European Commission

ERM
European Reference Materials

CERTIFICATION REPORT

The certification of the genetic identification of a *Hippoglossus hippoglossus* (Atlantic halibut) fish powder: EURM®-020

EUR 30097 EN

European Commission
Joint Research Centre
Directorate F – Health, Consumers and Reference Materials

Contact information
Reference materials sales
Address: Retieseweg 111, 2440 Geel, Belgium
E-mail: jrc-rm-distribution@ec.europa.eu
Tel.: +32 (0)14 571 705

JRC Science Hub
https://ec.europa.eu/jrc

JRC119890

EUR 30097 EN

ISBN 978-92-76-16230-8 (PDF)
ISSN 1831-9424 (online)
doi:10.2760/170544

Luxembourg: Publications Office of the European Union, 2020

Abstract

This report describes the production of EURM-020, a fish tissue material certified for the identification of the fish species *Hippoglossus hippoglossus* (Atlantic halibut). This material was produced following ISO 17034:2016 and is certified in accordance with ISO Guide 35:2017.
The material was produced from one fillet originating from a single fish which was genetically identified as *Hippoglossus hippoglossus* (Atlantic halibut). In facilitating storage, handling and shipment, the fish fillet was cut into small pieces, slowly dried using a freeze drying process and cryogenically milled to obtain a fine powder. The powder was bottled, and the bottles were closed under an argon atmosphere to ensure the conservation of the material. In storing the material, the bottles were placed at 4 °C.
The fish species present in EURM-020 was determined to be *Hippoglossus hippoglossus* (Atlantic halibut) via genetic identification based upon the sequencing results of two specific regions of mitochondrial DNA (also called DNA barcodes): the *cytochrome b* gene (*cytb*) and the *cytochrome c oxidase subunit I* gene (*COI*).
Between-unit homogeneity was established by evaluating the quality and quantity of the extracted DNA from multiple bottles. In addition, the two DNA barcodes were amplified by polymerase chain reaction (PCR) and analysed by bi-directional Sanger sequencing for each bottle sampled. The stability during dispatch and storage was assessed through quantification of extracted DNA by UV spectrophotometry, and gel-electrophoresis of the extracted DNA and PCR products of the two DNA barcodes. The process was conducted following ISO Guide 35:2017.
The material was characterised based on a comparison of measurements produced by two laboratories with demonstrated competencies and adhering to ISO/IEC 17025. Data analysis and species identification was completed at the JRC.
The material is certified for its identity which was verified with two different mitochondrial DNA based methods. It is unlikely that there is an alternative species that shares both the sequences of the *cytb* and *COI* gene barcode regions with the species in EURM-020. Consequently, there is no doubt regarding the certified identity, *Hippoglossus hippoglossus*, for EURM-020 and the uncertainties of the certified value relating to inhomogeneity, instability and characterisation are negligible.
The material is intended for the quality control or assessment of method performance. As with any reference material, it can be used for validation studies. EURM-020 is available in glass vials containing at least 200 mg of dried *Hippoglossus hippoglossus* (Atlantic halibut) powder. The minimum amount of sample to be used is 7 mg.

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A.M. Kortekaas, H. Leys, J. Seghers, S. Trapmann

European Commission, Joint Research Centre
Directorate F – Health, Consumers and Reference Materials
Geel, Belgium

Disclaimer

Certain commercial equipment, instruments, and materials are identified in this paper to specify adequately the experimental procedure. In no case does such identification imply recommendation or endorsement by the European Commission, nor does it imply that the material or equipment is necessarily the best available for the purpose.

Summary

This report describes the production of EURM-020, a fish tissue material certified for the identification of the fish species *Hippoglossus hippoglossus* (Atlantic halibut). This material was produced following ISO 17034:2016 [1] and is certified in accordance with ISO Guide 35:2017 [2].

The material was produced from one fillet originating from a single fish which was genetically identified as *Hippoglossus hippoglossus* (Atlantic halibut). In facilitating storage, handling and shipment, the fish fillet was cut into small pieces, slowly dried using a freeze drying process and cryogenically milled to obtain a fine powder. The powder was bottled, and the bottles were closed under an argon atmosphere to ensure the conservation of the material. In storing the material, the bottles were placed at 4 °C.

The fish species present in EURM-020 was determined to be *Hippoglossus hippoglossus* (Atlantic halibut) via genetic identification based upon the sequencing results of two specific regions of mitochondrial DNA (also called DNA barcodes): the *cytochrome b* gene (*cytb*) and the *cytochrome c oxidase subunit I* gene (*COI*).

Between-unit homogeneity was established by evaluating the quality and quantity of the extracted DNA from multiple bottles. In addition, the two DNA barcodes were amplified by polymerase chain reaction (PCR) and analysed by bi-directional Sanger sequencing for each bottle sampled. The stability during dispatch and storage was assessed through quantification of extracted DNA by UV spectrophotometry, and gel-electrophoresis of the extracted DNA and PCR products of the two DNA barcodes. The process was conducted following ISO Guide 35:2017 [2].

The material was characterised based on a comparison of measurements produced by two laboratories with demonstrated competencies and adhering to ISO/IEC 17025 [3]. Data analysis and species identification was completed at the JRC.

The material is certified for its identity which was verified with two different mitochondrial DNA based methods. It is unlikely that there is an alternative species that shares both the sequences of the *cytb* and *COI* gene barcode regions with the species in EURM-020. Consequently, there is no doubt regarding the certified identity, *Hippoglossus hippoglossus*, for EURM-020 and the uncertainties of the certified value relating to inhomogeneity, instability and characterisation are negligible.

The material is intended for the quality control or assessment of method performance. As with any reference material, it can be used for validation studies. EURM-020 is available in glass vials containing at least 200 mg of dried *Hippoglossus hippoglossus* (Atlantic halibut) powder. The minimum amount of sample to be used is 7 mg.

The following value for the nominal property was assigned:

	Fish powder	
	Certified value [2]	Uncertainty [3]
Taxon [1]	*Hippoglossus hippoglossus* [4] (Atlantic halibut)	Negligible

1) As obtained by bi-directional Sanger sequencing of two fish DNA identification barcodes, the mitochondrial *cytochrome b* gene (*cytb*) and the mitochondrial *cytochrome c oxidase subunit I* gene (*COI*) and subsequent comparison with reference sequences from GenBank® (NCBI), FishTrace (EC) and the Bar Code of Life Database System (BOLD).

2) Certified values are values that fulfil the highest standards of accuracy. The given value represents the consensus value of accepted sets of data, with each set obtained by a different laboratory or with a different method of determination. The certified value is traceable to reference DNA sequences in the GenBank (NCBI), FishTrace (EC) and BOLD databases. The identity at the species level, was defined as a minimum of 99 % similarity with the DNA sequences of the specimens (voucher samples, specimens with morphological identification) deposited in the GenBank (NCBI), FishTrace (EC) or BOLD databases.

3) The identity was verified with two different (genetic) methods. It is unlikely that there is an alternative species that shares the sequences of these independent genes. Consequently, there is no doubt regarding the certified identity, *Hippoglossus hippoglossus,* for EURM-020 and its related uncertainty is negligible.

4) *Hippoglossus hippoglossus* (Linnaeus, 1758)

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Glossary

BLAST	Basic Local Alignment Search Tool
BOLD	Barcode of Life Data system
CEN	European Committee for Standardization
COI	Mitochondrial *cytochrome c oxidase 1* gene
CRM	Certified reference material
cytb	Mitochondrial *cytochrome b* gene
DNA	Deoxyribonucleic acid
EC	European Commission
EDTA	Ethylenediaminetetraacetic acid
ERM®	Trademark of European Reference Materials
EU	European Union
EURM	European Reference Materials
ILC	Interlaboratory comparison
ISO	International Organization for Standardization
IU	International units
JRC	Joint Research Centre of the European Commission
mtDNA	Mitochondrial DNA
n	Number of replicates per unit
N	Number of samples (units) analysed
n.a.	Not applicable
NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction
PSA	Particle size analysis
QA	Quality assurance
QC	Quality control
RM	Reference material
RMP	Reference material producer
rpm	Revolutions per minute
RT	Room temperature
RM Unit	Reference Materials Unit of Directorate F
s	Standard deviation
$s_{extracts}$	Standard deviation of the extracts
SI	International System of Units
s_{meas}	Standard deviation of the measurement data

SOP	Standard Operating Procedure
$s_{\bar{x}\,\text{bottle}}$	Standard deviation of the mean of bottles
T	Temperature
TaqMan®	*Thermus aquaticus* (Taq) DNA polymerase-based technology for fluorescent signal generation during real-time PCR
TE	Buffer containing TRIS and EDTA
TRIS	Tris(hydroxymethyl)aminomethane
UV	Ultraviolet
VIM	International Vocabulary of Metrology – Basic and General Concepts and Associated Terms *[ISO/IEC Guide 99:2007]*
V-KFT	Volumetric Karl Fischer titration

1 Introduction

1.1 Background

To improve transparency in the fishery and aquaculture supply chain, Europe has implemented Regulation No. 1379/2013 on the common organisation of markets for fishery and aquaculture products. Article 35 sets out the rules on the mandatory marking or labelling of pre-packed or non-prepacked fishery and aquaculture products. For example indicating the commercial name of the species and its scientific name, the production method, the area where the product was caught or farmed, the category of fishing gear used in capturing fisheries, whether the product has been defrosted and date of minimum durability [4].

Labelling of fish species may be inadvertently wrong during identification; however, deliberate mislabelling for financial gain occurs when species of higher value are substituted with lower value species. Additionally, species from unsustainable or illegal fisheries may enter the market through substitution [5].

Inspections of the quality and authenticity of fresh or prepared fish products on the market are conducted to discourage operators from falsely labelling catches or products. These inspections are carried out following European regulations such as Regulation No. 1224/2009 which sets out a control regime, comprising the monitoring and inspection of the whole chain of fishery production and marketing [6] and Regulation No. 178/2002, which covers the labelling of fishery products to avoid misleading consumers and the traceability of food products [7].

The current technique used for the genetic identification of fish products is based on the sequencing of specific genes/regions of mitochondrial DNA. Mitochondrial DNA (mtDNA) has a high mutation rate, and as a consequence, significant amounts of sequence variation can be observed between closely related species [8]. Specific genes or parts of genes on the mtDNA that are reliable for species identification are called DNA barcodes. In addition, mtDNA is present in cells at higher concentrations compared to nuclear DNA and is, therefore, the preferred template [9].

The high mutation rate of mtDNA can also lead to sequence variations within one species, and the use of specific barcodes for species identification can only be reliable if the within-species variation is less than the between-species variation. The gap in the distribution of distances between species is named the “barcoding gap”. For the identification of fish species, the mitochondrial *cytochrome b* gene (*cytb*) and the mitochondrial *cytochrome c oxidase subunit I* gene (*COI*) have been identified as reliable barcodes [10, 11]. Although, genetic identification using a single barcode might lead to misidentification because two different species share the sequence of the barcode, it is unlikely that there are two different distinctive species that share the sequences on several independent genes [12].

Publicly available species-specific DNA sequences, used as reference for the identification of fish samples are available in databases such as Bar Code for Life Database System (BOLD), GenBank® (i.e. the National Center for Biotechnology Information's (NCBI) genetic sequence database) and FishTrace (i.e. a genetic and taxonomic reference database for species identification of European commercial marine fish species from the European Commission (EC)). They all contain data from “voucher specimen”, species identified via morphological and genetic methods, which are curated by museums in reference collections. With the exception of FishTrace, the databases also contain DNA sequences of samples not connected to reviewed publications and may not all have a direct link to morphological identification.

In developing and validating DNA related identification methods, laboratories can request tissue aliquots of voucher specimens from museums, although not an easy procedure. In 2018, the JRC decided to produce reference materials for fish identification of commercially important fish species, which can be used for the validation and quality control of genetic identification methods

for fish species. The reference material EURM-020 is a fish powder of *Hippoglossus hippoglossus* (Atlantic halibut) and is characterised based on the genetic identification by two DNA barcodes, *cytb* and *COI*. After extraction of the DNA, parts of both genes have been amplified by polymerase chain reaction (PCR) and sequenced using bi-directional Sanger sequencing. The EURM-020 material is produced as a fish powder which can be stored at 4 °C and dispatched under cooled conditions.

1.2 Choice of the material

EURM-020 is a fish powder prepared from a single fillet of *Hippoglossus hippoglossus* (Atlantic halibut). It is a dried powder to facilitate storage, handling and shipment. After reconstitution, the material closely resembles samples of fresh fish fillet.

1.3 Design of the project

The certification of the fish species present in EURM-020 was based upon genetic analysis performed by two independent laboratories, selected for their expertise in DNA sequencing. Both laboratories analysed two DNA barcodes, known to be reliable for fish identification, by PCR amplification and subsequent bi-directional Sanger sequencing. The results of the two independent laboratories were used for the certification of the genetic identification of the species which is a result of the unambiguous data of the accepted sets of data, each set obtained in a different laboratory or with a different method of determination. The traceability chain of the genetic identification was linked to reference sequences from both voucher specimens and peer reviewed publications in the BOLD, GenBank (NCBI) and FishTrace (EC) databases.

The material is certified for its identity. The material originates from a single fish and the identity was verified by two different genetic methods, therefore, there is no doubt regarding the identity. As a consequence, the uncertainties of the certified value relating to inhomogeneity, instability and characterisation are negligible.

2 Participants

2.1 Project management and evaluation

European Commission, Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, Geel, BE
(accredited to ISO 17034:2016 for production of certified reference materials, BELAC No. 268-RM)

2.2 Provider of raw material and processing

European Commission, Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, Geel, BE
(accredited to ISO 17034:2016 for production of certified reference materials, BELAC No. 268-RM)

2.3 Homogeneity study

European Commission, Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, Geel, BE

Eurofins Genomics Europe Applied Genomics GmbH, Ebersberg, DE
(*cytb* measurements under scope of ISO/IEC 17025:2005 accreditation, DAkkS, D-PL-13372-01-00)

2.4 Stability study

European Commission, Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, Geel, BE

2.5 Characterisation

European Commission, Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, Geel, BE

Eurofins Genomics Europe Applied Genomics GmbH, Ebersberg, DE
(*Cytb* measurements under scope of ISO/IEC 17025:2005 accreditation, DAkkS, D-PL-13372-01-00)

SYNLAB Analytics & Services United Kingdom Ltd, Cramlington, UK

3 Material processing and process control

3.1 Origin/Purity of the starting material

The JRC purchased one fish of the species, *Hippoglossus hippoglossus* (Atlantic halibut) from a local fishmonger. The fish was filleted, packed, labelled and stored at -20 °C until use. Confirmation of identity and an assessment of purity were performed during the characterisation and homogeneity studies. Genetic identification by bi-directional Sanger sequencing using two DNA barcodes, *cytb* and *COI*, confirmed that the identity of the species used for this EURM was *Hippoglossus hippoglossus*. Since only a single fish was used the fish powder produced could be considered pure. This was confirmed by good quality electropherograms generated during the characterisation and homogeneity studies.

3.2 Processing

During processing, contamination of the final EURM-020 material with foreign DNA was avoided by treating all contact surfaces with DNA degrading solution (DNA-Erase™, MP Biomedicals, Irvine, CA, USA) prior to use and by using clean laboratory clothing. An in-house validation study had previously proven that the solution degraded DNA effectively under the conditions used.

The *Hippoglossus hippoglossus* (Atlantic halibut) single fillet was thawed and the skin removed. The fillet was then cut into pieces of approximately 1 cm^3 and about 1.5 kg of fillet cubes were freeze-dried on trays using a program that started at -40 °C and increased gradually to 25 °C. The total freeze-drying process lasted 55 h (Epsilon 1-6D, Martin Christ, Osterode, DE). The dried cubes were then stored in stainless steel drums over liquid nitrogen prior to being cryogenically milled using a Palla VM-KT cryogenic vibrating mill (KHD, Köln, DE). The resultant powder was transferred in a plastic bag, placed in an airtight container and temporarily stored at 4 °C. The water content of the powder was further reduced by drying for 20 h under vacuum in the freeze-dryer at 20 °C, with an additional 20 h under vacuum at 25 °C. The powder was then manually sieved over a 1000 µm stainless steel mesh and the remaining powder mixed in a DynaMIX CM200 (WAB, Muttenz, CH) for 1 h to homogenise. The final powder, about 354 g, was packed in a plastic bag and stored in an airtight container at 4 °C.

The residual water mass fraction of the fish powder was 18.0 ± 1.6 g/kg, measured by volumetric Karl Fischer titration (V-KFT, 841 KFD Titrando, Metrohm, Herisau, CH), (N = 1, n = 3), with the expanded uncertainty calculated using a coverage factor k = 2.

The particle size distribution of the powder was measured based on the deconvoluted laser diffraction patterns (PSA, Sympatec, Clausthal-Zellerfeld, DE). The cumulative volume distribution of the particles derived from laser diffraction data was based on the equivalent spherical diameter, i.e. the diameter of the particles derived from the volume occupied upon their rotation. The mean particle diameter of the powder material was 94.7 µm ± 6.6 µm (s).

A feeder, FD – SPAc 4A (MCPI, Meythet, France), was used to fill 5 mL amber glass bottles with approximately 200 mg of the fish powder. Lyophilisation inserts were placed in the bottle-necks, the bottles were placed in a freeze-dryer to provide an argon atmosphere, (Epsilon 1-6D, Martin Christ, Osterode, DE) and were closed inside the freeze-dryer. Capping was executed using an HV 100 B

semi-automatic capping machine (Bausch & Ströbel, Ilshofen, DE) while labelling was completed manually. Each of the bottles was identified by a numbered label indicating the EURM code and the unit number according to filling order. Following an inventory and the selection of bottles for future analysis, according a random stratified sampling scheme, the remaining bottles were stored in the dark at 4 ± 3 °C.

3.3 Process control

To determine the residual mass fraction of water in EURM-020, 30 bottles were randomly selected for the V-KFT measurement. To reach the sample intake for V-KFT measurements, the content from 3 individual subsequent bottles from the random scheme, was used for one V-KFT measurement (N = 10, n = 1). The water mass fraction of EURM-020 was 33.4 ± 1.9 g/kg with the expanded uncertainty calculated using a coverage factor k = 2. The associated expanded uncertainty (U) has been estimated during validation of the V-KFT method on fish tissue.

The particle size distribution in EURM-020 was determined based on the deconvoluted laser diffraction pattern. Five randomly selected bottles covering the whole EURM-020 production batch were analysed in triplicate (N = 5, n = 3) and no particles larger than 860 µm were detected (Figure 1). The mean particle diameters and standard deviation of the mean, measured by laser diffraction, was 104.3 µm ($s_{\bar{x}}$ = 8.8 µm).

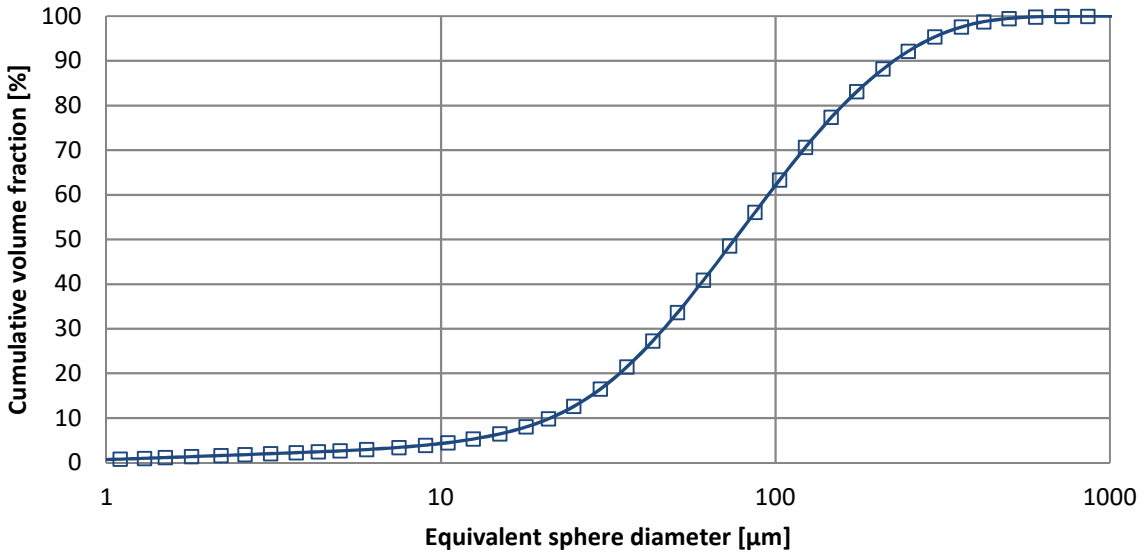

Figure 1: Volume-based cumulative distribution of equivalent sphere diameters in EURM-020 analysed by laser diffraction (N = 5, n = 3). The total particle volume for the material is set as 100 %.

4 Homogeneity

A key requirement for any reference material aliquoted into units is the equivalence between those units. The complete batch of the EURM-020 was produced from a single fish and it can therefore be concluded that the certified species identity is valid for all the units. The RM is intended to be used as a control material for genetic identification techniques for fish species. The suitability of EURM-020 depends on the quality of the DNA that can be extracted. A homogeneity study was carried out to confirm that DNA of sufficient quality can be extracted from all the units.

4.1 Between-unit homogeneity

The number of bottles selected corresponds to approximately the cube root of the total number of bottles produced. Consequently, 10 bottles were selected using a random stratified sampling scheme covering the whole batch for the between-unit homogeneity test. For this study, the batch was divided into 10 groups (with a similar number of bottles) and one bottle was selected randomly from each group.

The DNA extraction and the DNA quality checks were carried out at the JRC. Two independent replicates of between 7 to 8 mg were weighed into 2 mL low-DNA-binding microcentrifuge tubes. The replicates were divided over two extraction series using a randomised order. The material was reconstituted in 160 µL nuclease-free water (Promega) and vortexed for 10 seconds. Immediately, the DNA was extracted following the Animal Tissue Protocol from the DNeasy Blood & Tissue Kit (QIAGEN Benelux BV, Antwerpen, BE). The incubation time was 30 minutes, which was found to produce sufficient extracted DNA during the extraction method validation. At the end of the extraction the DNA was eluted in a single eluate of 100 µL of buffer AE. In checking the integrity of the DNA, gel-electrophoresis was conducted using a Gel-red pre-stained 1 % Agarose gel (PowerPac 3000 Electrophoresis Power Supply, Biorad). Some degree of DNA fragmentation was observed for all the samples, but the quality of the DNA was sufficient for PCR amplification and Sanger sequencing of the targeted DNA barcodes of 413 bp and 652 bp (expected length of the nucleotides between the primers respectively for the targets of the *cytb* and *COI* genes). The average extracted DNA concentration measured by UV spectrophotometry (ND-1000 Spectrophotometer, NanoDrop Technologies Inc., Wilmington, USA) was 128.0 ng/µL ($s_{extracts}$ = 25.7 ng/µL, $s_{xbottles}$ = 17.5 ng/µL) and was sufficient to provide template material for PCR amplification and sequencing. The results per bottle are summarised in Figure 1 in Annex B1.

The samples were diluted in TE low buffer (pH 8,1 mmol/L Tris and 0.01 mmol/L EDTA) to the target DNA concentration (20 ng/µL) and shipped on dry ice to the laboratory (secton 2.3) that performed the bi-directional Sanger sequencing of the mitochondrial *cytb* and COI genes after the PCR amplification and purification.

The primers used for PCR amplification and Sanger sequencing of the *cytb* region were taken directly from the publication by Wolf, Burgerer et al. [10]. The primers used for the *COI* amplification and sequencing were provided by the standard operating procedure for the genetic identification of fish species using DNA barcoding (mitochondrial *cytochrome c oxidase subunit I* sequencing) from the Labelfish Consortium [13, 14, 15]. For PCR amplification of the *COI* barcode, a cocktail of two forward primers or two reverse primers was used in the respective master-mix compositions. The M13-tail primers were used for the Sanger sequencing of the *COI* barcode. (Primers for *cytb* and *COI* are described Annex A1). The measurements were performed under repeatability conditions and in a randomised manner.

All sequencing data were analysed at the JRC and sequencing results for the two barcodes (*cytb* and *COI*) were analysed separately. Initially, the electropherograms were analysed for their quality using the SeqManPro application within Lasergene 15.1 software (DNASTAR, Madison, US) [37]. Primer sequences and regions of low quality at either end of the electropherogram were removed and ambiguous bases and no base calls were checked against the sense or anti-sense sequence and manually corrected where possible. Forward and reverse sequences were aligned per extraction to obtain a consensus sequence.

The 20 *cytb* consensus sequences were compared to a *cytb* barcode reference sequence for *Hippoglossus hippoglossus* (EU513792.1 in GenBank (NCBI) [16]) using the SeqManPro application within the Lasergene 15.1 software (DNASTAR, Madison, US) [37]. For *cytb,* 15 of 20 sequences covered the complete length (413 bp) of the barcode sequence for *Hippoglossus hippoglossus*. The subsequent five sequences were somewhat shorter due to poorer quality base calls at the extremes of the barcodes (384, 395, 397 (2) and 405 bp). No sequence differences were observed between

the samples, however, one mismatch was observed between all the 20 consensus sequences and the reference sequence.

The 20 *COI* consensus sequences were compared with the *COI* reference sequence for *Hippoglossus hippoglossus* (GBMTG1322.16 in BOLD (mined from NC_009709.1 in NCBI GenBank [17, 36])). The *COI* barcode sequence has a length of 652 bp. None of the 20 sequences obtained covered the complete length of the *COI* barcode, however, all sequences produced ranged between 618 bp to 645 bp. No sequence differences were observed between the sample sequences, however, one mismatch was observed between the 20 consensus sequences and the reference sequence.

The results of the homogeneity study showed that DNA of sufficient quality could be extracted from samples contained within each of the selected bottles. The sequencing results produced were of good quality, indicating no significant contamination with foreign DNA within each of the bottles sampled. The sequences of the extraction replicates and the consensus sequences per bottle have been identified as *Hippoglossus hippoglossus* at both barcode positions sequenced, *cytb* and *COI*. The between-bottle homogeneity and within-unit homogeneity is confirmed by the identical consensus sequences and the sequence identity results of above 99 % with the *Hippoglossus hippoglossus* reference sequences in GenBank (NCBI) for the *cytb* barcode using Basic Local Alignment Search Tool BLAST® software, (version BLASTN 2.9.0+, NCBI, National Library of Medicine, Bethesda, US) [18, 19] and above 99 % similarity results with the reference sequences for the *COI* barcode using the Barcode of Life Data Identification Systems v4 (BOLD) [20].

The results of the BLAST and BOLD searches are summarised in Table 1 to 2 in Annex B.

4.2 Within-unit homogeneity and minimum sample intake

The EURM-020 material is a pure powder that was produced from a fillet originating from a single fish and is therefore homogenous. A meaningful minimum sample intake at which the within-unit inhomogeneity no longer contributes to analytical variation can therefore not be established. Homogeneity, characterisation and stability measurements were performed with a sample intake between 7 and 8 mg and were found to produce DNA concentrations fit for PCR amplification and Sanger sequencing, therefore, the minimum sample intake is 7 mg.

5 Stability

Time, temperature, light (including ultraviolet radiation) and water content were regarded as the most relevant influences on the stability of the reference materials. The impact of ultraviolet or visible light was minimised by storing the material in brown glass bottles that reduce light exposure. In addition, materials are stored in the dark and dispatched in boxes, thus removing any possibility of degradation by light. The water content was adjusted to an optimum during processing. Consequently, only the influences of time and temperature had to be investigated.

Stability testing is necessary to establish the conditions for storage (long-term stability) as well as the conditions for dispatching the materials to customers (short-term stability). During transport, especially in the summer, temperatures of up to 60 °C can be reached, and stability under these conditions must be demonstrated if the samples are to be transported without any additional cooling.

The stability studies were carried out using an isochronous design [34]. In this approach, samples were stored for a given length of time at different temperature conditions. At the specified time points, the samples were moved to conditions where further degradation can be assumed to be negligible (reference conditions). At the end of the isochronous storage, the samples were analysed simultaneously under repeatability conditions. Analysis of the material (after various exposure times and temperatures) under repeatability conditions greatly improves the sensitivity of the stability tests.

EURM-020 is certified for its genetic identity. As a result, stability behaviour cannot be investigated using a quantitative method and consequently, the inability to estimate stability uncertainties. However, an indication of stability was obtained through a smell-test of the powder upon opening the bottle and analysing the concentration of the extracted DNA and using gel-electrophoresis to examine the quality of the DNA in the extracts. Additionally, the quality of the extracted DNA was tested by performing PCR using the *cytb* and *COI* PCR methods and subsequently comparing the length of the obtained PCR products for each method with the expected PCR product lengths. All stability testing was conducted at the JRC and methods for DNA extraction and PCR were validated at the JRC.

5.1 Short-term stability study

For the short-term stability study, samples were stored at 4 °C, 18 °C and 60 °C for 0, 1, 2 and 4 weeks (at each temperature). The reference temperature was set at -70 °C. Two units per storage time and temperature, were selected using a random stratified sampling scheme. When the stability sample bottles were opened, the smell of the fish powder was described and compared with the smell of the samples stored at the reference temperature. From each bottle, two replicates of between 7 to 8 mg were weighed into 2 mL low-DNA-binding microcentrifuge tubes and were reconstituted in 160 µL nuclease-free water (Promega) and vortexed for 10 seconds. Immediately following reconstitution, the DNA was extracted by following the Animal Tissue Protocol of the DNeasy Blood & Tissue Kit (QIAGEN Benelux BV, Antwerpen, BE). The incubating time was kept at 20 minutes, incubation times between 20 and 30 minutes were found to produce sufficient extracted DNA during the extraction method validation. At the end of the extraction the DNA was eluted in a single eluate of 100 µL of buffer AE. The extracted DNA concentration was measured using UV-spectrophotometry (ND-1000 Spectrophotometer, NanoDrop Technologies Inc., Wilmington, USA). The DNA integrity was assessed by gel-electrophoresis on a Gel-red pre-stained 1 % Agarose gel (PowerPac 3000 Electrophoresis Power Supply, Biorad). PCR product size was assessed through, *cytb* and *COI* specific PCR (C1000 Touch Thermal Cycler, Bio-Rad, NL), using the methods described in Annex A.2 and A.3. PCR product sizes were estimated by comparison to a 100 bp DNA ladder (Invitrogen) using gel-electrophoresis. The measurements were performed under repeatability conditions, the sample replicates were extracted on separate occasions, and a randomised sample sequence was used.

The data were evaluated individually for each temperature. The short-term stability study results for samples stored at 4 °C and 18 °C showed that these samples were stable at these temperatures. The results of the smell test on the powder after the opening of the bottle, the concentration of extracted DNA measured by UV spectrophotometry, gel-electrophoresis assessment of extracted DNA and the *cytb* and *COI* PCR products did not indicate matrix or DNA degradation. The samples stored at 4 °C and 18 °C showed similar results with the samples stored at the reference temperature -70 °C. However, samples stored at 60 °C were found to be unstable at this temperature. These samples smelt different in comparison to samples stored at the reference temperature -70 °C, after opening, indicating decomposition of the fish powder at 60 °C. This was confirmed by the significant decrease in the concentration of extracted DNA from samples stored at 60 °C for one week and the further decline in the samples stored for four weeks. Gel-electrophoresis of the DNA extracts also showed reduced DNA concentrations. Gel-electrophoresis of the *cytb* and *COI* PCR products still showed bands at the expected sizes, the bands were weaker suggesting poor amplification of the degraded template material.

Extracted DNA concentration results measured by UV spectrophotometer for each temperature are shown in Figures 1-3 of Annex C. The results of the smell test, DNA quality tests and gel-electrophoresis are not presented.

The results of the short-term stability study showed that there was significant degradation of the material at 60 °C but no significant degradation was observed at 18 °C and below, therefore, cooled shipment is necessary to maintain sample integrity.

5.2 Long-term stability study

For the long-term stability study, samples were stored at -20 °C and 4 °C for 0, 3, 6, 9 and 12 months (at each temperature) and the isochronous reference temperature was -70 °C. Two samples per storage time and temperature were selected using a random stratified sampling scheme. All the samples were assessed for the smell of the fish powder after opening. From each bottle the DNA of two replicates was extracted and DNA concentration and gel-electrophoresis assessment of extracted DNA and *cytb* and *COI* PCR products were performed as described in Section 5.1.

The long-term stability data were evaluated individually for each temperature. The results of the smell test, the extracted DNA concentration, gel-electrophoresis of the extracted DNA and the *cytb* and *COI* PCR products, showed that for all time points, the tested temperature conditions (4 °C and -20 °C) showed no significant difference to the material stored at the reference temperature. The higher variability of the extracted DNA concentration at time point 6 months at 4 °C was considered as an artefact. It can therefore be concluded that long-term storage at -20 °C or 4 °C has no detrimental impact on the material.

The results of extracted DNA concentration measured by UV spectrophotometer for each temperature of the 12-month stability study are shown in Figures 1 and 2 of Annex D. The results of the smell test, DNA quality tests and gel-electrophoresis are not presented.

The EURM-020 bottles are closed under an argon atmosphere, and the long-term stability test showed that the matrix and DNA are stable when stored at -20 °C and 4 °C. The material can, therefore, be stored at 4 °C. The stability of the material after opening has not been tested.

After the certification study, the material will be included in the JRC's regular stability monitoring programme, to further characterise the stability of the material.

6 Characterisation

The material characterisation is the process of determining the property value of the reference material.

This was based on a comparison between two expert laboratories, i.e. the property of the material was determined in parallel in different laboratories to demonstrate the absence of measurement bias. Due to the nature of the analyte; however, both participants used PCR amplification and bi-directional Sanger sequencing method for the measurements.

6.1 Selection of participants

Two laboratories were selected based on criteria that comprised both technical competence and quality management aspects. Each participant was required to operate a quality system. Having a formal accreditation was not mandatory, but meeting the requirements of ISO/IEC 17025 was obligatory. Where measurements are covered by the scope of accreditation, the accreditation number is stated in the list of participants (Section 2).

6.2 Study setup

The sample preparations, DNA extraction and extracted DNA quality checks, were all performed at the JRC. DNA extracts were sent to both laboratories that independently performed *cytb* and *COI* PCR amplification and Sanger sequencing (bi-directional). The final result (assignation of taxon), is not influenced through the use of a common DNA extraction site, hence the datasets are considered independent.

For the characterisation study, both laboratories received two DNA extracts from each of five bottles of EURM-020. They were requested to provide two independent results, two per bottle and thus two per method (*cytb* and *COI*) per bottle. The five bottles for the material characterisation were selected using a random stratified sampling scheme and covered the whole batch of reference material. For one laboratory, the five bottles for the characterisation study were selected from the homogeneity samples as these had been selected using the same random stratified sampling scheme and therefore reduced the number of samples required and the overall costs of the project. The certified value for this EURM is the assigned identification which is a qualitative property. For such cases, it is not necessary to follow an intermediate precision sample scheme (replicates measured on different days). Consequently, the sample preparation and measurements were completed under repeatability conditions, in a randomised order in a single day.

6.3 Methods used

DNA extraction and extracted DNA quality checks were all performed at the JRC. From each bottle two replicates of between 7 to 8 mg fish powder were weighed into 2 mL low-DNA-binding microcentrifuge tubes. The replicates were divided over two extraction series using a randomised order. The material was reconstituted in 160 µL nuclease-free water (Promega) and vortexed for 10 seconds. Immediately after reconstitution, the DNA was extracted, following the Animal Tissue Protocol from the DNeasy Blood & Tissue Kit (QIAGEN Benelux BV, Antwerpen, BE). The incubating time was 30 minutes. During in-house validation of the extraction method an incubation time between 20 and 30 minutes proved to produce sufficient DNA for PCR amplification and sequencing. The DNA was eluted in a single eluate of 100 µL of buffer AE. The extracted DNA concentration was measured by UV-spectrophotometry (ND-1000 Spectrophotometer, NanoDrop Technologies Inc., Wilmington, USA) and results are shown in Figure 1, Annex E2. The average DNA concentration of the 20 extracts was 128.1 ng/µL ($s_{extracts}$ = 22.6 ng/µL, $s_{\bar{x}bottles}$ = 15.5 ng/µL). The DNA integrity was checked by gel-electrophoresis on a Gel-red pre-stained 1 % Agarose gel (PowerPac 3000 Electrophoresis Power Supply, Biorad). Although some degree of DNA fragmentation was observed for all samples, the quality of the DNA was sufficient for PCR amplification and Sanger sequencing of the targeted DNA barcodes of 413 bp and 652 bp. The samples were diluted with TE low buffer (pH 8,1 mmol/L Tris and 0.01 mmol/L EDTA) to the target DNA concentration and shipped on dry ice conditions to the laboratories listed in Section 2.3.

For the PCR amplification and sequencing both laboratories used the *cytb* primers as described in Table 1 in Annex A1 [10]. The laboratories used the *COI* primers as described in Table 2 in Annex A1 [13, 14, 15], according to the Standard Operating Procedure (SOP) for the genetic identification of fish species using DNA barcoding (mitochondrial *cytrochrome 1* sequencing) prepared by the Labelfish Consortium in 2016. For the *COI* PCR amplification, one laboratory used a cocktail of the two *COI* forward or two *COI* reverse primers in the forward and reverse PCR master-mix compositions respectively, while the second laboratory prepared four different PCR master-mix compositions using single *COI* primers. The PCR chemistry used and thermocycler conditions were slightly deviating from the SOP for both laboratories.

Successful amplification of the *cytb* and *COI* barcode regions were confirmed by either gel-electrophoresis or capillary electrophoresis of the PCR products. The purified PCR products of *cytb* and *COI* were bi-directionally Sanger sequenced by each of the laboratories using their own sequencing protocols.

All sequencing data were analysed at the JRC using the SeqManPro application within Lasergene 15.1 software (DNASTAR, Madison, US) [37]. The data for the two barcodes (*cytb* and *COI*) were analysed separately.
The resulting consensus sequences were uploaded to GenBank's BLAST [18, 19] and BOLD [20] identification tools for genetic identification of the fish powder. The reference sequences for the *cytb* barcode in the alignment hit table in the output from BLAST were checked for voucher status, related publication and morphological identification. Reference sequences that had suspicious genetic and morphological identification were no longer used for identification. The reference

sequences in the hit list from the BOLD identification tool for the *COI* barcode were checked similarly.

For each barcode, *cytb* and *COI*, the consensus sequences per extract were aligned per bottle to obtain one sequence per bottle. A consensus sequence per laboratory was aligned from the five consensus sequences per barcode. A final consensus sequence for the *cytb* and the *COI* barcode was aligned with the sequences from both the laboratories and are given in FASTA format in Annex E1.

6.4 Evaluation of results

The characterisation study resulted in two datasets for each of the *cytb* and *COI* barcode, thus four datasets in total. The taxon assignment results obtained by the JRC by analysing the data from the participants, grouped per laboratory and barcode method are displayed in Table 1 in Annex E1.

6.4.1 Technical evaluation

The sequencing data obtained from each laboratory were first checked for compliance with the requested analysis protocol and for their validity based on technical reasons. The following criteria were considered during the evaluation:

- Valid PCR-sequencing procedure
- Compliance with the analysis protocol: analytical sequence according to the prepared scheme
- PCR products showing a single band of about 464 and 738 bp in size for *cytb* and *COI* respectively as visualised on a gel
- Quality of electropherograms; Bi-directional sequences, check for ambiguous bases in the contig and sequence length (at least 360 and 600 bp for *cytb* and *COI* respectively to obtain optimum length for differentiation between *Hippoglossus hippoglossus* and the sister species *Hippoglossus stenopelis*)

The successful amplification of the DNA using the requested methods and the use of the specific primers as described in Annex A1 for the barcodes, *cytb* and *COI,* was checked by gel-electrophoresis or capillary electrophoresis. The PCR products showed a single band of the expected length of approximately 464 bp and 738 bp for *cytb* and *COI* respectively. The PCR products were purified, and Sanger sequenced according to Standard Operating Procedures meeting the requirement of ISO/IEC 17025. The sequences obtained were clean and reached suitable lengths for species identification, > 384 bp for *cytb* and > 634 bp for *COI*. The two independent data sets per method (*cytb* and *COI*) gave identical sequence results. The identification resulted in the same taxon identification for all characterisation samples for both methods (*cytb* and *COI*) and from both laboratories. The individual laboratories have species identification methods under their scope of accreditation. However, from the requested methods and the related primers, only the *cytb* method of one laboratory was covered under their scope of accreditation. For the other lab, this specific *cytb* method was new. Both laboratories used the primers mentioned in the SOP for the *COI* method, but the chemistry and PCR conditions were slightly changed; thermocycler temperatures were optimised and different PCR master mixes were used. This implies that for the *cytb* method for one laboratory and for the *COI* method for both laboratories the PCR amplification was not fully validated. The quality of the subsequent results were, however, controlled and checked using gel- or capillary electrophoresis results and the quality of the electropherograms produced in sequencing. Additionally, the similarity of all the sequencing results between the laboratories gave a good indication that the methods used were valid for the genetic identification of the species in this EURM. Based on the above mentioned, it was concluded that the datasets were technically valid.

6.4.2 Evaluation of the genetic identification for the *cytb* barcode

The two data sets for the *cytb* barcode, five bottles per laboratory and two DNA extracts per bottle, were analysed separately. Alignment was performed using the SeqManPro application within Lasergene 15.1 software (DNASTAR, Madison, US) [37]. The forward and reverse sequences were analysed and aligned to one consensus sequence per extraction replicate. Regions of low quality at either end of the electropherogram were removed and, where possible, ambiguous bases and no base calls were checked against the sense or anti-sense sequence and manually corrected.

The 10 consensus extraction sequences per laboratory were compared to the reference sequence for *Hippoglossus hippoglossus* (EU513792.1 in GenBank [16]). From the *cytb* analysis of the two sets of 10 extraction sequences, 17 of the obtained consensus sequences covered the complete length (413 bp) of the *cytb* barcode. The three remaining samples were shortened due to poorer quality base calls at the extremes of the barcodes (384, 397 and 405 bp). No sequence differences were observed between the samples however, there was one mismatching base between the consensus sequences and the reference sequence for *Hippoglossus hippoglossus* (EU513792.1 in GenBank [16]). The mismatching nucleotide is highlighted in the *cytb* barcode sequence given in Annex E1.

Additionally, all samples were aligned to a reference sequence of the sister species *Hippoglossus stenopelis* (EU513796.1 in NCBI GenBank [16]). All the sequences deviated for the same seven bases with this reference, including the above mentioned mismatching base.

The two replicate sequences per bottle were aligned to obtain a consensus sequence per bottle and further used for species identification by using Megablast in the Standard Nucleotide Basic Local Alignment Search Tool BLAST® software, (version BLASTN 2.9.0+) (National Center for Biotechnology Information (NCBI), U.S. National Library of Medicine, Bethesda, US) [18, 19]. The BLASTN algorithm compares nucleotide sequences pairwise; searching for local regions of similarity in nucleotide sequences against the nucleotide database and calculates the statistical significance of matches [18]. Sequence similarity in BLAST refers to identity: similarity between two sequences; exactly the same nucleotide at the same position in the aligned sequences [38, 39, 40] and is expressed as % identity (Amount of identical nucleotides / length of the shortest of the compared sequences).

The length of these consensus sequences for all bottles covered the whole length of the *cytb* barcode (413 bp). Nucleotide BLAST produced an alignment hit table with 100 nucleotide sequence database entries of different species within the family Pleuronectidae (righteye flounders), of which, the three species with the highest similarity are: *Hippoglossus hippoglossus, Hippoglossus stenolepis, Reinhardtius hippoglossoides*. The 12 *Hippoglossus hippoglossus* reference sequences hits varied in overall length between 402 bp as a part of the *cytb* gene and the whole length of the mitochondrial DNA.

All consensus sequences of the bottles used for the characterisation study showed for the *cytb* method a very high similarity with 11 *Hippoglossus hippoglossus* reference sequences ranging from 99.8 % to 99.3 % identity (Table 1). For the sister species *Hippoglossus stenolepis* (Pacific halibut) it ranged from 98.3 % to 97.8 % and for *Reinhardtius hippoglossoides* (Greenland halibut), from 92.7 % to 91.0 %. One *Hippoglossus hippoglossus* reference sequence (Genbank MH031842.1) [21] showed deviating results for the similarity with the samples. Detailed information in the related publication mentions that this *Hippoglossus hippoglossus* was revealed as *Hippoglossus stenolepis* and according to the authors statement, they *'could not make any strong inference about the taxonomic status of this species of halibut because it lacks a voucher specimen*' [21]. Therefore, this reference sequence was excluded for the identification of the EURM-020 samples. The sequences of the characterisation samples deviated up to three nucleotides with the 11 reference sequences for *Hippoglossus hippoglossus* in the hit-list. Therefore, they could not reach 100 % identity. A summary of the similarity results and reference sequences used is given in Table 1. All the consensus sequences from the bottles had at least seven different bases compared to *Hippoglossus stenolepis* and at least 30 compared to *Reinhardtius hippoglossoides.*

The identification for the *cytb* barcode for the consensus sequences per bottle resulted in 99.8 % to 99.3 % identity with *Hippoglossus hippoglossus*. Due to mismatching nucleotides within the sequences of the individual reference sequences used for the *cytb* barcode identification and the different lengths of the sequences compared, outcomes vary between reference sequences. They are given here in a range of identity percentage instead of a fixed percentage. The identity percentage never exceeded 99.8 % for the *cytb* barcode because of the same mismatching nucleotide in the EURM-020 samples compared with all the *Hippoglossus hippoglossus* reference sequences used.

Table 1: Average sequence alignment similarity results of the characterisation samples of EURM-020, expressed as % identity, compared with 11 *Hippoglossus hippoglossus* reference sequences in GenBank for the *cytb* barcode using BLASTN 2.9.0+ (NCBI).

GenBank accession code of *cytb* reference sequence	**Average identity [%] of EURM-020 with *cytb* reference sequence**	**Taxon identity of *cytb* reference sequence**	**Nucleotide mismatches of samples of EURM-020 with reference sequence**	**Voucher ID**
EU513791.1 [16]	99.8	*Hippoglossus hippoglossus*	1	n.a.
AM749125.1 [17]	99.8	*Hippoglossus hippoglossus*		n.a.
AM749124.1 [17]	99.8	*Hippoglossus hippoglossus*		n.a.
AM749122.1 [17]	99.8	*Hippoglossus hippoglossus*		n.a.
AF413798.1 [24]	99.8	*Hippoglossus hippoglossus*		n.a.
EU492150.1 [22]	99.8	*Hippoglossus hippoglossus*		MNHN 2005-1582
EU492256.1 [23]	99.8	*Hippoglossus hippoglossus*		NRM 53139
EU513790.1 [16]	99.5	*Hippoglossus hippoglossus*	2	n.a.
AM749123.1 [17]	99.5	*Hippoglossus hippoglossus*		n.a.
HQ283277.1 [30]	99.5	*Hippoglossus hippoglossus*		n.a.
AF413799.1 [24]	99.3	*Hippoglossus hippoglossus*	3	n.a.

6.4.3 Evaluation of the genetic identification for the *COI* barcode

The two data sets for the *COI* barcode, five bottles per laboratory and two DNA extracts per bottle, were analysed separately. From each extract, the forward and reverse sequences were analysed and aligned to one consensus sequence per sample using the SeqManPro application within Lasergene 15.1 software (DNASTAR, Madison, US) [37]. Regions of low quality at either end of the electropherogram were removed and, ambiguous bases and no base calls were checked against the sense or anti-sense sequence and manually corrected where possible.

The 10 consensus extraction sequences per laboratory were compared to the *Hippoglossus hippoglossus* reference sequence (GBMTG1322.16 in BOLD/ mined from NC_009709.1 in GenBank [17, 36]). From the two sets of 10 sample sequences for the *COI* analysis from each lab, the length of the sequences was found to be between 626 bp and 652 bp. Of those 20 sequences, 17 were shorter than the complete *COI* barcode region (652 bp) due to poorer quality base calls at the extremes. No sequence differences were observed between the samples. However, there was one mismatching base between the consensus sequences and the reference sequence. The mismatching nucleotide is highlighted in the *COI* barcode sequence given in Annex E1.

The sequences were also aligned to a reference sequence of the sister species *Hippoglossus stenopelis*. All extraction sequences showed the same 13 deviating bases compared to the reference (GBMTG908_16 in BOLD, mined from NC_009710 in NCBI GenBank [17]) of the sister species *Hippoglossus stenolepis*.

The two sample sequences per bottle were aligned to obtain a consensus sequence per bottle and further used for species identification. For three of the consensus sequences, the entire region of the COI barcode (652 bp) was covered. For the remaining seven consensus sequences, the lengths varied from 634 bp to 651 bp. Identification for the *COI* method was performed using the BOLD Identification Systems v4 [20]. The search database for animal identification used, the Public Record Barcode Database, contains all published *COI* records from BOLD with a minimum sequence length of 500 bp and is a library with a collection of records from the published projects section of BOLD. In BOLD, nucleotide sequences are first translated into amino acids sequences, verified that they originate from the COI protein and, after passing some additional quality checks, a linear search of the reference library is followed [20]. Sequence similarity in BOLD refers to the ratio to which nucleotide or protein sequences are related and takes substitutions into account; expressed as similarity percentage [39, 40]. For sequences showing less than 1 % divergence to a reference sequence, the BOLD identification system delivers a species identification [20]. The BOLD identification application generated a hit list of the order Pleuronectiformes (flatfish) and family Pleuronectidae of 99 individuals of species. The species that gave the highest similarity are: *Hippoglossus hippoglossus, Hippoglossus stenolepis* and *Reinhardtius hippoglossoides.* The 25 *Hippoglossus hippoglossus* reference sequence hits varied in length between 606 bp and 1563 bp for the *COI* gene region.

All consensus sequences of the bottles used for the characterisation study for the *COI* barcode had comparable similarity ranges using the BOLD identification application. The sequence similarity for *Hippoglossus hippoglossus* ranged between 99.8 % and 99.4 % and the samples were all identified as *Hippoglossus hippoglossus* (Table 2). For the sister species *Hippoglossus stenolepis* similarity ranged from 98.3 % to 97.6 % and for *Reinhardtius hippoglossoides* from 93.1 % to 92.7 %. One of the 25 *Hippoglosssus hippoglossus* reference sequences in the hit list (GBMIN131002-17 in BOLD, KX164003 in Genbank [33]) showed a deviating alignment with the samples. Upon closer investigation, the neighbour-joining tree (Kimura 2-parameter distance model for *COI*-5P marker) in BOLD [20, 31, 32] showed that this reference sequence is clustered within the *Hippoglossus stenopelis* sister species. Because of the lack of a publication and therefore proof for morphological taxonomy for this reference sequence it has been excluded from further analysis of the data.

Using the SeqManPro application within the Lasergene 15.1 software [37] revealed that within the *COI* barcode region of the characterisation samples, there was one deviating nucleotide, compared to all 24 *Hippoglossus hippoglossus* reference sequences in the hit list from the BOLD application. The sequences of the characterisation samples deviated up to four nucleotides with the reference sequences in the hit list (Table 2). All the characterisation samples had at least 11 different bases compared to *Hippoglossus stenolepis* and at least 44 compared to *Reinhardtius hippoglossoides*.

Also, a BLAST search for the *COI* barcode gave comparable results for the sample replicate sequences, from 99.8 % to 99.3 % identity with *Hippoglossus hippoglossus*.

For the *COI* barcode, the identification of the consensus sequences per bottle resulted in 99.8 % to 99.4 % similarity with *Hippoglossus hippoglossus*. Due to mismatching nucleotides within the sequences of the individual reference sequences used for the *COI* barcode, identification outcomes vary between reference sequences. They are given here in a range of similarity instead of a fixed percentage. The similarity for the *COI* barcode was never higher than 99.8 % because of the same mismatching nucleotide in the EURM-020 samples compared with all reference sequences.

Table 2: Average sequence alignment similarity results of characterisation samples of EURM-020, expressed as % similarity, compared with 24 *Hippoglossus hippoglossus* reference sequences for the *COI* barcode using the BOLD system v4 identification tool (Animal Public Record Barcode Database)

BOLD process ID of *COI* reference sequence	**GenBank accession code of *COI* reference sequence**	**Average similarity [%] of EURM-020 with *COI* reference sequence**	**Taxon identity of *COI* reference sequence**	**Nucleotide mismatches of samples EURM-020 with reference sequence**	**Voucher ID**
GBMNA14652-19	AM749122.1 [17]	99.8	*Hippoglossus hippoglossus*	1	n.a.
GBGC3118-07	AM749123.1 [17]	99.8	*Hippoglossus hippoglossus*		n.a.
SCFAC777-06	KC015478.1 [25]	99.8	*Hippoglossus hippoglossus*		06-604
SCFAC105-05	KC015480.1 [25]	99.8	*Hippoglossus hippoglossus*		05-406-006d4
SCAFB738-07	KC015482.1 [25]	99.8	*Hippoglossus hippoglossus*		07-126
GBGCA8489-15	KJ128511.1 [26]	99.8	*Hippoglossus hippoglossus*		NRM:53139
GBMTG1322-16	NC_009709.1 [17][36]	99.8	*Hippoglossus hippoglossus*		n.a.
GBGC3116-07	AM749125.1 [17]	99.7	*Hippoglossus hippoglossus*	2	n.a.
FOAE640-06 [27]	n.a.	99.7	*Hippoglossus hippoglossus*		BW-A2852/ CSIRO H 4303-12
GLF055-14 [28]	n.a.	99.7	*Hippoglossus hippoglossus*		GLF055
SCFAC836-06	KC015479.1 [25]	99.7	*Hippoglossus hippoglossus*		06-889
SCFAC657-06	KC015481.1 [25]	99.7	*Hippoglossus hippoglossus*		06-739
SCFAC110-05	KC015483.1 [25]	99.7	*Hippoglossus hippoglossus*		05-399-006e3
SCFAC648-06	KC015484.1 [25]	99.7	*Hippoglossus hippoglossus*		06-724
BNSF511-12	KJ204922.1 [29]	99.7	*Hippoglossus hippoglossus*		MT02932
BNSF534-12	KJ204923.1 [29]	99.7	*Hippoglossus hippoglossus*		MT02955
BNSF645-12	KJ204924.1 [29]	99.7	*Hippoglossus hippoglossus*		MT03047
BNSFI074-12	KJ204925.1 [29]	99.7	*Hippoglossus hippoglossus*		MT04123
GBGC3117-07	AM749124.1 [17]	99.5	*Hippoglossus hippoglossus*	3	n.a.
SCAFB551-07	KC015477.1 [25]	99.5	*Hippoglossus hippoglossus*		06-786
GBGC7349-09	EU513652.1 [16]	99.4	*Hippoglossus hippoglossus*	4	n.a.
GBGC7348-09	EU513653.1 [16]	99.4	*Hippoglossus hippoglossus*		n.a.
GBGC7347-09	EU513654.1 [16]	99.4	*Hippoglossus hippoglossus*		n.a.
FOAE641-06 [26]	n.a.	99.4	*Hippoglossus hippoglossus*		BW-A2853

7 Value Assignment

A certified value was assigned.

Certified values are values that fulfil the highest standards of accuracy. Two datasets were used to assign the certified value, the identity, of EURM-020 and is based on two genetic identification methods using two different mitochondrial DNA barcodes, *cytb* and *COI* measured by bi-directional Sanger sequencing. The certified value, the taxon assignment for EURM-020, refers to reference sequences in the GenBank (NCBI) and FishTrace (EC) databases for *cytb* and the BOLD database for *COI*.

The length of the resultant sequence of EURM-020 for the *cytb* barcode was 413 bp and for the *COI* barcode 652 bp. The sequences of EURM-020 for both barcodes are given in FASTA format in Annex E1.

The assigned taxon, for all characterisation samples from both datasets for the *cytb* barcode, was *Hippoglossus hippoglossus,* based on the highest scores of similarity, 99.8 % identity, with the *Hippoglossus hippoglossus* reference sequences retrieved by the BLAST search tool (version BLASTN 2.9.0+NCBI) [18, 19]). The *cytb* sequences of the characterisation samples mismatched one nucleotide with the reference sequences with the highest similarity scores. The assigned taxon is linked to the reference sequences in the GenBank (NCBI) and FishTrace (EC) databases (Table 3).

Table 3: *Hippoglossus hippoglossus* reference sequences for the *cytb* barcode in GenBank used for taxon assignment of EURM-020.

GenBank accession code of *cytb* reference sequence	**Average identity [%] of EURM-020 with *cytb* reference sequence**	**Taxon identity of *cytb* reference sequence**	**Nucleotide mismatches of samples of EURM-020 with reference sequence**	**Voucher ID**
EU513791.1 [16]	99.8	*Hippoglossus hippoglossus*	1	n.a.
AM749125.1 [17]	99.8	*Hippoglossus hippoglossus*		n.a.
AM749124.1 [17]	99.8	*Hippoglossus hippoglossus*		n.a.
AM749122.1 [17]	99.8	*Hippoglossus hippoglossus*		n.a.
AF413798.1 [24]	99.8	*Hippoglossus hippoglossus*		n.a.
EU492150.1 [22]	99.8	*Hippoglossus hippoglossus*		MNHN 2005-1582
EU492256.1 [23]	99.8	*Hippoglossus hippoglossus*		NRM 53139

The taxon assignment for all characterisation samples from both datasets for the *COI* barcode, was *Hippoglossus hippoglossus*, based on the highest scores, 99.8 % similarity with *Hippoglossus hippoglossus* reference sequences retrieved by the Data Identification Systems v4 of BOLD [20]. The *COI* sequences of the characterisation samples mismatched one nucleotide with the reference sequences with the highest similarity scores. The assigned taxon is linked to the reference sequences in the BOLD Animal Public Record Barcode Database (Table 4).

Table 4: *Hippoglossus hippoglossus* reference sequences for the *COI* barcode in the BOLD Animal Public Record Barcode Database used for taxon assignment of EURM-020.

BOLD process ID reference sequence	**GenBank accession code of *COI* reference sequence**	**Average similarity [%] of EURM-020 with *COI* reference sequence**	**Taxon identity of *COI* reference sequence**	**Nucleotide mismatches of samples EURM-020 with reference sequence**	**Voucher ID**
GBMNA14652-19	AM749122.1 [17]	99.8	*Hippoglossus hippoglossus*	1	n.a.
GBGC3118-07	AM749123.1 [17]	99.8	*Hippoglossus hippoglossus*		n.a.
SCFAC777-06	KC015478.1 [25]	99.8	*Hippoglossus hippoglossus*		06-604
SCFAC105-05	KC015480.1 [25]	99.8	*Hippoglossus hippoglossus*		05-406-006d4
SCAFB738-07	KC015482.1 [25]	99.8	*Hippoglossus hippoglossus*		07-126
GBGCA8489-15	KJ128511.1 [26]	99.8	*Hippoglossus hippoglossus*		NRM:53139
GBMTG1322-16	NC_009709.1 [17][36]	99.8	*Hippoglossus hippoglossus*		n.a.

The species identity for EURM-020 was defined by a minimum 99 % sequence similarity with the DNA sequences of the specimens (voucher samples and specimens with morphological identification), deposited in the GenBank (NCBI), FishTrace (EC) and BOLD databases. It is unlikely that there is an alternative species that shares the sequences of the two independent genes used in this study, therefore, there is no doubt regarding the certified identity, *Hippoglossus hippoglossus*, for EURM-020. Consequently, the uncertainty is negligible.

8 Metrological traceability and commutability

8.1 Metrological traceability

Identity

The genetic identity is a clearly defined molecular property. The genetic identity was confirmed by two different mtDNA barcode methods, *cytb* and *COI*, and is traceable to DNA reference sequences of which the morphological identity of the related species was executed, in the GenBank (NCBI), FishTrace (EC) and BOLD databases.

8.2 Commutability

The commutability of a CRM defines its fitness for use and is, therefore, a crucial characteristic when applying different measurement methods. When the commutability of a CRM is not established, the results from routinely used methods cannot be legitimately compared with the certified value.

The CRM is prepared from fresh fish tissue that was dried and milled to a powder. The certified value established, was obtained by PCR amplification and bi-directional Sanger sequencing of two different DNA barcodes, *cytb* and *COI*. After reconstitution, the material closely resembles samples of fresh fillet. The analytical behaviour will, therefore, be the same as is the case for fresh fish tissue samples tested with these DNA barcodes using PCR sequencing.

9 Instructions for use

9.1 Safety information

The usual laboratory safety measures apply.

The material is for in vitro use only.

9.2 Storage conditions

The materials should be stored at 4 ± 3 °C in the dark. Care should be taken to avoid any changes in the moisture content once the units are opened since, the material is hygroscopic. The user should close any bottles immediately after taking a sample.

Please note that the European Commission cannot be held responsible for changes that occur during storage of the material at the customer's premises, especially for opened bottles.

9.3 Preparation and use of the material/Reconstitution

To prepare for use, the material must be reconstituted according to the following procedure: Weigh 7 to 8 mg of the fish powder into a 2 mL low-DNA-binding microcentrifuge tube and add 160 µL nuclease-free water, vortex for 10 seconds and immediately continue with a DNA extraction protocol.

9.4 Minimum sample intake

EURM-020 is a pure fish powder originating from a single fish; therefore, the minimum sample intake for this material is not linked to the within-unit homogeneity. Nevertheless, to obtain a significant amount of DNA, it is recommended to use a minimum sample intake of 7 mg when using the extraction method used in this study.

9.5 Use of the certified value

The main purpose of this material is to assess method performance using PCR and/or sequencing, i.e. for checking the accuracy of analytical results. As with any reference material, it can also be used for validation studies.

10 Acknowledgements

The authors would like to acknowledge the support received from various contributors of the JRC, Directorate F, among them are: O. De Rudder for the delivery of the raw material for the processing of this EURM, P. Conneely and M.-F. Tumba-Tshilumba for the KFT and PSA measurements, A. Lieven and V. Paracchini for sequencing measurements of the raw material for this EURM, and F. Vanderveken for the set-up of the required isochronous studies.

Furthermore, the authors would like to thank L. Deprez and A. Held (JRC, Directorate F) for reviewing the certification report, as well as the experts of the Reference Material Review Panel "Biological Macromolecules and Biological/Biochemical Parameters", G. Berben (Centre wallon de Recherches agronomiques, Gembloux, Belgium) and H. Hird (Manchester, UK) for their constructive comments.

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Annexes

Annex A: Summary of methods used

Annex A.1: Primers for PCR-sequencing for the *cytb* and *COI* genes used for homogeneity and characterisation studies:

Table 1: PCR and sequencing primers *cytb* [1)]

Primer name	**Sequence 5' – 3'**	**Reference**
Forward primer L14735	AAA AAC CAC CGT TGT TAT TCA ACT A	[10]
Reverse primer H15149ad	GCN CCT CAR AAT GAY ATT TGT CCT CA	[10]

1) The expected size of the PCR product is 464 bp

Table 2: PCR and sequencing primers *COI* [1)]

Primer name, PCR	**Sequence 5' – 3'** [2) 3)]	**Reference**
VF2_t1	TGTAAAACGACGGCCAGTCAA CCA ACC ACA AAG ACA TTG GCA C	[14, 13]
FishF2_t1	TGTAAAACGACGGCCAGTCGA CTA ATC ATA AAG ATA TCG GCA C	[14, 13]
FishR2_t1	CAGGAAACAGCTATGACACT TCA GGG TGA CCG AAG AAT CAG AA	[14, 13]
FR1d_t1	CAGGAAACAGCTATGACACC TCA GGG TGT CCG AAR AAY CAR AA	[15, 13]
Primer name, sequencing	**Sequence 5' – 3'**	**Reference**
M13_F	TGTAAAACGACGGCCAGT	[35]
M13_R	CAGGAAACAGCTATGAC	[35]

1) The expected size of the PCR product is 738 bp
2) Primers with highlighted M13 tails
3) One laboratory performed the sequencing of the homogeneity and characterisation samples by using a cocktail of two forward or two reverse primers in the respective forward and reverse master-mix compositions. The second laboratory that performed the sequencing of the second set of characterisation samples used four separate PCR master-mix compositions with single primers.

Annex A.2 Method for *cytb* specific PCR used for stability measurements:

Table 1: Primer sequences *cytb* PCR [1)]

Primer name, PCR	**Sequence 5' – 3'**	**Reference**
L14735	AAA AAC CAC CGT TGT TAT TCA ACT A	[10]
H15149ad	GCN CCT CAR AAT GAY ATT TGT CCT CA	[10]

1) The expected size of the PCR product is 464 bp

Table 2: Cycling conditions *cytb* PCR (C1000 Touch Thermal Cycler, Bio-Rad, NL)

Section	**Temperature °C**	**Time**	**Cycles**
Hot start	95	11 min	1
Denaturation	95	45 s	35
Annealing	50	80 s	
Elongation	72	90 s	
Final elongation	72	30 min	1
Cooling	10	∞	1

Table 3: Master-mix composition for *cytb* PCR reaction

Reagent	**Concentration Stock**	**Concentration Final solution**	**µl per reaction**	**Supplier**
Buffer	10 X	1 x	5.00	AmpliTaq Gold PCR buffer, ThermoFisher scientific, Gent, BE
Primer F: L14735	7.5 µM	0.5 µM	3.33 [1)]	
Primer R: H15149ad	7.5 µM	0.5 µM	3.33 [1)]	
dNTPs	10 mM	250 µM	1.25	dNTP mix, 10 mMol each, ThermoFisher scientific, Gent, BE
Polymerase	5 U/µL	0.05 U/µL	0.50	AmpliTaq Gold DNA polymerase, ThermoFisher scientific, Gent, BE
$MgCl_2$	25 mM	3.0 mM	6.00	$MgCl_2$, AmpliTaq Gold DNA polymerase kit, ThermoFisher scientific, Gent, BE
Water			27.59 [1)]	Promega Benelux, Leiden, NL
DNA	~20 ng/µL	~60 ng/rxn	3.00	DNA concentration according Nanodrop
Total volume (µl)			50.00	

1) For the stability study one PCR reaction was prepared using a cocktail of the forward and reverse primer together in one master-mix composition.

Annex A.3 Method for *COI* specific PCR according standard operating procedure from the Labelfish Consortium used for stability measurements [13]:

Table 1: Primer sequences *COI* PCR [1)]

Primer name, PCR	**Sequence 5' – 3' [2)]**	**Reference**
VF2_t1	TGTAAAACGACGGCCAGTCAA CCA ACC ACA AAG ACA TTG GCA C	[14, 13]
FishF2_t1	TGTAAAACGACGGCCAGTCGA CTA ATC ATA AAG ATA TCG GCA C	[14, 13]
FishR2_t1	CAGGAAACAGCTATGACACT TCA GGG TGA CCG AAG AAT CAG AA	[14, 13]
FR1d_t1	CAGGAAACAGCTATGACACC TCA GGG TGT CCG AAR AAY CAR AA	[15, 13]

1) The expected size of the PCR product is 738 bp
2) Primers with highlighted M13 tails

Table 2: Cycling conditions *COI* PCR (C1000 Touch Thermal Cycler, Bio-Rad, NL)

Section	**Temperature °C**	**Time**	**Cycles**
Hot start	94	2 min	1
Denaturation	94	30 s	35
Annealing	52	40 s	
Elongation	72	60 s	
Final elongation	72	10 min	1
Cooling	10	∞	1

Table 3: Master-mix composition for *COI* PCR reaction

Reagent	**Concentration Stock**	**Concentration Final solution**	**µl per reaction**	**Supplier**
D(+)-Trehalose dihydrate	10 %	5 %	10.00	Alfa Aesar ,VWR, Leuven, BE
Buffer	10 X	1 x	2.00	Reaction buffer, Bioline, GCbiotech, Waddinxveen, NL
Forward primers VF2_t1 and FishF2_t1	10 µM	0.1 µM each	0.20 each [1)]	
Reverse primers FishR2_t1 and FR1d_t1	10 µM	0.1 µM each	0.20 each [1)]	
dNTPs	10 mM	200 µM	0.40	dNTP mix, Bioline, GCbiotech, Waddinxveen, NL
Polymerase	5 U/µL	0.025 U/µL	0.10	Biotaq DNA polymerase, Bioline, GCbiotech, Waddinxveen, NL
$MgCl_2$	50 mM	2.5 mM	1.00	$MgCl_2$, Bioline
Water			2.70 [1)]	Promega Benelux, Leiden, NL
DNA	~20 ng/µL	~60 ng/rxn	3.00	DNA concentration according Nanodrop
Total volume (µl)			20.00	

1) For the stability study, one PCR reaction was prepared using a cocktail of the two forward and two reverse primers together in one master-mix composition.

Annex B: Results of the homogeneity measurements

Annex B1: Average extracted DNA concentration per bottle

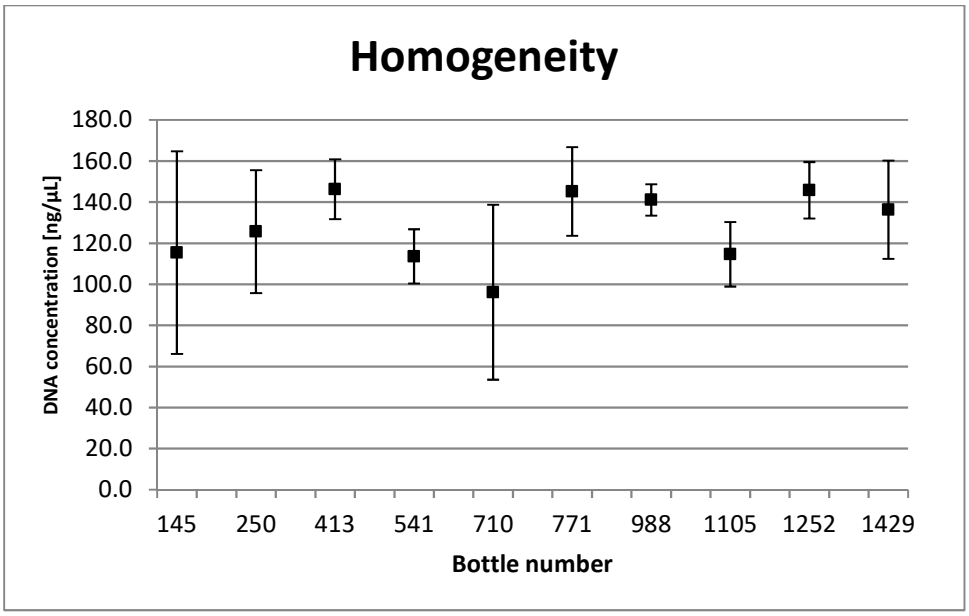

Figure 1: Extracted DNA concentration [ng/µL] per bottle measured for the homogeneity study of EURM-020. Each data point represents the average of 2 extraction duplicates from each bottle, N = 1, n = 2 with error bars expressed as s_{meas}. Measurements were conducted by using UV spectrophotometry (Nanodrop).

Annex B2: Results of the homogeneity measurements: *cytb*

Table 1: Homogeneity results EURM-020 of the species identification for the *cytb* barcode using BLASTN 2.9.0+ (NCBI). Maximum and minimum identity results of the sequence of the extraction replicates per bottle, *N* = 10, *n* = 2, compared with 11 different *Hippoglossus hippoglossus* reference sequences in GenBank (NCBI).

Bottle Number-replicate	**Species identification**	**Maximum identity [%]** [1)]	**Minimum identity [%]** [1)]
145-1	*Hippoglossus hippoglossus*	99.8	99.3
145-2	*Hippoglossus hippoglossus*	99.7	99.2
250-1	*Hippoglossus hippoglossus*	99.8	99.3
250-2	*Hippoglossus hippoglossus*	99.7	99.0
413-1	*Hippoglossus hippoglossus*	99.7	99.2
413-2	*Hippoglossus hippoglossus*	99.7	99.2
541-1	*Hippoglossus hippoglossus*	99.8	99.3
541-2	*Hippoglossus hippoglossus*	99.8	99.3
710-1	*Hippoglossus hippoglossus*	99.7	99.0
710-2	*Hippoglossus hippoglossus*	99.8	99.3
771-1	*Hippoglossus hippoglossus*	99.8	99.3
771-2	*Hippoglossus hippoglossus*	99.8	99.3
988-1	*Hippoglossus hippoglossus*	99.8	99.3
988-2	*Hippoglossus hippoglossus*	99.8	99.3
1105-1	*Hippoglossus hippoglossus*	99.7	99.0
1105-2	*Hippoglossus hippoglossus*	99.8	99.3
1252-1	*Hippoglossus hippoglossus*	99.7	99.2
1252-2	*Hippoglossus hippoglossus*	99.8	99.3
1429-1	*Hippoglossus hippoglossus*	99.8	99.3
1429-2	*Hippoglossus hippoglossus*	99.8	99.3

1) % Identity = Amount of identical nucleotides / minimum aligning length.

Annex B3: Results of the homogeneity measurements: *COI*

Table 1: Homogeneity results EURM-020 species identification for the *COI* barcode using the BOLD system v4 identification tool. Maximum and minimum similarity results of the sequence of the extraction replicates per bottle, *N* = 10, *n* = 2, compared with 24 *Hippoglossus hippoglossus* reference sequences in the BOLD Animal Public Record Barcode Database.

Bottle Number-replicate	**Species identification**	**Maximum similarity [%] [1)]**	**Minimum similarity [%] [1)]**
145-1	*Hippoglossus hippoglossus*	99.8	99.4
145-2	*Hippoglossus hippoglossus*	99.8	99.4
250-1	*Hippoglossus hippoglossus*	99.8	99.4
250-2	*Hippoglossus hippoglossus*	99.8	99.4
413-1	*Hippoglossus hippoglossus*	99.8	99.4
413-2	*Hippoglossus hippoglossus*	99.8	99.4
541-1	*Hippoglossus hippoglossus*	99.8	99.4
541-2	*Hippoglossus hippoglossus*	99.8	99.4
710-1	*Hippoglossus hippoglossus*	99.8	99.4
710-2	*Hippoglossus hippoglossus*	99.8	99.4
771-1	*Hippoglossus hippoglossus*	99.8	99.4
771-2	*Hippoglossus hippoglossus*	99.8	99.4
988-1	*Hippoglossus hippoglossus*	99.8	99.4
988-2	*Hippoglossus hippoglossus*	99.8	99.4
1105-1	*Hippoglossus hippoglossus*	99.8	99.4
1105-2	*Hippoglossus hippoglossus*	99.8	99.4
1252-1	*Hippoglossus hippoglossus*	99.8	99.4
1252-2	*Hippoglossus hippoglossus*	99.8	99.4
1429-1	*Hippoglossus hippoglossus*	99.8	99.4
1429-2	*Hippoglossus hippoglossus*	99.8	99.4

1) The statistics related to the aligned sequences in BOLD, is expressed in similarity percentage.

Annex C: Short-term stability study results

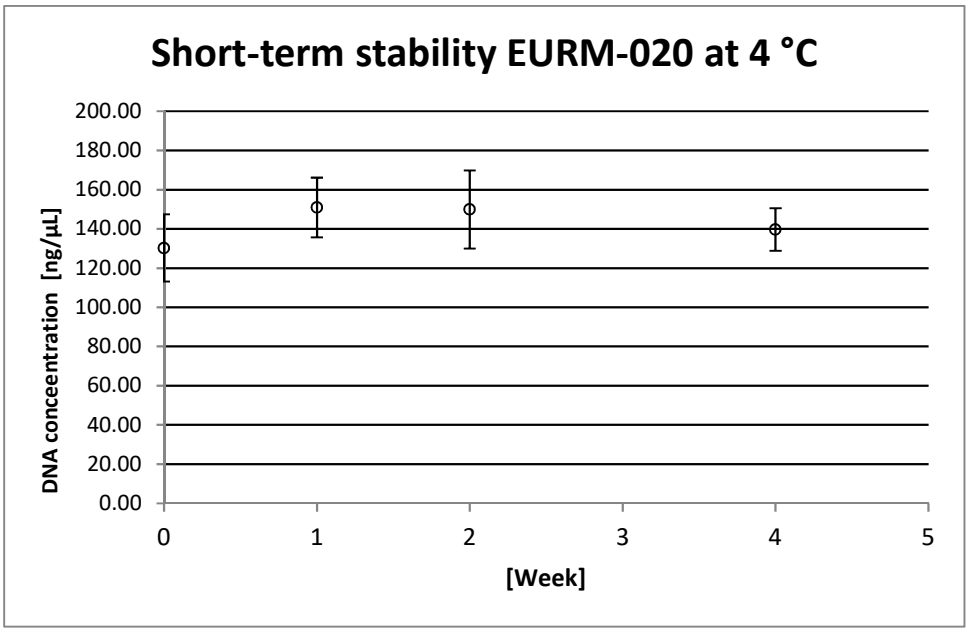

Figure 1: Extracted DNA concentration [ng/µL] of the samples included in the short-term stability study of EURM-020 at 4 °C. Average of 2 samples of each 2 extraction duplicates, N = 2, n = 2 with error bars expressed as s_{meas}. Measurements performed by UV spectrophotometry (Nanodrop).

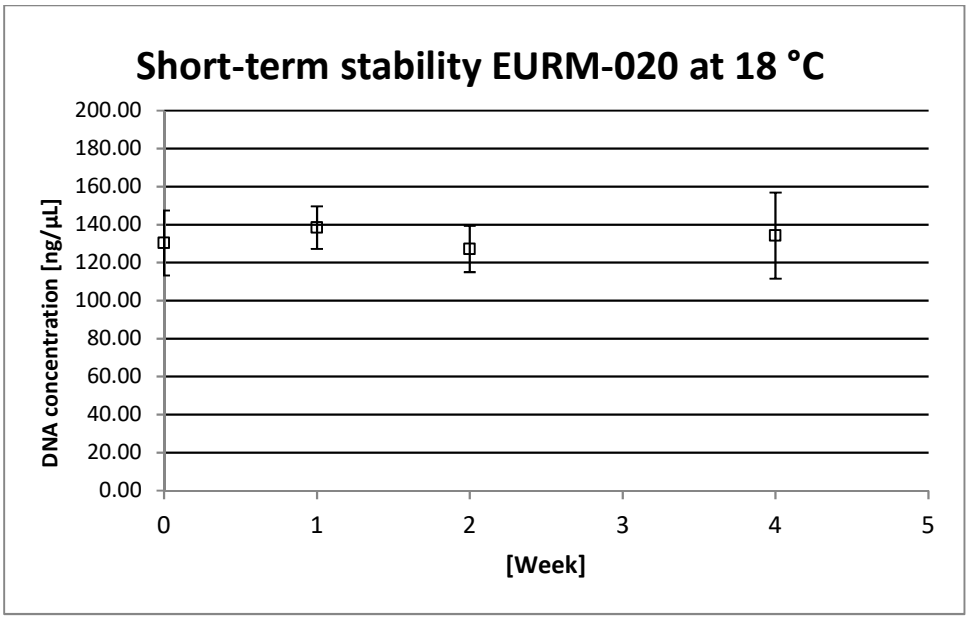

Figure 2: Extracted DNA concentration [ng/µL] of the samples included in the short-term stability of EURM-020 at 18 °C. Average of 2 samples of each 2 extraction duplicates, N = 2, n = 2 with error bars expressed as s_{meas}. Measurements performed by UV spectrophotometry (Nanodrop).

Annex C: Short-term stability study results

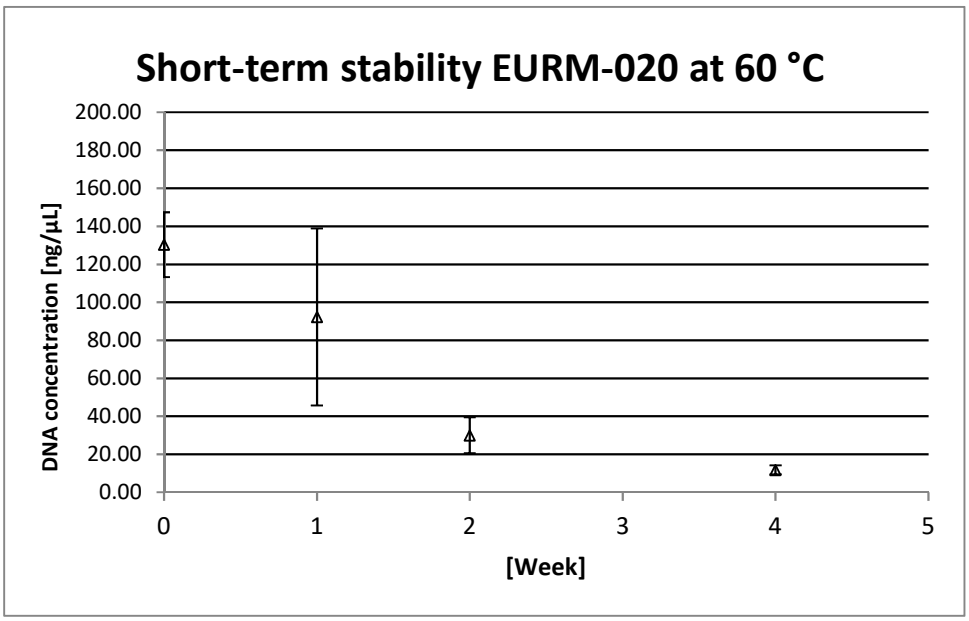

Figure 3: Extracted DNA concentration [ng/µL] of the samples included in the short-term stability of EURM-020 at 60°C. Average of 2 samples of each 2 extraction duplicates, N = 2, n = 2 with error bars expressed as s_{meas}. Measurements performed by UV spectrophotometry (Nanodrop).

Annex D: Long-term stability study results

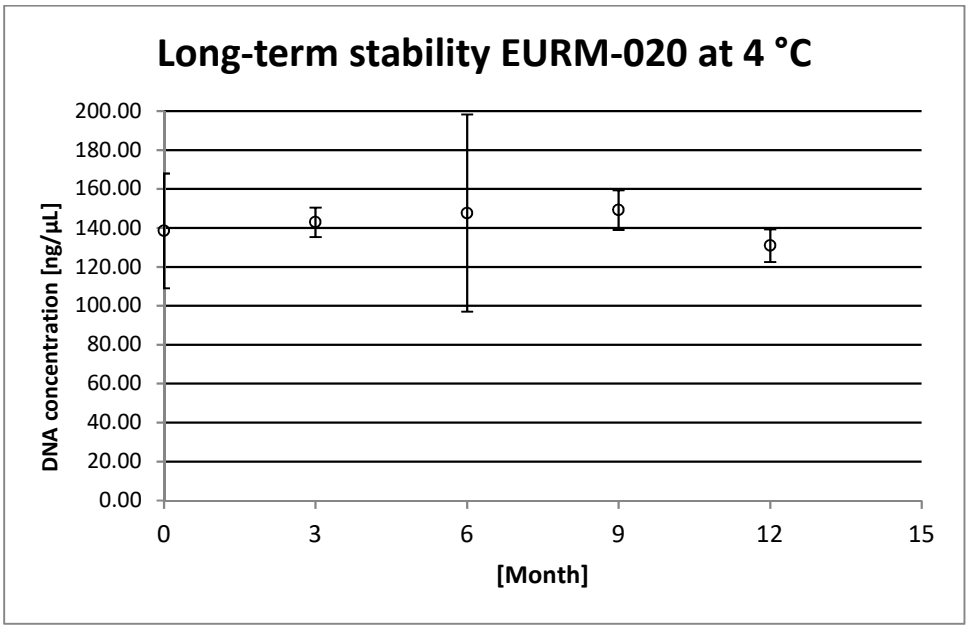

Figure 1: Extracted DNA concentration [ng/µL] of the samples included in the long-term stability of EURM-020 at 4°C. Average of 2 samples of each 2 extraction duplicates, N = 2, n = 2 with error bars expressed as s_{meas}. Measurements performed by UV spectrophotometry (Nanodrop).

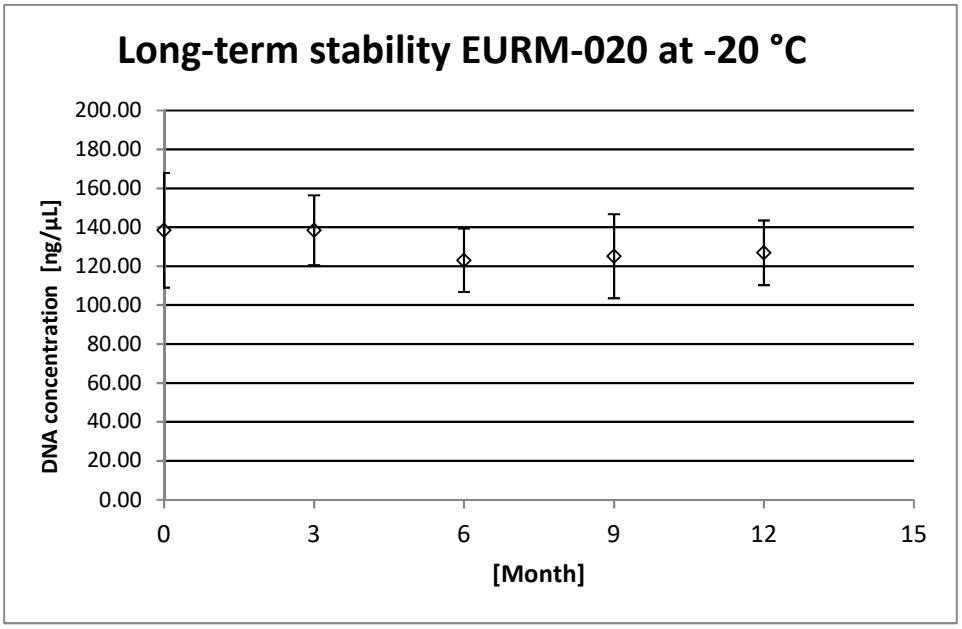

Figure 2: Extracted DNA concentration [ng/µL] of the samples included in the long-term stability of EURM-020 at -20°C. Average of 2 samples of each 2 extraction duplicates, N = 2, n = 2 with error bars expressed as s_{meas}. Measurements performed by UV spectrophotometry (Nanodrop).

Annex E: Results of the characterisation measurements

Annex E1: Taxon assignment and sequences

Table 1: Taxon assignment characterisation samples per laboratory and assay.

Laboratory code	replicate 1 **Taxon assignment**	replicate 2 **Taxon assignment**	replicate 3 **Taxon assignment**	replicate 4 **Taxon assignment**	replicate 5 **Taxon assignment**
L1 *cytb*	*Hippoglossus hippoglossus*	*Hippoglossus hippoglossus*	*Hippoglossus hippoglossus*	*Hippoglossus hippoglossus*	*Hippoglossus hippoglossus*
L2 *cytb*	*Hippoglossus hippoglossus*	*Hippoglossus hippoglossus*	*Hippoglossus hippoglossus*	*Hippoglossus hippoglossus*	*Hippoglossus hippoglossus*
L1 *COI*	*Hippoglossus hippoglossus*	*Hippoglossus hippoglossus*	*Hippoglossus hippoglossus*	*Hippoglossus hippoglossus*	*Hippoglossus hippoglossus*
L2 *COI*	*Hippoglossus hippoglossus*	*Hippoglossus hippoglossus*	*Hippoglossus hippoglossus*	*Hippoglossus hippoglossus*	*Hippoglossus hippoglossus*

Sequence of EURM-020, *Hippoglossus hippoglossus*, *cytb* barcode

>EURM-020, *Hippoglossus hippoglossus*, *cytb*, 413 bp

CAAGAACCCTAATGGCCAGTCTACGTAAATCCCACCCTCTTCTAAAAATCGCAAACGATGCTTTAGTCGACCTCC
CCGCCCCCTCTAATATCTCAGTTTGATGGAACTTTGGGTCTCTTTTAGGACTCTGTTTAATTACCCAAATTGCGA
CCGGCTTATTTCTAGCCATACACTACACATCAGACATTGCTACTGCCTTCACCTCCGTGGCCCACATCTGTCGAG
ACGTCAACTACGGCTGACTTATCCGAAGCATTCATGCCAACGGCGCATCATTCTTTTTCATTTGCCTCTACCTTC
ATATTGGCCGAGGACTATACTATGGCTCTTACCTCTATAAAGAAACATGAACTGTTGGGGTTATTCTTCTCCTTC
TCGTAATAATAACAGCCTTCGTTGGATACGTCCTCCCT

The highlighted base shows the mismatch in all EURM-020 samples included in the characterisation study with the *Hippoglossus hippoglossus* reference sequences for *cytb* in GenBank (NCBI) used for the identification of EURM-020 (Table 3, Section 7).

Sequence of EURM-020, *Hippoglossus hippoglossus*, *COI* barcode

>EURM-020, *Hippoglossus hippoglossus, COI*, 652 bp

CCTCTATCTCGTATTTGGTGCCTGAGCCGGAATAGTGGGGACAGGCCTAAGTCTGCTTATTCGGGCAGAACTAAG
CCAACCCGGGGCTCTCCTGGGAGACGACCAAATTTATAATGTGATCGTCACCGCACACGCCTTTGTAATAATCTT
TTTTATAGTAATACCCATTATGATTGGGGGGTTCGGAAACTGGCTTATTCCACTAATAATTGGGGCCCCAGACAT
GGCGTTCCCTCGAATGAATAATATGAGTTTCTGACTTCTTCCCCCCTCCTTTCTCCTCCTCTTAGCCTCTTCAGG
TGTTGAAGCCGGAGCAGGTACCGGATGAACCGTGTACCCCCCACTAGCTGGCAATTTAGCCCACGCCGGGGCATC
CGTAGACCTGACAATCTTCTCACTTCACCTTGCAGGAATTTCATCAATTCTGGGGGCAATTAACTTTATTACTAC
CATCATTAACATGAAACCCACAACAGTCACTATGTACCAAATTCCGTTATTTGTTTGAGCCGTTCTTATTACAGC
CGTACTTCTTCTTCTGTCCCTGCCCGTTTTAGCCGCAGGGATTACAATGCTACTAACAGACCGCAACCTTAACAC
GACCTTCTTTGACCCTGCCGGAGGAGGTGACCCCATTCTCTACCAACACCTA

The highlighted base shows the mismatch in all EURM-020 samples included in the characterisation study with the *Hippoglossus hippoglossus* reference sequences for *COI* in the BOLD system used for the identification of EURM-020 (Table 4, Section 7).

Annex E2: Additional information

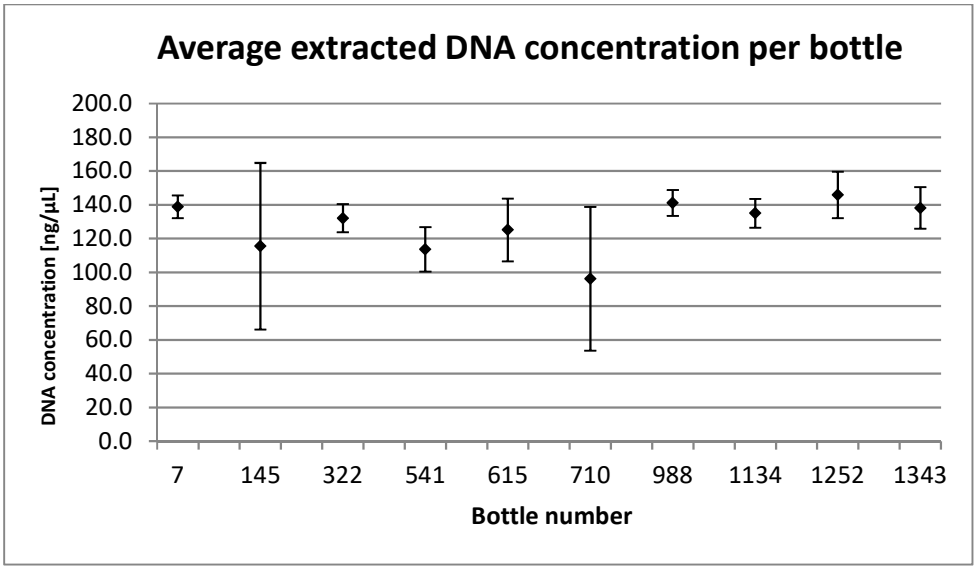

Figure 1: Extracted DNA concentration [ng/µL] of the samples per bottle for the characterisation study of EURM-020. Average of duplicate extractions N = 1, n = 2, with error bars expressed as s_{meas}. Measurements were performed by UV spectrophotometry (Nanodrop).

European Commission

EUR 30097 EN – Joint Research Centre – Directorate F – Health, Consumers and Reference Materials
Title: **The certification of the genetic identification of a *Hippoglossus hippoglossus* (Atlantic halibut) fish powder: EURM®-020**
Author(s): A.M. Kortekaas, H. Leys, J. Seghers, S. Trapmann
Luxembourg: Publications Office of the European Union
2020 –38 pp. – 21.0 x 29.7 cm
EUR – Scientific and Technical Research series – ISSN 1831-9424
ISBN 978-92-76-16230-8
doi:10.2760/170544

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KJ-NA-30097-EN-N

Publications Office
of the European Union

doi:10.2760/170544

ISBN: 978-92-76-16230-8