GMO*Ident* RT (IPC)
Accelerated Growth Salmon

Test kit for qualitative event-specific real-time PCR detection of AquAdvantage® Salmon

Cat. No. 5421230601

Includes reagents and control DNA for 96 real-time PCR reactions

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13. TECHNICAL SERVICE

If you have any questions or experience any difficulties regarding the GMO*Ident* Kit or Eurofins GeneScan Technologies 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 Technologies. 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 your local distributor.

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

GMO*Ident* RT (IPC) Accelerated Growth Salmon

Kit for the qualitative event-specific real-time PCR detection of DNA from AquAdvantage® Salmon

1. Introduction

The GMO*Ident* RT (IPC) Event Accelerated Growth Salmon kit is a qualitative real-time PCR kit for detection of the genetically modified salmon AquAdvantage® which shows an accelerated growth and was developed by the company Aquabounty.

The method utilized in this kit uses the PCR system described in the method published by Debode et al.:

http://www.euginius.eu/euginius/pages/gmo_method_listview.jsf;jsessionid=5EA7464373FB770B3175B2A0B3AC44C7?gmoname=AquAdvantage+Salmon

with an additional internal positive control (IPC). The GMO*Ident* RT (IPC) Accelerated Growth Salmon test kit employs a real-time PCR system validated for either Applied Biosystems® 7500/Fast or AgilentMx3005P/ Mx3000P™ or Bio-Rad CFX96 Touch™.

GMO*Ident* RT (IPC) Accelerated Growth Salmon is part of Eurofins GeneScan Technologies' comprehensive product lines for screening, identification and quantification of genetically modified organisms, called GMO*Screen*, GMO*Ident,* and GMO*Quant*.

1.1 Test Principle

The first step in the detection and identification of GMO salmon is the isolation of DNA from samples. The choice of the appropriate DNA isolation procedure is crucial and depends on the sample type.
We recommend the use of either our DNA*Extractor* kits (cat. no. 5224700610, -710) or our GENE*Spin* kit (cat. no. 5224400605), combined with our DNA Cleaning Columns (cat. no. 5224400310).

The second step involves the detection of a specific DNA sequence by amplification with the real-time Polymerase Chain Reaction (RT-PCR).
The GMO specific reaction amplifies a DNA sequence which is specific for AquAdvantage® Salmon.
A signal in the RT-PCR indicates that GM material is present in the sample.

The IPC (internal positive control). PCR system is used for heterologous detection of inhibitory effects.

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2. Components of the Kit

The GMO*Ident* RT (IPC) Accelerated Growth Salmon kit contains reagents for a total of 96 PCR reactions including controls:

MasterMix QL RT IPC (LR) GS-11.002, Accelerated Growth Salmon:

Mix prior to use in empty vial!

2x - 1 mL composed of BasicMix (BM) and OligoMix (OM) as follows:

- 650 µL BM (NR)
  GS-P-26.012 • EFGi TP 2x (NR) 1.5U
- 390 µL OM QL RT IPC (LR) •
  GS-P 11.002 • GH2/AFP- G/T-D

2x **Positive control DNA**:
50 µL plasmid DNA pGSE682 (10 copies/µL)

1x **DNA stabilization buffer**:
150 µL (for NTCs)

Important Note:
Store all reagents light protected at –20°C.
Never store components of the kit together with samples or amplicons. Never store or use them in areas where PCR or gel electrophoresis are performed.

3. 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, aliquot reagents at first use.
Refer to the reagent label for specific instructions regarding the correct storage.

4. Material and Equipment not included in the Kit

– water, DNase-free
– Vortex mixer
– Micropipettes (variable 1-1000 µL) and filter tips
– PCR optical tubes or plates 0.2 mL and optical caps or seals

– Agilent Mx3000P™ /Mx3005P™, Bio-Rad CFX96 Touch™ , Applied Biosystems® 7500/Fast with required filter sets.

5. Sample Preparation

5.1 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. To achieve a limit of detection of 0.01%, 62.2 ng of salmon DNA per reaction must be analysed. In case inhibitors are present in the DNA, dilution of the sample DNA is feasible. Dilution factors depend on the degree of inhibition and on the total DNA amount. Excessive

dilution will compromise the practical LOD (pLOD) and should be avoided.
Each sample should be treated in duplicate.
(Two independent DNA extracts yielding an “a” and a “b” sample DNA are taken. Each DNA extract is analysed with a single determination.)
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.

6. PCR

6.1 Special precautions during PCR analysis

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

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The 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 or at least dedicate different 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 GMO*Ident* Kits and materials for DNA extraction together with samples or amplicons.

Always perform extraction controls and PCR controls (NTCs).

6.2 General Information

PCR is performed in a final volume of 25 µL in 0.2 mL reaction tubes/plates according to the RT-PCR cycler instructions.

For setup and evaluation information for your individual cycler, please send an email to kits@eurofins.com or see our website www.eurofins.com/kits.

6.3 PCR Setup

6.3.1 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.

20 µL MasterMix for low ROX (LR) cyclers consist of 12.5 µL BasicMix + 7.5 µL OligoMix per reaction.

The composed MasterMix can be stored up to 4 hours in the refrigerator, but must not be used longer.

The following reactions are required for a RT PCR run:
Add approx. 5% of volume as pipetting error compensation or use complete tubes.

Samples and extraction controls	1	8	23	46
NTCs	2	2	2	2
Pos. controls	2	2	2	2
Samples/extraction controls (duplicates)	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

Table 3: Number of reactions and volumes of reagents needed for 1, 8, 23 or 46 samples (incl. extraction controls).

1. Thaw only the volumes of BasicMix and OligoMix needed for analysis. Shake thoroughly.
2. Remove the required volumes and transfer into a fresh tube. Freeze the rest.
3. Mix thoroughly.

6.4 Plate Setup

The following plate document (table 4) shows a possible distribution of 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	ECa	ECb

Table 4:
Plate layout for 45 samples plus extraction control (EC);
NTC = no template control; C+ = positive control;
a and b = DNA extract duplicates

Every run requires 2 no template controls (NTC) and 2 positive controls (C+). It is highly recommended to test the samples in duplicate.

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6.5 Programming of the cycler

The GMOIdent RT (IPC) Accelerated Growth Salmon was validated for Agilent Mx3000P™ /Mx3005P™, Bio-Rad CFX96 Touch™, Applied Biosystems® 7500/Fast.

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 and to our Application Notes for use of our kits on your cycler (www.eurofins.com/kits).

For the plate template, you may use our templates for ABI 7500, CFX96 Touch™ and Agilent Mx3000P™ /Mx3005P™ (send an e-mail mentioning your cycler model to kits@eurofins.com to receive a copy) or program your own template with the following settings.
If you wish to use the Eurofins GeneScan Technologies evaluation sheet with automated data import, please use the detector/assay names given below.

ABI 7500 (7500 Fast)

System	Detector	Reporter	Quencher
GHc/AFP-G/T-D	1102a	FAM™	NONE
IPC	1102i	VIC™	NONE

Passive Reference: ROX

Agilent/Stratagene Mx3005P (3000P)

System	Detector	Filter
GHc/AFP-G/T-D	1102a	FAM™/SYBR® Green I
IPC	1102i	HEX™/JOE™/VIC™
ROX	ROX	Texas Red®/ROX™

The fluorescence signals are scanned in the following order: (1) ROX™, (2) FAM™, (3) HEX™.

Bio-Rad CFX96 Touch™

System	Detector	Reporter
GHc/AFP-G/T-D	1102a	FAM™
IPC	1102i	VIC™

A reference dye is not used.

6.6 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

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

ABI7500
Ramping : Default rate, 100%

Bio-Rad CFX96 Touch™
Ramping : Default maximum rate

7. RESULTS

7.1 Evaluation

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

Threshold:

ABI 7500/7500 Fast and Agilent Mx 3005/3000P™:
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.

Bio-Rad CFX96 Touch™:
Auto calculated threshold can be used but should be checked visually.

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Baseline:

ABI 7500/7500 (Fast): Set manually, 3-15

Agilent Mx3005/3000P™: Adaptive

Bio-Rad CFX96 Touch™:
Baseline Subtracted Curve Fit and Fluorescence Drift Correction should be applied.

For more details, please see our Application Notes for use of our kits on your cycler (www.eurofins.com/kits).

7.2 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

Threshold cycle (Ct/Cp) Cut-offs	**GHc/AFP-G/T -D** Cut-off-1: Mean Ct (C+) -10 **IPC** Cut-off-1: Mean Ct (NTC) - 3 **GHc/AFP-G/T-D** Cut-off-2: Mean Ct (C+) +8 **IPC** Cut-off-2: Mean Ct (NTC) +3
Endpoint fluorescence (EPF)- Limits	**GHc/AFP-G/T-D**: Mean EPF (C+) x 0.2 **IPC:** Mean EPF (NTC) x 0.33
Outliers	Maximum acceptable outliers (C+): 0 Maximum acceptable outliers (NTC): 0

Inhibition control, scoring of IPC

Threshold cycle (Ct/Cp) = Ct and Endpoint fluorescence = EF	**Results**
Ct_{IPC} Cut-off-1 ≤ Ct_{IPC} sample ≤ Ct_{IPC} Cut-off-2 **and** EPF_{IPC} sample ≥ EPF_{IPC} Limit	Sample valid (not inhibited)
Ct_{IPC} sample < Ct_{IPC} Cut-off-1 **and** EPF_{IPC} sample ≥ EPF_{IPC} Limit	Check amplification!
Ct_{IPC} sample > Ct_{IPC} Cut-off-2 **or** EPF_{IPC} sample < EPF_{IPC} Limit	Sample inhibited
No Ct_{IPC}	Sample inhibited
***Note*:** It is generally recommended to check the sample amplification curves for sigmoid amplification. In case of a Ct/ Cp value of 40.0 or "Check amplification!" the linear scale amplification plot must be checked critically for presence or absence of a sigmoid PCR amplification signal. If a sigmoid curve is observed, the sample is valid; If not, the sample is inhibited.	

Test reaction GMO

Threshold cycle (Ct/Cp) =Ct	**Endpoint fluorescence dRn/dR= EPF**	**Result**
Ct_{GMO} Cut-off-1 ≤ Ct_{GMO} sample ≤ Ct_{GMO} Cut-off-2	EF_{GMO} sample ≥ EF_{GMO} Limit	Reaction positive
Ct_{GMO} sample ≤ Ct_{GMO} Cut-off-2	EF_{GMO} sample < EF_{GMO} Limit	Check amplification!
Ct_{GMO} sample < Ct_{GMO} Cut-off-1	EF_{GMO} sample ≥ EF_{GMO} Limit	Check amplification!
Ct_{GMO} sample > Ct_{GMO} Cut-off-2	EF_{GMO} sample ≥ EF_{GMO} Limit	Reaction positive
Ct_{GMO} sample > Ct_{GMO} Cut-off-2	EF_{GMO} sample < EF_{GMO} Limit	Reaction negative
No Ct_{GMO}	-	Reaction negative
***Note*:** In case of a Ct value of 40.0 or "Check amplification!" the linear scale amplification plots must be carefully checked for presence or absence of a sigmoid PCR amplification signal. If a sigmoid curve can be observed, the sample is positive; if not, the sample is negative.		

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Final result from combination of inhibition control and test reaction

IPC	**GMO**	**Final result**
Sample valid	Reaction positive	Positive
Sample valid	Reaction negative	Negative
Sample inhibited	Reaction positive	Positive
Sample inhibited	Reaction negative	Inhibited

Evaluation of the IPC
Calculate the MEAN Ct/Cp Value from NTC. Refer to data from IPC-detector. To calculate the Ct cut-off-1, subtract 3 Ct/CP, to calculate the Cut-off-2 add 3 Ct. Calculate the MEAN Endpoint fluorescence (EPF) value from NTC. Refer to data from IPC-detector. The Endpoint fluorescence cut-off is 33% of the MEAN Endpoint fluorescence.

Evaluation of GMO
Calculate the MEAN Ct/Cp Value from positive Control. Refer to data from Accelerate Growth Salmon (GHc/AFP-G/T-D) detector. To calculate the Ct/Cp cut-off-1, subtract 10 Ct/Cp, to calculate the Cut-off-2, add 8 Ct/Cp.

Calculate the MEAN Endpoint fluorescence value from positive control. Refer to data from Accelerate Growth Salmon detector. The Endpoint fluorescence cut-off is 20% of the MEAN Endpoint fluorescence.

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 GMO concentration close to the LOD.

8. Limit of detection, quantification

The absolute limit of detection (LOD_{abs}) for the method has been validated as ≤ 10 copies of Accelerated Growth Salmon 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.

9. PRODUCT USE LIMITATIONS

The Eurofins GeneScan Technologies GMO*Ident* 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 the materials described in this text.

10. Troubleshooting

Result	Possible mistakes/reasons	Possible verification and measures
No PCR signal from IPC in sample	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, not even from positive controls	Wrong PCR program MasterMix not properly prepared	Check and correct PCR program. Check and correct MasterMix preparation.

* 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).

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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 for a period equal to or shorter of one year from the date of shipment of the product(s) or 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 also provided in our price lists.

12. Important Notes

- The PCR and 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 this process. Use of these products is recommended for persons that either have a license to perform PCR 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.