**TITLE:** Identification of Atlantic cod (*Gadus morhua*), ling (*Molva molva*) and Alaska pollock (*Gadus chalcogrammus*) by PCR-ELISA using duplex PCR

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**ABSTRACT**

Species-specific PCR-ELISA assays for the identification of Atlantic cod (*Gadus morhua*), Alaska pollock (*Gadus chalcogrammus*) and Ling (*Molva molva*) in food products have been developed. The method, comprising a set of primers common to the first two species, a set of primers for *Molva molva* and a probe for each species, was designed using *ND4* and *cytochrome b* genes as molecular markers. The sensitivity and selectivity were then determined for each assay. These assays were afterwards used to analyze DNA extracted from commercial fish products. The presence of the target species was successfully detected in all analyzed samples, demonstrating the applicability of this method to the analysis of food products.

**KEYWORDS**: *Gadus chalcogrammus*; *Gadus morhua*; *Molva molva*; Species identification; PCR-ELISA

## INTRODUCTION

The Gadidae family includes a large number of species, many of them of great economic value, as *Gadus morhua* which is marketed worldwide. However, due to several factors such as overfishing and climate change, some natural cod stocks are threatened and the total world catch is less than 1 million tons, while in 1970 was 3.5 million tons[1].

Gadoid species can be identified in a relatively simple manner based on their morphological characteristics in fresh and unprocessed fish, however they are usually commercialized with some degree of processing, that being, frozen, gutted, filleted, smoked, canned, etc. Therefore morphological characteristics that differentiate the species are, in many cases, absent. This situation favors that some species are eventually replaced by others species, either accidentally or intentionally. This produces mislabeled fishery products which impacts directly or indirectly on the consumer, environment and industry. These consequences include human health risks [2-4], overexploitation and depletion of some of the most consumed and valued species by consumers[5-7.] Other consequences are the economic losses for the consumers[5] due to the addition of undeclared cheaper fish species or the sell of species that have a defined fishing quota, and are captured over them, under the name of other species. For all this legislation is needed to control the correct labeling of fishery products[8-13]. European Union labeling regulations (EC No 13/2000) specify that the commercial and scientific names should be included on the label of seafood products.

From this point of view, molecular DNA based techniques are the most suitable option as an alternative to morphological analysis. Different genetic authentication techniques based on DNA have been proposed during the last decades for gadoids identification: Polymerase Chain Reaction (PCR) [13, 14], (PCR)-Restriction Fragment Length Polymorphism (RFLP) [16, 17], Real-time PCR [15,18-21], Forensically Informative Nucleotide Sequencing (FINS) [22], Single-Stranded Conformation Polymorphism analysis (SSCP) [23] and Single Nucleotide Polymorphism (SNPs)[24].

One of the advantages of PCR-ELISA methodology is that it can be applied to fresh, frozen or processed products, and that it only requires a conventional PCR system (thermo-cycler), since the results can be evaluated with the naked eye. This approach has been used in the detection of viruses [25], bacteria [26-28] and fish [29, 30].

The main objective of the present study was to develop a specific molecular technique to identify *G.morhua*, *G.chalcogrammus* and *M.molva* in seafood products.

## MATERIALS AND METHODS

### *Fish samples*

Thirteen specimens of each of the Gadiformes target species were collected: *Gadus morhua, Gadus chalcogrammus* and *Molva molva*.

In addition 20 representative specimens of other Gadiformes, Lophiiformes, Perciformes and Cupleiformes orders were included in this study. Scientific and common names of these species are listed in Table 1.

During the validation step of the developed methodology, 19 commercial cod samples were used, purchased in different local markets (Table 2). All samples were analyzed in triplicate.

### *DNA extraction*

DNA was extracted from 0.3 g of thawed muscle and tissue of commercial samples which were digested overnight in a thermo shaker at 56ºC with 860 µL of lysis buffer (1% SDS, 150 mM NaCl, 2 mM EDTA, and 10mMTris-HCl at pH8), 100 µL of 5M guanidium thiocyanate (Sigma-Aldrich), and 40 µL of proteinase K (20 mg/ml) (Gibco Invitrogen, LifeTechnologies). After 3 h, extra proteinase K (40 µL) was added to the solution, and it was left overnight. After digestion, DNA was isolated employing the Wizard DNA Clean-Up System kit (Promega) following the manufacturer's instructions. DNA was quantified by NanoDrop 2000 Spectrophotometer (Thermo Scientific) at 260 nm and the ratio 260/280, respectively. The 260/280 ratio was between 1.8 and 2.0. DNA concentration was adjusted to 50 ng/µL. The purified DNA was stored at -20 ºC.

### *Design of PCR-ELISA systems*

An extensive number of partial DNA sequences (nuclear and mitochondrial), and a wide number of fish species were analyzed (data not shown) during the study.

Two PCR systems were designed to amplify two mitochondrial fragments: a small fragment of *cytochrome b* gen and a fragment of *ND4* gene. Specific sets of primers were designed for each system to include the previously identified polymorphisms among the target species and other

77 gadoid using Oligo Analyzer v. 1.0.3 (Freeware, Teemu Kuulasmaa, Finland). The 5' biotin-
78 labeled probes Gmor_ND4-P, Gchal_ND4-P and Mmol_Cytb-P were designed to hybridize with
79 the specific polymorphism to *G.morhua*, *G.chalcogrammus and M.molva* PCR amplification
80 products, respectively (Figure 1). HPLC purification grade primers and probes were synthesized
81 by *Sigma Genosys*. Primer and probe sequences designed and used are listed in Table 3.

82 ***PCR amplification and DIG labeling conditions***

83 Primers and magnesium chloride concentrations were optimized to perform PCR DIG-labeling
84 reactions in a final volume of 25 µL containing: molecular grade water, 1.5mM final
85 concentration magnesium chloride (Bioline), DIG dNTPs (Roche) final concentrations of 200 µM
86 of each of dATP, dCTP, dGTP, 190 µM dTTP, 10 µM DIG-11dUTP, primers T/G_ND4-F and
87 T/G_ND4-R (final concentration 0.24µM), primers Mmol_Cytb-F and Mmol_Cytb-R (final
88 concentration 0.36 µM), BIOTAQ™ DNA polymerase (final concentration 0.5 *U*), and 50ng of
89 DNA template. Thermal cycling parameters were as follows: 95°C for 5 min; followed by 25
90 cycles of 95°C for 20 s, 63°C for 30 s, 72°C for 30 s; and a terminal extension step of 72°C for 5
91 min. Negative controls (molecular grade water) were included in each set of reactions. These
92 PCR reactions were carried out in an Applied Biosystems 2720 thermocycler.
93 The amplification products were tested in a 2% agarose gel (*Pronadisa*),
94 containing RedSafe™ 1X (*iNtRON Biotechnology*) in 0,5X TBE buffer (*Sigma*). DNA fragments
95 were visualized using the Gel Documentation System Gel Doc XR System and the software
96 Quantity One® v 4.5.2 (*Bio-Rad*).
97 The PCR products were purified using the Nucleospin Extract II kit *(Macharey-Nagel)* according
98 to the manufacturer´s instructions. The concentration and purity were estimated by means of
99 UV spectrometry at 260 nm using a NanoDrop 2000 Spectrophotometer *(Thermo Scientific).*
100 In order to verify the correct assignation of the specimens used in the method set-up, a new
101 PCR product was amplified and sequenced on an ABI Prims 310 Genetic Analyzer *(Applied*
102 *Biosystems).* This analysis was carried out using the primers L14735 and H1549D [31]. The
103 resulting *cytochrome b* nucleotide sequences were also analyzed by FINS[32] and to confirm,
104 were compared with those present in the NCBI database using *Megablast.*

Once the correct performance of each PCR for the two designed systems was verified individually, a duplex PCR for the two systems was set up. Primer concentrations and thermal protocol were the same as the ones previously used in simplex PCR.

***Detection of DIG-labeled PCR products by ELISA***

The PCR ELISA DIG Detection kit (Roche) was used for the detection of labeled PCR products according to the manufacturer's instructions. A volume of 10 µL of each DIG-labeled PCR product (5ng/ µL) were denatured in 20 µL of denaturation solution at 25ºC for 10 min; this solution and 170 µL hybridization buffer, containing the biotynilated-probe at a concentration of 5,3nM, were then transferred to the wells of a streptavidin-coated microtitre plate strip, and the plate was incubated at 59ºC for 3h. The microtitre plate wells then were washed and Anti-DIG-POD (Anti-Digoxigenin-peroxidase-conjugate) working solution (200µL) was added to each well; the microtitre plate was incubated afterwards at 37ºC for 30 min. After another washing step, peroxidase substrate, ABTS (2, 2-azino-di-3-ethylbenzithiazoline sulfonate), was added and the plate was incubated at 37ºC for 30 min to allow color development. The absorbance of the contents of each well was read in a SynergyMx spectrophotometer (BioTek Instruments), at a wavelength of 405 nm

In order to obtain the method's detection limit, mixtures of three species' DNA template were diluted in molecular biology grade water obtaining 50ng to 50pg per µL. These dilutions were used in the PCR ELISA reaction and the labeled products were detected using the three probes.

Positive reactions were determined by a calculation of cut-off values as follow: cut-off=4x absorbance at 405nm of the mean of ten replicates of negative detection control.

***Validation of the methodology with commercial samples***

Once the methods were optimized, they were applied to 19 commercial samples purchased in markets and shops from different places in Spain.

## RESULTS AND DISCUSSION

The aim of the present study was the development and evaluation of a PCR-ELISA technique for the authentication of *G. morhua, G. chalcogrammus* and *M. molva* species in seafood products. Due to their low cost, when Ling (*M. molva*) and Alaska Pollock (*G.chalcogramma*) lose their morphological characteristics, they are highly susceptible to being used in fishery products for the replacement of more expensive fish species such as cod (*G. morhua*) [5].

### *PCR-ELISA system design*

For the correct design of the PCR-ELISA system, a large number of DNA sequences of nuclear and mitochondrial genes from a large number of fish species were analyzed previously. Mitochondrial DNA has been extensively studied and used as a marker for phylogenetic classification species of fish as well as for their detection in commercial products. This is because in the cells, mitochondrial DNA is more abundant than the nuclear, have a higher mutation rate and contains more sequence diversity[33, 34].

In order to achieve sufficient sensitivity to accurately identify the species of Atlantic cod, ling and Alaska pollock in processed samples, one of the objectives of this work was to find a system (comprising primers and probe) as short as possible.

This system must be designed in a region of DNA that contains sufficient interspecific variability and low or null intraspecific variability in order for the primers and biotin-labeled probe be specifically bound and avoiding false negatives and false positives[35].

The hybridization of the biotin-labeled probe with specific PCR products allows the detection of the target species, therefore the increasing the number of interspecific differences the increasing the specificity. It is shown that this specificity is also dependent on the length, hybridization temperature and GC content of the capture probe. Sails et al.[26] in their work of identifying *Campylobacter jejuni* and *Campylobacter coli* observed that short capture probes (18-20 nucleotides) with nucleotide differences in the central region and high annealing temperatures (up to 55 ° C) can identify a single nucleotide difference in the sequence target.

The designed systems consisted of a 160 bp fragment (Gmor/Gchal_ND4 system) and an 81bp fragment (Mmol_cytb system) (Figure 1).

The sequences of the selected primers and probes are listed in Table 3.

***Specificity of PCR-ELISA assay***

Detection of PCR products by simple electrophoresis in agarose gel may not be sufficiently specific, giving rise to false positives if a verification of the sequence isn´t performed by hybridization to specific probes, this may occur due to the difficulty in determining the exact size of the amplicons and the possible occurrence of artifacts with the same size that PCR products[36]. However the results of this assay showed that specific PCR products were always obtained and that non false positives were produced in any case (Figure 2). The designed PCR systems allow to obtain a certain degree of specificity, for instance if primer pair Gmor/Gchal_ND4 is used, a 160bp DIG-labeled PCR product will be produced only when mitochondrial DNA used as template is from *G.chalcogrammus* and *G.morhua.*

Similarly, primer pair Mmol_Cytb generates an 81 bp DIG-labeled PCR product only when template DNA is from *M. molva.*

Both primer pairs were tested for their specificity using 50ng/µL DNA of all species listed in Table 2.

Thanks to the joint use of specific sets of primers and probes, a highly specific hybridization is achieved, allowing the high specificity necessary to verify the DNA sequence of the PCR products and to avoid false positives and correct identification of the target species was achieved.

***Detection of DIG labeled PCR products***

For the determination of the specificity of detection of the three labeled probes, these were tested for specific identification of labeled PCR products from the three species problem by measuring their absorbance at 405 nm (Table 4), considering positive those samples whose absorbance at 405 nm was higher than 0.604. This cutoff value was calculated as follows: cutoff=4xabsorbance at 405nm of the mean of twelve replicates of negative detection control (0.151±0.009). Furthermore the results showed a positive green coloration detectable to the naked eye.

With the purpose of evaluating the sensitivity and detection limit of the PCR-ELISA developed, a series of dilutions from 16ng to 60 pg, of the extracted sample of positive control DNA were amplified and subsequently used in an assay for PCR-ELISA and a convectional agarose gel electrophoresis assay in order to compare the signals obtained in both assays (Table 5, Figure

3). These assays showed that PCR-ELISA was at least 100 fold more sensitive than the detection method based on gel. The minimum amount of DNA template detectable in the PCR-ELISA assay with the Gmor_ND4-P probe was 123 pg and with Gchal_ND4-P and Mmol_Cytb-P probes it was 416 pg, while with the conventional agarose gel electrophoresis method, the minimum amount of DNA template easily visually detected was 16,7 ng. The data here presented indicate that the PCR-ELISA technique based on the detection of Cytochrome b and ND4 gene products is highly sensitive.

Although there are other molecular detection techniques that require less time, such as TaqMan real time PCR, PCR-ELISA can also be considered as a rapid technique, requiring less than a day's work to obtain the results[27]. The PCR-ELISA approach can be immediately adopted by most identification laboratories because the results can be visually read, eliminating the need for expensive equipment, and a large number of samples can be handled.

***PCR amplification and sequencing***

The success of the methodology used depends on correct design of specific probes labeled with biotin that will be linked through an antigen-antibody reaction to a microplate with streptavidin, and the incorporation, during the PCR process, of a triphosphate nucleotide labeled with DIG (*digoxigenin-11-dUTP*), allowing the amplicons detection.

The annealing temperature was adjusted in order to allow the correct amplification of problem species, while avoiding the amplification of other closely related species (Figure 2). Finally, this parameter was adjusted to 63ºC, since it allows the efficient amplification of *G .morhua, G. chalcogrammus* DNA with Gmor/Gchal_ND4 primers and *M. molva* in Mmol_Cytb primers. Each primer set was independently tested and optimized first.

With the aim of reducing the number of steps of the identification methodology, PCR amplification of the two designed systems were grouped in a duplex PCR[37], this also allows the identification of three species, even when mixed, in a single step[38]. The conditions were the same used in simplex PCR. In these conditions, similar concentrations of PCR products were obtained in both duplex PCR.

The main advantage of multiplex PCR strategy is the reduction of the number of reactions required, decreasing labor time and total cost. It is particularly justified when a mix of species is suspected in the sample or when the assurance of the presence/absence of the three target

219 species is desired (Figure 4). The duplex PCR allows to verify if *Gadus morhua/chalcogrammus*
220 and/or *Molva molva* DNA is present in a tissue or DNA mixture.
221 Alternatively, the individual use of each particular system is also possible, allowing
222 determination of the presence of any of the three studied species.
223 The set of primers L14735 and H1549D was used as amplification control in PCR, to avoid the
224 occurrence of false negative amplifications due to problems associated with the samples, such
225 as presence of inhibitors, poor quality or fragmented DNA associated with the transformation
226 process.
227 In order to confirm the correct identification based on morphological characters, 460 bp
228 fragment from each of the specimens used in this study were amplified, sequenced and
229 analyzed by FINS. Moreover, in order to verify that these sequences were correctly assigned to
230 particular species, they were compared with those present in the NCBI database using
231 Megablast.

232 ***Validation of the methodology with commercial samples***

233 Once the method surpassed the validation step, it was applied to 19 commercial samples
234 (Table 2) following the flowchart proposed (Figure 4), allowing us to evaluate and broadly
235 determine the labeling situation of these products in the Spanish market. All samples analyzed
236 were correctly identified. The positive and negative results were clearly visualized without the
237 need of a spectrophotometer or ELISA plate reader (Figure 5). Four analysed samples
238 contained different species than those indicated in the label; one of them was identified as a
239 mixture *Gadus morhua and Molva molva*. This mislabeling would not have been possible to
240 detect using DNA sequencing analysis (FINS) (Table 2).

241 **In conclusion**, the technique described in this article represents a useful tool to unequivocally
242 authenticate the species *G. morhua, M. molva* and *G. chalcogrammus.*
243 In no case were obtained false negatives or false positives. The purpose of this study was the
244 development and evaluation of a PCR-ELISA technique for the detection of *G. morhua, M.*
245 *molva* and *G. chalcogrammus* in fish products, which has been successfully accomplished.
246 This methodology proves useful for enforcing labeling regulations in the authentication of fresh
247 or elaborated fish products and could be used as a routine analysis in food control laboratories.

We indeed identified the species *Molva molva* in these products, whereas, according to law, cod can only be manufactured with the species *Gadus morhua* and *Gadus macrocephalus*. *Molva molva* is a less economically valued species and thus frequently used in transformed food products where substitution is impossible to detect by simple visual inspection.

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**FIGURE CAPTIONS**

**Figure 1**. Location and position of primers and probes designed in this work.

**Figure 2**. Agarose gel electrophoresis of amplification products obtained by a duplex PCR from DNA extracts of reference samples. Line 1 *G. morhua*, line2: *G. macrocephalus*, line 3: *G. ogac*, line4: *M.aeglefinus*, line 5: *M. merlangius,* line 6: *M. poutassous,* line 7: *P. pollachius,* line 8: *P. virens,* line 9: *T. luscus,* 10: *B. brosme,* line 11: *M. molva,* line 12: *M. dypterigia,* line 13: *M. magellanicus,* line 14: *M. bilinearis,* line 15: *M. capensis,* line 16: *M. polli,* line 17: *M. hubbsi,* line 18: *M. merluccius,* line 19: *M. paradoxus,* line 20: *M. senegalensis,* line 21: *L. piscatorius ,* line 22: *E. encrasicolus,* line 23: *S. aurira,* line 24: *S. pilchardus,* line 25: *T. alalunga,* line 26: *T. obesus,* line 27: negative control

**Figure 3**. Agarose gel electrophoresis of amplification products obtained by a duplex PCR from mixtures of *G. morhua*, *G. chalcogrammus* and *M. Molva* DNA extracts, ranging 16 ng to 60 pg of DNA of each DNA template.

**Figure 4**. Flow diagram of the proposed method.* *unlikely to occur, if this occurs it is recommended to check the Elisa test components including a positive control.** *160 bp PCR product but double negative hybridization of both Gadus probes is unlikely to occur, if this occurs it is recommended to check the Elisa test components including positive controls.*

**Figure 5.** PCR-ELISA results of commercial products. Wells 1-23 correspond to samples described in Table2. Wells 24-26 correspond to negative samples. Positive and negative results could be clearly visualized without the need of a spectrophotometer or ELISA plate reader.

**TABLES**

**Table 1.** List of reference species used for the study.

| Species | N | Common name |
|---|---|---|
| **Orden gadiformes** | | |
| **family Gadidae** | | |
| *Gadus morhua* | 13 | Atlantic Cod |
| *Gadus chalcogrammus* | 13 | Alaska pollock |
| *Gadus macrocephalus* | 1 | Pacific Cod |
| *Gadus ogac* | 1 | Greenland Cod |
| *Melanogrammus aeglefinus* | 1 | Haddock |
| *Merlangius merlangius* | 1 | Whiting |
| *Micromesistimus poutassous* | 1 | Blue whiting |
| *Pollachius pollachius* | 1 | Pollack |
| *Pollachius virens* | 1 | Saithe |
| *Trisopterus luscus* | 1 | Pouting |
| **family Merlucidae** | | |
| *Macruronus magellanicus* | 1 | Patagonian grenadier |
| *Merluccius bilinearis* | 1 | Silver hake |
| *Merluccius capensis* | 1 | Shallow-water Cape hake |
| *Merluccius polli* | 1 | Benguela hake |
| *Merluccius hubbsi* | 1 | Argentina hake |
| *Merluccius merluccius* | 1 | European hake |
| *Merluccius paradoxus* | 1 | Deep-water Cape hake |
| *Merluccius senegalensis* | 1 | Senegalese hake |
| **family Lotidae** | | |
| *Brosme brosme* | 1 | Tusk |
| *Molva dypterigia* | 1 | Blue ling |
| *Molva molva* | 13 | Ling |
| **Order Lophiiformes** | | |
| **family Lophiidae** | | |
| *Lophius piscatorius* | 1 | Angler |
| **Order Cupleiformes** | | |
| **family Engraulidae** | | |
| *Engraulis encrasicolus* | 1 | European anchovy |
| **family Clupeidae** | | |
| *Sardinella aurira* | 1 | Round sardinella |
| *Sardina pilchardus* | 1 | European pilchard |
| **Order Perciformes** | | |
| **family Scombridae** | | |
| *Thunnus alalunga* | 1 | Albacore |
| *Thunnus obesus* | 1 | Bigeye tuna |

**Table 2**.List of commercial products used for the study and results of the PCR-ELISA assay of commercial products.

| | Commercial name | Scientific name | Remarks information | FINS identification | PCR amplification | | PCR-ELISA | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Mmol_ Cytb | Gmor/Gchal_ ND4 | *Gchal_ ND4-P* | *Gmor_ ND4-P* | *Mmol_ Cytb-P* |
| 1 | Cod omelette | *Not available* | | *Mixed DNA* | + | + | - | + | + |
| 2 | Cod liver | *Not available* | Canned | *G. morhua* | - | + | - | + | - |
| 3 | Nordic cod | *G. morhua* | Smoked | *G. morhua* | - | + | - | + | - |
| 4 | Iceland cod | *G. morhua* | Salted | *G. morhua* | - | + | - | + | - |
| 5 | Cod cheeks | *G. morhua* | Desalted | *G. morhua* | - | + | - | + | - |
| 6 | Cod | *Not available* | Croquettes | *G. morhua* | - | + | - | + | - |
| 7 | Cod fritter | *Not available* | Frozen | *G. morhua* | - | + | - | + | - |
| 8 | Cod filet | *G. morhua* | Frozen | *G. morhua* | - | + | - | + | - |
| 9 | Iceland cod | *G. morhua* | Frozen | *G. morhua* | - | + | - | + | - |
| 10 | Cod | *Gadus spp* | Salted | *G. morhua* | - | + | - | + | - |
| 11 | Ling loins | *Molva molva* | Frozen | *Molva dypterigia* | - | - | | | |
| 12 | Cod liver | *Not available* | Canned | *G. morhua* | - | + | - | + | - |
| 13 | Minced cod | *G.morhua* | Salted | *Molva molva* | + | - | - | - | + |
| 14 | Alaska pollock | *G. chalcogrammus* | Salted | *G. chalcogrammus* | - | + | + | - | - |
| 15 | Alaska Pollock loins | *G. chalcogrammus* | Frozen | *G. chalcogrammus* | - | + | + | - | - |
| 16 | Alaska Pollock | *G. chalcogrammus* | Salted | *G. chalcogrammus* | - | + | + | - | - |
| 17 | Alaska Pollock | *G. chalcogrammus* | Frozen | *G. chalcogrammus* | - | + | + | - | - |
| 18 | Ling roe | *Molva molva* | Dry-salted | *Molva molva* | + | - | - | - | + |
| 19 | Ling loins | *Molva molva* | Salted | *Molva dypterigia* | - | - | | | |
| 20 | *G.morhua* | | Reference sample | | - | + | - | + | - |
| 21 | *G.chalcogrammus* | | Reference sample | | - | + | + | - | - |
| 22 | *M.molva* | | Reference sample | | + | - | - | - | + |
| 23 | *G.morhua+G.chalcogrammus +M.molva* | | Mix DNA | | + | + | + | + | + |

**Table 3.** Primers and PCR-ELISA probes used in this work

| | | | | | |
|---|---|---|---|---|---|
| ***Gmor/Gchal_ND4-R*** | *Gmor/Gchal (160)* | *ND4* | 5´-TAACAAATGCTCTCGTGTGTAG-´3 | 22 | |
| ***Gmor_ND4-P*** | | | 5´-[Biotin]GCCTACTCCCTGTATATATTCCT-3´ | 23 | |
| ***Gchal_ND4-P*** | | | 5´-[Biotin]TTCACTGTACATATTTCTGATGAG-3´ | 24 | |
| ***Mmol_Cytb-F*** | *Mmol(81)* | *Cyt b* | 5´-GGGTTCTCGCACTTCTATTTTCA-3´ | 23 | |
| ***Mmol_Cytb-R*** | | | 5´-ATGTTAGTCCTCGTTGTTTAGAGGTATG-3´ | 28 | |
| ***Mmol_Cytb-P*** | | | 5´-[Biotin] TAGTTCTCATAGTAGTCCCCTTC-3´ | 23 | |
| ***L14735*** | *Kocher (460)* | *tRNAglu-cytb* | 5´-GCICCTCARAATGAYATTTGTCCTCA-3´ | 26 | |
| ***H15149D*** | | | 5´-AAAAACCACCGTTGTTATTCAACTA-3´ | 25 | |
| | | | | | |
| | | | | | |
| | | | | | |

**Table 4.** Comparative specificity of the different probes for the detection of *M. molva*, *G. chalcogrammus* and *G.morhua.* ***No****: number of replicates.*

| Sample | No | Absorbance readings at 405nm ± standard deviation |
|---|---|---|
| *Molva molva* | 13 | 0,818+-0,07 |
| *Gadus chalcogrammus* | 13 | 1,904+-0,50 |
| *Gadus morhua* | 13 | 2,39+-0,8 |
| Negative | 12 | 0,151-+0,00 |

**Table 5.** PCR-ELISA readings at 405 nm of different amounts of purified PCR products

| DNA template | Absorbance readings at 405nm | | |
|---|---|---|---|
| | *G. morhua* Probe | *G. chalcogrammus* Probe | *M. molva* Probe |
| 16,7 ng | 2,59 | 1,74 | 1,72 |
| 1,7 ng | 2,27 | 1,68 | 1,67 |
| 833 pg | 2,6 | 1,92 | 2,04 |
| 416 pg | 1,86 | 0,64 | 0,68 |
| 250 pg | 1,31 | 0,29 | 0,31 |
| 123 pg | 0,81 | 0,29 | 0,29 |
| 63 pg | 0,47 | 0,21 | 0,20 |
| 0 | 0,11 | 0,12 | 0,13 |

**Figure 1**

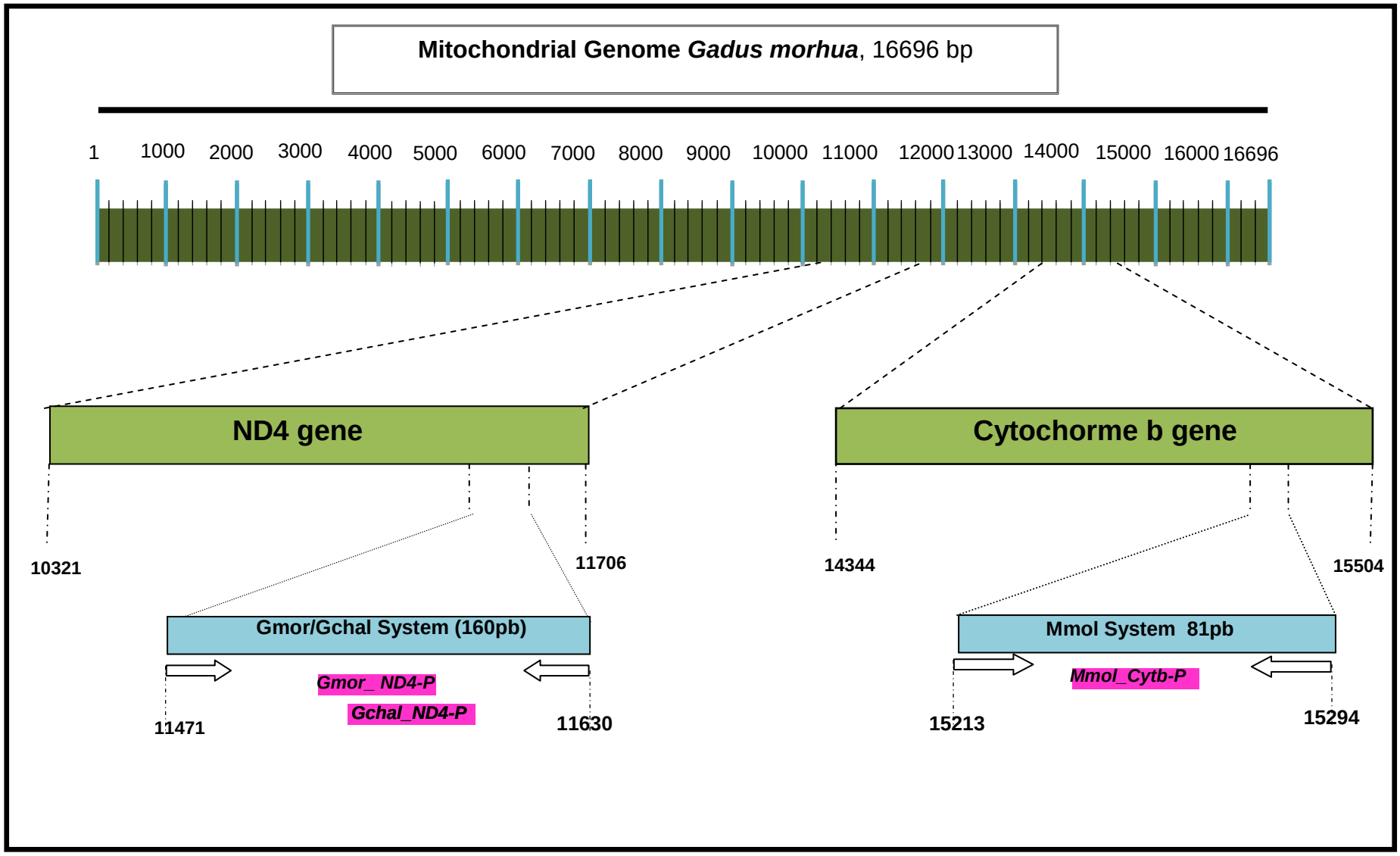

Mitochondrial Genome *Gadus morhua*, 16696 bp
1
1000
2000
3000
4000
5000
6000
7000
8000
9000
10000
11000
12000
13000
14000
15000
16000
16696
ND4 gene
Cytochorme b gene
10321
11706
14344
15504
Gmor/Gchal System (160pb)
Mmol System 81pb
Gmor_ ND4-P
Gchal_ND4-P
Mmol_Cytb-P
11471
11630
15213
15294

**Figure 2**

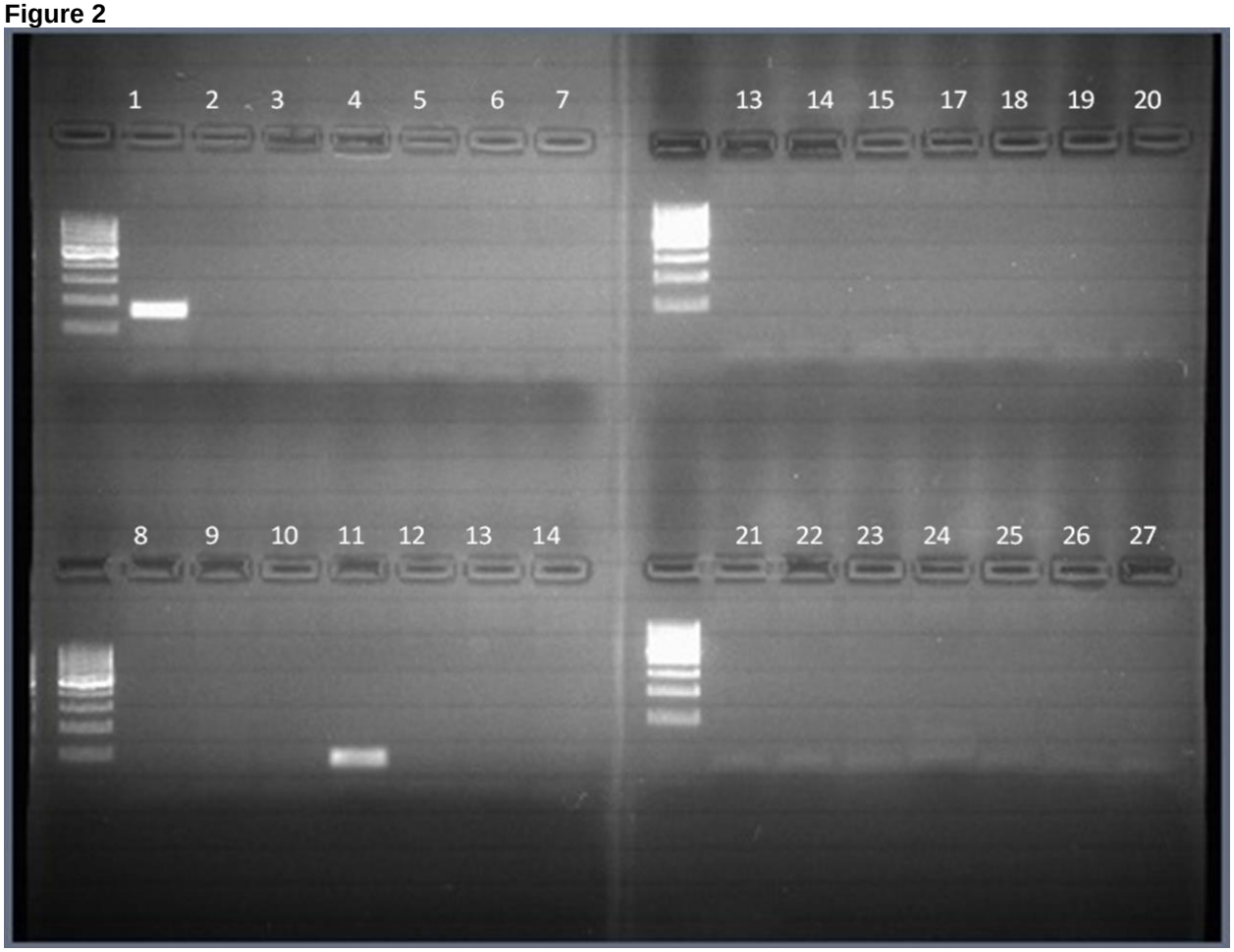


**Figure 3**

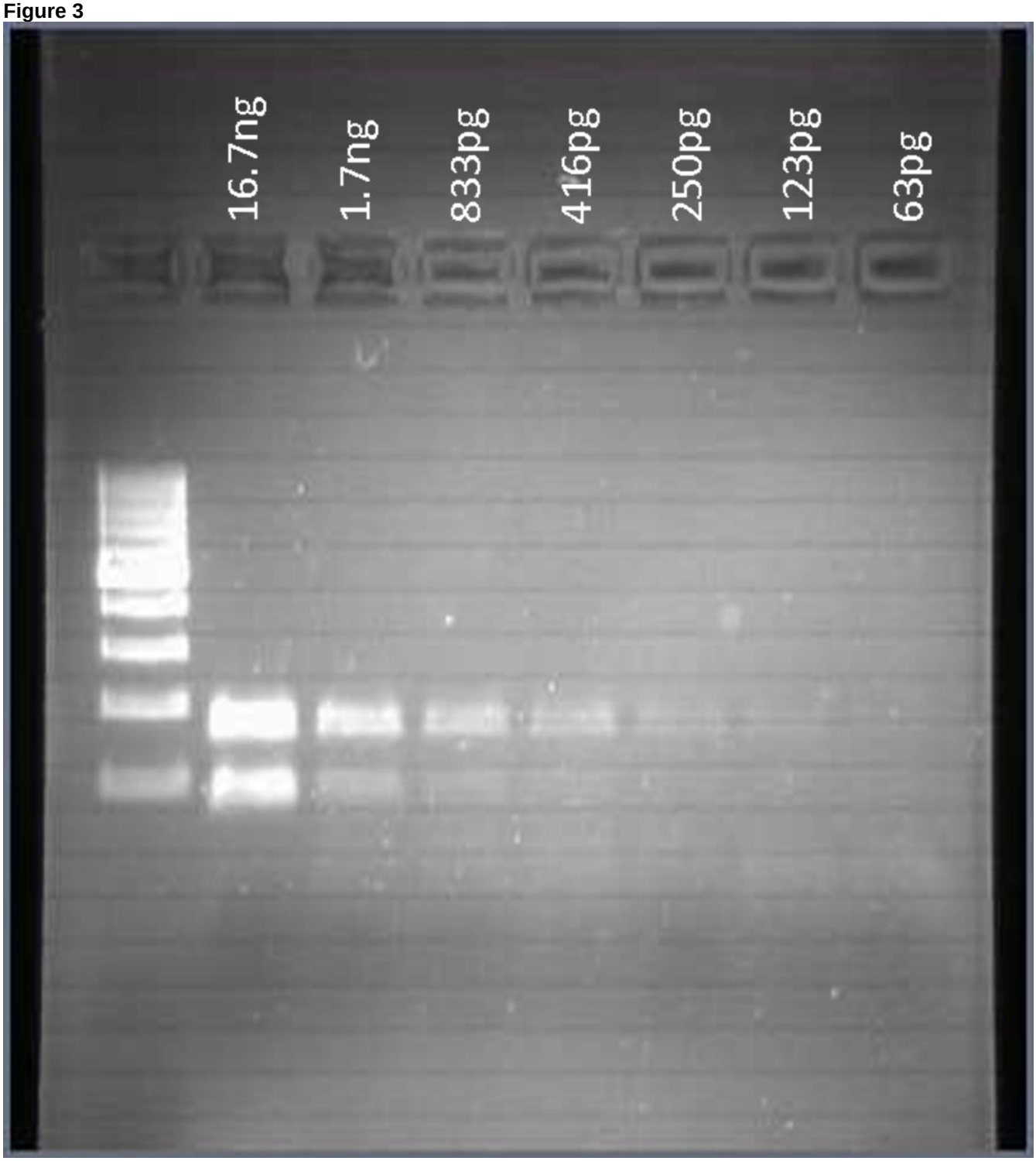


**Figure 4**

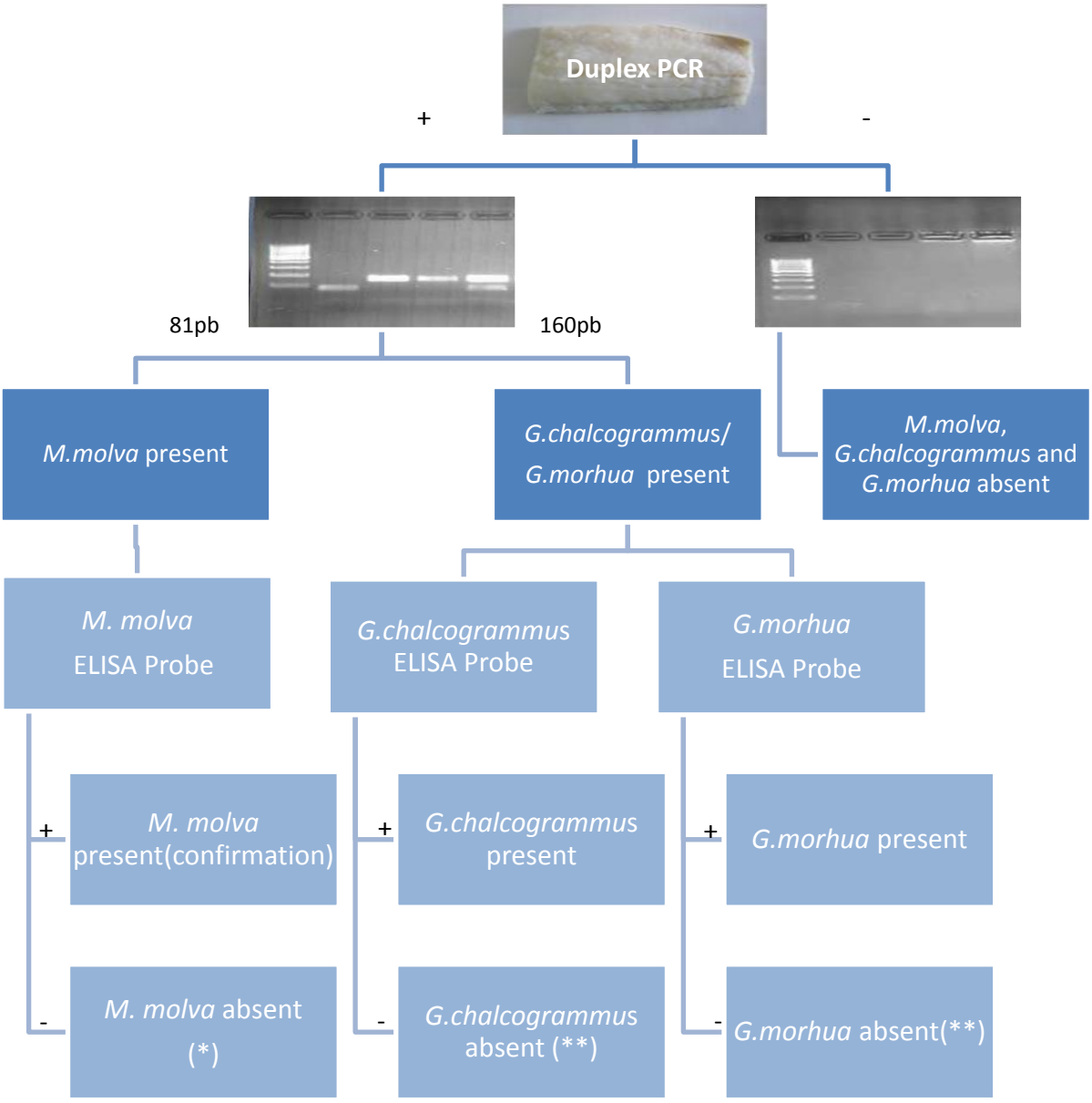
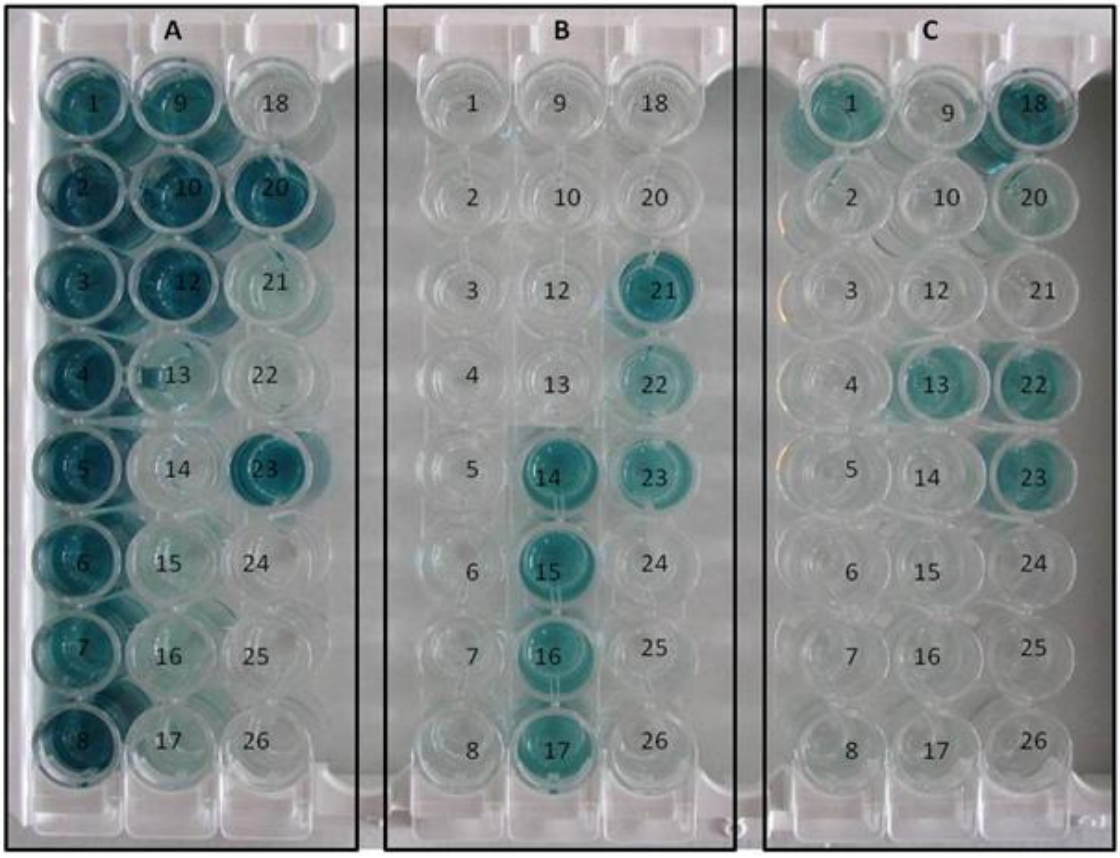

Duplex PCR
+
-
81pb
160pb
M.molva present
G.chalcogrammus/ G.morhua present
M.molva, G.chalcogrammus and G.morhua absent
M. molva ELISA Probe
G.chalcogrammus ELISA Probe
G.morhua ELISA Probe
+
M. molva present(confirmation)
-
M. molva absent (*)
+
G.chalcogrammus present
-
G.chalcogrammus absent (**)
+
G.morhua present
-
G.morhua absent(**)

**Figure 5**




*G. morhua* Probe

| A405nm | A405nm | A405nm |
|---|---|---|
| **1** 1,935 | **9** 1,92 | **18** 0,156 |
| **2** 3,075 | **10** 2,708 | **20** 2,684 |
| **3** 2,252 | **12** 2,928 | **21** 0,385 |
| **4** 2,995 | **13** 0,433 | **22** 0,229 |
| **5** 2,873 | **14** 0,179 | **23** 2,377 |
| **6** 2,382 | **15** 0,357 | **24** 0,144 |
| **7** 1,643 | **16** 0,399 | **25** 0,157 |
| **8** 2,937 | **17** 0,298 | **26** 0,153 |

*G. chalcogrammus* Probe

| A405nm | A405nm | A405nm |
|---|---|---|
| **1** 0,151 | **9** 0,146 | **18** 0,154 |
| **2** 0,158 | **10** 0,15 | **20** 0,156 |
| **3** 0,149 | **12** 0,166 | **21** 1,81 |
| **4** 0,154 | **13** 0,143 | **22** 0,544 |
| **5** 0,158 | **14** 1,336 | **23** 1,159 |
| **6** 0,159 | **15** 1,68 | **24** 0,144 |
| **7** 0,156 | **16** 1,217 | **25** 0,151 |
| **8** 0,161 | **17** 1,141 | **26** 0,141 |

*M. molva* Probe

| A405nm | A405nm | A405nm |
|---|---|---|
| **1** 0,989 | **9** 0,145 | **18** 1,534 |
| **2** 0,193 | **10** 0,177 | **20** 0,37 |
| **3** 0,142 | **12** 0,186 | **21** 0,167 |
| **4** 0,152 | **13** 0,918 | **22** 1,236 |
| **5** 0,196 | **14** 0,17 | **23** 1,165 |
| **6** 0,144 | **15** 0,2 0,04 | **24** 0,152 |
| **7** 0,151 | **16** 0,192 | **25** 0,15 |
| **8** 0,196 | **17** 0,164 | **26** 0,145 |

**TOC Graphic**