

Tabel 8 - Negative results for Target and IC

POSSIBLE CAUSES	SOLUTIONS
The PCR conditions do not comply with the protocol	Check the PCR conditions (see above) and repeat PCR with correct settings, if necessary
The PCR was inhibited	Follow the manufacturer's instructions of the DNA extraction method and/or diluted the sample (1:10)
The reagents storage conditions didn't comply with the instructions	Check the storage conditions and the expiration date (see the kit label) of the reagents and use a new kit, if necessary

If additional support is needed, please contact BPMR.

15. PERFORMANCE CHARACTERISTICS

15.1. Specificity

No amplification signal was observed for the 16 fish food samples of *Gadus morhua* (Atlantic cod) and *Gadus chalcogrammus* (synonym: *Theragra chalcogramma*, Alaska pollack) species. No false positives were obtained, either in the right conditions or in the deviations used for Robustness, decreasing the stringency and increasing PCR reagents.

15.2. Analytical Sensitivity





Serially diluted *Gadus macrocephalus* DNA was performed in order to evaluate the Limit of Detection (LoD). The LoD was 100pg of target DNA, thus, the method can detect 0.2% of *Gadus macrocephalus* DNA in 50 ng of total DNA. No false negatives were obtained, either in the defined PCR conditions or in the deviations used for Robustness, increasing the stringency.




16 REFERENCES

¹ Lockley AK, Bardsley RG, 2000. Trends Food Sci. Technol., 11: 67-77.

² Tetzlaff C & Made D, 2017. European Food Res. Technol., 243: 849-857.

17. EXPLANATION OF SYMBOLS

 Expiry Date
 Batch Code
 Catalogue Number
 Temperature Limit

 Consult Instructions for Use
 Contains sufficient for <n> tests
 Manufacturer

BPMR – Production and Development, Lda.

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SUPREME REAL TIME DETECTION KIT Pacific cod (*Gadus macrocephalus*)

TABLE OF CONTENTS

1 Fish Information	10 Assay Principle
2 Intended Use	11 Contents
3 Trademarks and Disclaimer	12 Experimental Protocol
4 Limited License Agreement	13 Data Analysis
5 Storage Conditions and Stability	14 Troubleshooting
6 Quality Control	15 Performance Characteristics
7 Warnings and Precautions	16 References
8 Materials required but not provided in the product	17 Explanation of Symbols
9 Equipment required but not provided in the product	

1. FISH INFORMATION

According to the European Commission Directive 2002/86/EC, food ingredients have to be declared. The fish species must be disclosed on the label with both commercial and scientific denominations in order to verify the origin and traceability of the fish and to analyze the quality control of handling and cleaning processes of production lines.

Methods for species detection based on the identification of proteins are not reliable for application in highly processed and heated products due to protein deterioration. DNA is more stable than proteins during processing¹. Real time PCR techniques are suitable for amplify small fragments of DNA high sensitivity and specificity².

2. INTENDED USE

The SUPREME REAL TIME DETECTION KIT Pacific Cod (*Gadus macrocephalus*) enables a qualitative detection of Pacific Cod DNA in food samples by Real Time PCR, after a sample processing step. This DNA is present in the mitochondrial genome and it may be found in commercial seafood and processed fishery. The test may be used with raw or processed cooked meal fish and can detect 0.2% of *Gadus macrocephalus* DNA in 50 ng of total DNA

3. TRADEMARKS AND DISCLAIMER

ABI PRISM® 7500 Fast

Registered names, trademarks, etc. used in this document, even when not specifically marked as such, are not to be considered unprotected by law. BPMR kit handbooks and user manuals can be requested from BPMR or your local distributor.

4. LIMITED LICENSE AGREEMENT

Use of this product signifies the agreement of any purchaser or user of the SUPREME REAL TIME DETECTION KIT Pacific Cod (*Gadus macrocephalus*) to the following terms:

- The kit must be used solely in accordance with the respective Instructions for Use. BPMR grants no license under any of its intellectual property to use or incorporate the enclosed components of this Kit with any components not included within this Kit except as described in the Instructions for Use.
- This Kit and its components are licensed for one-time use and may not be reused, refurbished, or resold.

5. STORAGE CONDITIONS AND STABILITY

The kit must be stored at -20°C. The kit must be shipped refrigerated, using coolers, and should be stored immediately on receipt. Repeated thawing and freezing should be avoided, as this may reduce the sensitivity. It should be protected from exposure to light. The kit is stable until the expiration date stated on the outer package and on the labels of the vials. Components stored under conditions other than those stated on the labels may not perform properly and may adversely affect the assay results.

6. QUALITY CONTROL

Each lot of the kit is tested against predetermined specifications to ensure consistent product quality.

7. WARNINGS AND PRECAUTIONS

- These products are exclusively for *in vitro* use.
- Molecular Biology procedures, such as DNA extractions and PCR amplification, require qualified staff to prevent the risk of erroneous results, especially due to sample contamination or degradation of the nucleic acids contained in the samples.
- It is strongly recommended to have dedicated areas, materials and equipment for the DNA extraction, preparation of the PCR and post-PCR procedures. Workflow in the laboratory should proceed in a uni-directional manner, from the Extraction Area to the Amplification and Detection Area. Do not return samples, equipment and reagents in the area where you performed previous step.
- The user should always pay attention to the following:
 - Read all the instructions provided with the product before running the assay.
 - Do not mix reagents from different batches.
 - Do not use reagents from other manufacturer's products.
 - Wear disposable gloves, laboratory coats and eye protection when handling specimens and reagents.

- Use sterile pipette tips with filters.
 - Store and extract positive material (specimens, controls and amplicons) separately from all other reagents and add it to the reaction mix in a spatially separated facility.
 - Thaw all components thoroughly at room temperature before starting an assay. When thawed, mix the components and centrifuge briefly.
 - Avoid contact of specimens and reagents with the skin, eyes and mucous membranes. If these solutions come into contact, rinse immediately with water and seek medical advice immediately.
5. Material Safety Data Sheets (MSDS) are available on request.
6. Waste must be treated and disposed of in compliance with the appropriate safety standards.

8. MATERIALS REQUIRED BUT NOT PROVIDED IN THE KIT

Disposable powder-free gloves
DNA extraction kit (Ex: Extraction Kit from Food, ref: BIOPEXT-0609)
Sterile pipette tips with filters
Tubes/Strips and accessories specific for the Real Time PCR Instrument
PCR grade water
optional: DNA Standard SUPREME Pacific cod (quantitative results) (ref: DNA0057)

9. EQUIPMENTS REQUIRED BUT NOT PROVIDED IN THE PRODUCT

Laminar Air Flow Cabinets/PCR Cabinets Micropipettes
Microcentrifuge
Real Time PCR instrument - channels for FAM (520 nm) and ROX (610 nm)
Freezer, refrigerator

10. ASSAY PRINCIPLE

The kit was designed comprising two reactions: a duplex PCR reaction, allowing the simultaneous detection of the *Gadus macrocephalus* DNA and an Internal Control (IC). The IC is used for evaluation of PCR inhibitors in the sample, or for evaluation of problems occurred during PCR preparation/amplification.

The kit includes Master-Mix containing primers and TaqMan® probes, labelled with non-fluorescent quenchers, Positive Control, Internal amplification Control, Uracil-DNA Glycosylase (UDG) to prevent DNA contamination with PCR products.

The signal for the detection of the targets is detected in the FAM channel, and the one for the IC in the ROX channel.

11. CONTENTS

Table 1 - Components provided by the kit.

CONTENTS	COMPOSITION	UNITS	REACTIONS/ KIT
Mix (Blue Cap)	Buffer, dNTPs, Primers and Probes	1 tube 2 tubes	10 reactions 100 reactions
Positive Control (Red Cap)	Target DNA	1 tube	10 reactions 100 reactions
Enzymes (White Cap)	DNA Polymerase + UDG	1 tube	10 reactions 100 reactions
Negative Control (Clear Cap)	Nuclease free water	1 tube	10 reactions 100 reactions

12. EXPERIMENTAL PROTOCOL

12.1. Preparation of PCR reactions

Thaw the Master-Mix, Control Positive and samples DNA. Vortex and centrifuge briefly the tubes. Prepare a PCR mix reaction in a 1,5mL tube, as described in Table 2. To calculate the volume of reagents, we recommended take in account the number of samples (at least in duplicate), positive control and negative controls*, plus 10% (e.g. for 10 samples, prepare a volume for 11). Mix thoroughly and centrifuge briefly.

* We recommended using a PCR negative control for each PCR run (this tube contains no DNA but all reagents) and an extraction negative control for each run of extraction carried out (in this tube no sample is added and is submitted to the same extraction process as the other samples).

Table 2 - Pipetting Scheme for the preparation of PCR reactions

REAGENTS	NUMBER OF SAMPLES	
	1	10 (10 + 1)
Mix	13 µL	143 µL
Enzymes	2 µL	22 µL
Total	15 µL	165 µL

- Add 15 µL of the PCR mix reaction into each PCR tube/well.
- Add 5 µL of DNA sample at 10 ng/µL; 5µL of DNA Positive Control and 5 µL of Negative Controls into the appropriate PCR tubes/wells.
- Close the tubes/strips, and centrifuge briefly.
- Optional (not included): Prepare the DNA Standard SUPREME Pacific cod (DNA0057).
- Place the reactions into the Real Time PCR instrument.

12.2. Programming the Instrument

The following instruction must be followed in order to setup the amplification program

- Reagents – TaqMan®, FAM and ROX Reporters, MGB Quenchers
- Ramp speed – Standard
- Reaction Volume – 20 µL
- Passive Dye Reference – None

Table 3 - Probes information

REACTION	TARGET	SELECTION OF CHANNELS
Pacific cod	Detection of Pacific cod DNA	FAM
	Internal Control (IC)	ROX

Table 4 - PCR program:

PHASE	TEMPERATURE	TIME	ACQUISITION MODE	CYCLES
Incubation	37°C	15 min.	No	1x
UDG Inactivation	95°C	5 min.	No	1x
DNA Amplification	94°C	15 sec.	No	40x
	60°C	30 sec.	Collect Data on hold	
	72°C	15 sec.	No	

13. DATA ANALYSIS

Positive Control: The result must always be positive for both FAM (amplification of target DNA) and ROX (amplification of Internal Control (IC)) channels. A sigmoid curve should be observed with Ct values ranging from 25 to 35.

Negative Controls: Amplification must be detected only in the ROX (amplification of IC) channel. The experience should be considered invalid and the assay repeated, if a positive result in the FAM channel was observed. A positive result indicates a possible contamination occurred during the DNA extraction and/or PCR preparation.

Samples: A sample is stated positive for *Gadus macrocephalus*, if amplification in the FAM channel was observed. A sample is stated negative for *Gadus macrocephalus*, if amplification in the FAM channel was not observed and if the amplification was observed in the ROX (amplification of IC) channel.

A negative result in the ROX channel and a negative result in the FAM channel indicates the presence of inhibitors in the sample. The PCR reaction must be repeated. It should be noted that, a negative result in the ROX channel could occur when a high concentration of the target DNA (in the FAM channel) is detected, because the PCR reagents are exhausted before amplification of the IC begins.

Table 5 - Interpretation of Data

SAMPLE RESULTS		INTERPRETATION OF RESULTS
Target (FAM channel)	IC (ROX channel)	
-	+	Negative (No target DNA detected)
-	-	Invalid (PCR inhibitors in the sample)
+	+	Positive (Target DNA detected)
+	-	Positive (High amount of target DNA)

	CONTROL RESULTS		INTERPRETATION OF RESULTS
	Target (FAM channel)	IC (ROX channel)	
Positive control	+	+	Expected result
	-	-	Invalid (PCR amplification failure*)
PCR negative control	+	+	Invalid (PCR contamination*)
	-	+	Expected result
Extraction negative control	+	+	Invalid (Extraction contamination*)
	-	+	Expected result

* Repeat the assay and/or the DNA extraction

14. TROUBLESHOOTING

Table 6 - Negative results in Positive control

POSSIBLE CAUSES	SOLUTIONS
Error in the preparation of the reaction mixture	Check the instructions for use and pipetting scheme for the preparation of the reaction mixtures (Table 2)
Dispensing error on the tubes/strips	Pipette carefully the PCR reactions Check the volumes of reaction mixture dispensed Check the volumes of positive control dispensed
Instrument setting error	Check the thermocycler settings on the instrument (Table 4) Check if the selected fluorescence channels for analysis are according to the instructions (Table 3) Check the position of the positive control reaction on the block of the instrument
The reagents storage conditions didn't comply with the instructions or the kit has expired	Check the storage conditions and the expiration date (see the kit label) of the reagents and use a new kit, if necessary

Table 7 - Positive results in Negative Controls

POSSIBLE CAUSES	SOLUTIONS
Contamination during PCR preparation	Repeat the PCR preparation Close the tubes/ wells directly after addition of the samples to be tested Pipette the Positive Control at last Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol or special DNA decontamination reagents
Contamination during DNA extraction preparation	Repeat the DNA extraction procedure Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol or special DNA decontamination reagents