 0

For more information, please s. chapter "Evaluation of the IPC" and "Evaluation of target"
** NTC = C+ of IPC*

Evaluation of the IPC result considering the lower and upper IPC cut-off and the corresponding EPF limit (inhibition control)

Cq (IPC) and EPF (IPC)	Results
Cq sample ≤ Cq Cut-off 2 and EPF sample ≥ EPF Limit	IPC valid
Cq sample > Cq Cut-off 2 or EPF sample < EPF Limit	IPC invalid
Cq sample < Cq Cut-off 1	IPC invalid
No Cq	IPC invalid

Evaluation of the preliminary target result considering the lower and upper target cut-off and the corresponding EPF limit

Cq (target)	EPF (target)	Preliminary target result
Cq sample ≤ Cq Cut-off 2	EPF sample ≥ EPF Limit	positive
Cq sample ≤ Cq Cut-off 2	EPF sample < EPF Limit	check amplification!
Cq sample < Cq Cut-off1	EPF sample ≥ EPF Limit	check amplification!
Cq sample > Cq Cut-off 2	EPF sample < EPF Limit	negative
Cq sample > Cq Cut-off 2	EPF sample ≥ EPF Limit	positive
No Cq	-	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 the curve is sigmoid, the result is positive; if not, the result is negative.

Evaluation of the final result for one test sample replicate by combination of preliminary target result and IPC result

Preliminary target result	IPC result	Final result
positive	valid	positive
positive	invalid	positive
negative	valid	negative
negative	invalid	inhibited

Note: If the preliminary target result is positive, the IPC result is irrelevant. The target result remains positive.

7.2 Procedure

Evaluation of the IPC

- Calculate the mean Cq value of the IPC from the reactions containing DNA stabilisation buffer instead of DNA (NTC). Refer to data from IPC-detector.
    - To calculate the Cq cut-off-1, subtract 3 Cq
    - To calculate the Cq cut-off-2 add 3 Cq.
- Calculate the mean endpoint fluorescence (EPF) value from NTC. Refer to data from IPC-detector.
    - The endpoint fluorescence limit is 33 % of the mean endpoint fluorescence.

Evaluation of fish

- Calculate the mean Cq value of the positive control.
    - To calculate the Cq cut-off-1, subtract 5 Cq,
    - To calculate the Cq cut-off-2 add 10 Cq
- Calculate the mean endpoint fluorescence value from positive control. Refer to data from target detector.
    - The endpoint fluorescence limit is 20 % of the mean endpoint fluorescence.

7.3 Ambiguous Results

If independently extracted replicates 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 target concentration close to the LOD.

7.4 Limit of Detection

For validation data as LOD_{abs} and $LOD_{rel,}$ please refer to the corresponding validation sheet.

The limit of detection for the analysis of individual samples depends on several factors: matrix properties, 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 depending strongly on the type of sample and the DNA extraction procedure.

Quantification of the target is not possible with this kit.

8 TROUBLESHOOTING

Result	**Possible 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. Too much DNA inhibits the PCR. Check dilution.
No PCR signals from positive controls	Wrong PCR program. Wrong or no positive DNA was used.	Check and correct PCR program. Repeat with a new aliquot of positive control DNA.

**Repeat extraction of DNA from the sample. If necessary, clean DNA further e.g. with our DNA cleaning columns.*

9 REFERENCES

[1] Validation Sheet DNAnimal Screen Fish IPC, module GS-P-08.036

[2] Gold Standard Diagnostics Evaluation (Excel™) Sheet for qualitative testing assays (contact your local sales support)

10 PRODUCT WARRANTIES, SATISFACTION GUARANTEE

Gold Standard Diagnostics Budapest ("GSDB") warrants the products manufactured by it will be free of defects in materials and workmanship when used in accordance with the applicable instructions before the expiration date marked on the product packaging and when stored under the storage conditions recommended in the instructions and/or on the package. GSDB makes no other warranty, expressed or implied. There is no warranty of merchantability or fitness for a particular purpose.

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11 IMPORTANT NOTES

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12 VERSION CONTROL

Version	Date	Changes
V5.1	13.12.2022	• Adaptation to Gold Standard Diagnostics layout for DNAnimal • Change of contact address • Deletion of the chapter "Product Use Limitations" • Add chapter "Version Control" • Change of file name • No product or assay-specific changes

TECHNICAL SUPPORT SERVICE

For technical assistance and more information, please contact Gold Standard Diagnostics Budapest's customer service or your local distributor.

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V5.1 / 13.12.2022