

# Collaborative trial validation of RT-PCR methods for the detection and quantification of the allergenic foods fish and peanut

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## Abstract

The analysis of food for allergens is an inherent part of consumer protection against health risks. Before application, these methods require validation by collaborative studies to ensure comparable results between laboratories. In this article, we present results of collaborative trial studies for validating RT-PCR methods for the detection and quantification of two food allergens, fish and peanut. Reference materials of different food matrices, spiked with fish and peanut, respectively, were analysed. Defined food matrix DNA standards (fish, peanut) as well as copy number based DNA solutions (fish) were applied for calibration and quantification. A limit of detection of approximately 10–20 mg/kg dried fish (i.e. approx. 50 mg fresh weight/kg) could be confirmed within a collaborative trial performed by 13 participating labs. Trueness and precision data were comparable to former collaborative studies suggesting that quantification is possible, if the fish species is known and appropriate calibration material is available. For peanut, we used a multicopy target-sequence, which allows a very sensitive detection of this allergen. Within the collaborative trial of 14 laboratories, a peanut concentration of 0.5 mg/kg (i.e. approx. 0.1–0.2 mg peanut protein/kg) could be detected reliably in a processed cookie matrix. The method revealed acceptable trueness and good precision data and is therefore suitable for quantification of the allergenic ingredient peanut. Both methods will be included into the Official Collection of Methods of Analysis in Germany in 2019 as a national standard method.

**Keywords** Allergens · Fish · Peanut · RT-PCR · Inter-laboratory validation · Standard methods

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## 1 Introduction

In developed countries, about 4–8% of people suffer from food allergies against at least one allergen. They are more frequent in children than in adults and appear to be increasing in frequency (Sicherer and Sampson 2014).

To ensure the availability of information about the presence of relevant allergens in food, labelling regulations have been implemented in many countries. Within the European Union, labelling is mandatory for food ingredients based on fish and peanut (amongst others), according to the regulation (EU) No. 1169/2011 (Regulation 2011/1169/EC).

In food surveillance, reliable methods for the detection and, if required, quantification of allergens are needed. In routine analysis, protocols based on real-time polymerase chain reaction (RT-PCR) and immunological methods have been used predominantly (EFSA 2014).

In case of positive results, the quantification of the respective allergens or allergenic ingredients may be necessary for risk assessment, allergen management, and surveillance (The Allergen Bureau; Waiblinger and Schulze 2018). The implementation of legal threshold values, e.g. at what threshold labelling of food allergens is mandatory, is currently under discussion. At present, such threshold values have not been defined within the European Union. An important prerequisite for setting thresholds for food allergens is the availability of standardized quantification methods. In previous studies, we have described the inter-laboratory validation of single and multiplex RT-PCR methods for the quantification of the allergenic ingredients soybean, white and brown mustard, celery and wheat in sausages (Siegel et al. 2012; Waiblinger et al. 2017) and of the allergenic ingredients sesame, almond, Brazil nut and lupine in baked products and sauce powder (Waiblinger et al. 2014).

These collaborative trials were planned and organized by the working group “Food Allergens” of the Federal Office of Consumer Protection and Food Safety within the scope of the official method collection according to § 64 of the German Food and Feed Code (LFGB). In this article, we present the results of 2 collaborative trials of published RT-PCR methods following a similar approach: One study describes the validation of a RT-PCR method for the detection of fish specific genomic DNA sequences (Tetzlaff and Maede 2017). The second validation study shows the detection of peanut using a RT-PCR method targeting a multicopy sequence from mitochondrial DNA (Ladenburger et al. 2018).

## 2 Materials and methods

### 2.1 Spiked (= incurred) food material

The composition of the materials used in the collaborative trial is compiled in Table 1. Materials of different food matrices were artificially contaminated (incurred) with fish and peanut, respectively. Details on the production of the materials and information about the allergenic ingredients as well as on the homogeneity testing of these materials are described elsewhere (Ladenburger et al. 2018; Siegel et al. 2013; Tetzlaff and Maede 2017; Waiblinger et al. 2017). The allergen peanut was added to sausage meat of the type “Lyoner” (i.e. “Bologna type sausage”, French: Saucisse de Lyon), rice cookies, wheat cookies and milk powder. For the allergen fish, defined amounts of cod (*Gadus morhua*) or carp bream (*Abramis brama*) were added to the ingredients for the production of rice cookies, sauce powder type hollandaise, shrimp salad or sausage meat of the type “Lyoner”. Artificially contaminated wheat cookies

used for peanut detection were provided by DLA Proficiency Testing (Ahrensburg, Germany). All materials were stored frozen until analysis.

### 2.2 DNA extraction

A cetyltrimethylammonium bromide (CTAB) extraction protocol with a precipitation step (Siegel et al. 2013) was used for extraction of DNA from all materials: 2.0 g of the homogenized sample was mixed with the CTAB extraction buffer and consecutively incubated with amylase and *proteinase* K solution. Lysates were purified by mixing with chloroform and DNA was selectively precipitated using the cationic detergent CTAB and lowering the NaCl concentration. The precipitate was dissolved by adding 1.2 M NaCl, incubated with RNase solution and further purified with chloroform. Finally, the DNA was pelleted using isopropanol. The precipitate was washed with ethanol solution (70%), centrifuged and dissolved in 50 µl 0.2 × TE solution. The DNA extracts were stored at 2–8 °C until analysis. 5 µl of the DNA samples were used for RT-PCR.

For the peanut validation study, DNA extraction was performed by one lab (see Sect. 2.9).

### 2.3 DNA calibration standard (fish)

For the calibration of results in copy numbers, calibrator DNA based on genome copies was prepared before the collaborative trial “fish”. Genomic DNA was extracted from the fish species cod (*Gadus morhua*) using a CTAB-based protocol (Waiblinger et al. 2017). The copy number of the target DNA was measured by Droplet Digital<sup>TM</sup> PCR (QX200<sup>TM</sup>, Bio-Rad Laboratories, Cressier, CH) using the same primers as described in this work. The DNA concentration was determined and adjusted to 1000 copies of the target sequence per 5 µl. The DNA extract was stored at 2–8 °C until analysis. Before analysis, the DNA solution was serially diluted to yield 1000 (undiluted), 316, 100, 31.6 and 10 copies per 5 µl. Copy number calibrator DNA was not prepared for the collaborative trial “peanut” due to the multicopy target used for quantification.

### 2.4 Matrix standard DNA

For calibration of results in mg/kg, we used DNA prepared by twofold extraction (see Sect. 2.2). The determination of fish was performed with 400 mg/kg sausage material and the determination of peanut with the 400 mg/kg rice cookie material (Table 1). For each material, both DNA extracts were pooled. The DNA extracts were stored at 2–8 °C until analysis. Before the setup of the peanut PCR, rice cookie DNA was diluted with 0.2 × TE-solution to yield DNA-

**Table 1** Materials used in the collaborative trials

| Material                          | Spiked allergenic ingredient (mg/kg) |                    |              | Validation study |
|-----------------------------------|--------------------------------------|--------------------|--------------|------------------|
|                                   | Cod (dried)                          | Carp bream (dried) | Peanut flour |                  |
| Sausage “Lyoner” 0                | 0                                    | 0                  | 0            | Fish             |
| Sausage “Lyoner” 10               | 10                                   | 0                  | 10           |                  |
| Sausage “Lyoner” 50               | 50                                   | 0                  | 50           |                  |
| Rice cookie 10.6                  | 10.6                                 | 0                  | 10.6         |                  |
| Sauce powder 20                   | 20                                   | 0                  | 20           |                  |
| Shrimp salad 15                   | 0                                    | 15                 | 0            | Peanut           |
| Sausage “Lyoner” 400 <sup>a</sup> | 400                                  | 0                  |              |                  |
| Rice cookie 0                     | 0                                    | 0                  | 0            |                  |
| Rice cookie 5.3                   | 5.3                                  | 0                  | 5.3          |                  |
| Rice cookie 20.8                  | 20.8                                 | 0                  | 20.8         |                  |
| Wheat cookie 0.5                  | 0.5                                  | 0                  | 0            |                  |
| Wheat cookie 5                    | 5                                    | 0                  | 0            |                  |
| Sausage “Lyoner” 5                | 5                                    | 0                  | 5            |                  |
| Milk powder 5                     | 5                                    | 0                  | 5            |                  |
| Rice cookie 400 <sup>a</sup>      | 400                                  | 0                  | 400          |                  |

<sup>a</sup>Material used for extraction of matrix standard DNA, see Sects. 2 and 2.4

solutions corresponding to the following hypothetical concentrations: 400 (undiluted DNA), 100, 25, 6.25, 1.56 and 0.5 mg peanut/kg.

## 2.5 RT-PCR

A RT-PCR system with two forward and three reverse primers and a fluorescent hydrolysis probe (TaqMan<sup>TM</sup>) was used for the amplification and detection of genomic DNA sequences of the *hoxc 13* gene specific for fish (Tetzlaff and Maede 2017). For peanut, another TaqMan<sup>TM</sup> RT-PCR method targeting a multicopy sequence from mitochondrial DNA close to the coding region of the ATPase subunit 6 (*atp6*) was used (Ladenburger et al. 2018). The respective primers and probes are compiled in the supplementary material (Table S1) and the compositions of the PCR reaction mixes for the PCR systems in Table S2.

The Quantitect Multiplex Mastermix (QIAGEN, Hilden, Germany) was used for each RT-PCR according to the manufacturer’s instructions. Primer and probes were synthesized by TIB MolBiol (Berlin, Germany). The temperature-time-protocol was: initial denaturation for 15 min at 95 °C for 1 cycle, followed by 45 cycles of 30 s at 95 °C and 60 s at 60 °C for fish PCR; the same temperature-time-protocol was run for peanut PCR except for a shorter denaturation step of 15 s at 95 °C. In both protocols, the fluorescence signal was measured during elongation.

## 2.6 Specificity testing and comparison between species/cultivars

The specificity of the methods is described by Tetzlaff and Maede (2017) and Ladenburger et al. (2018). Additionally, specificity was randomly tested before the collaborative study by the organizing laboratory. Specificity testing was done with non-target organisms/taxa and response testing was performed with target-organisms or cultivars. DNA was extracted from test material (fish fillets, seafood matrix and kernels/seeds respectively) using a CTAB-based protocol (Waiblinger et al. 2017).

- (a) Fish system, exclusivity test with non-target species/taxa:

Norway lobster (*Nephrops norvegicus*), common mussel (*Mytilus edulis*), pig (*Sus scrofa*), great scallop (*Pecten ssp.*), white pacific shrimp (*Litopenaeus vannamei*), black tiger shrimp (*Penaeus monodon*), northern shrimp (*Pandalus borealis*), squid (*Loligo vulgaris*), wheat (*Triticum aestivum*) and soy (*Glycine max.*).

Fish system, inclusivity test with target species/taxa:

cod (*Gadus morhua*), alaska pollack (*Gadus chalcogrammus*), plaice (*Pleuronectes platessa*), pollack (*Pollachius virens*), haddock (*Melanogrammus aeglefinus*), mackerel (*Scomber scombrus*), herring (*Clupea harengus*), hake (*Merluccius merluccius*), yellowfin tuna (*Thunnus albaceres*), trout (*Salmo trutta*)

- (b) Peanut-system, exclusivity test with non-target species/taxa:

almond, white bean, Brazil nut, broccoli, cashew, celery, emmer, hazelnut, blue lupin, white lupin, maize, white mustard, parsley, green pea, pig, pistachio, potato, rice, sesame, soy, tomato, walnut, wheat.

Peanut-system, inclusivity test with target species/cultivars: Peanuts kernels (native) from nine different cultivars/origins ( $n = 1$ ) and seven different market samples from roasted and unroasted peanuts.

The response of obtained amplifications was determined by using an external standard curve for cod (fish PCR) and with a market sample of roasted peanuts for the peanut PCR as reference (= 100%). Each response value was calculated as the ratio between the concentration of DNA of the analysed species/cultivar and the reference. Before the testing by PCR systems, the concentration of extracted DNA was measured photometrically and adjusted to a concentration of 20 ng/ $\mu$ l for fish PCR and 10 ng/ $\mu$ l for peanut PCR. For peanut response testing, the DNA-dilutions were analysed in parallel by RT-PCR amplifying a peanut-specific DNA sequence from nuclear *arah2* gene (Hird et al. 2003; Ladenburger et al. 2018).

## 2.7 In-house testing of the sensitivity

In order to confirm the previously published data by Tetzlaff and Maede (2017) ahead of the collaborative trial, the sensitivity of the fish PCR method was estimated in the organizing laboratory using a dilution series of cod DNA. The original concentration of this DNA solution given in copy numbers per  $\mu$ l was determined by digital PCR. Following the procedure described by Tetzlaff and Maede (2017), this DNA was serially diluted to yield the levels 20, 10, 5, 2, 1 and 0.1 copies per 5  $\mu$ l.

## 2.8 Collaborative trial

The reliability of the methods was tested in collaborative trials with 13 (fish) and 14 (peanut) participating labs (Table S3, supplementary material). They are all members of the working group “Food Allergens” (see Sect. 1). Materials from sausage artificially contaminated with allergens were used as mentioned before (see Sect. 2.1). In contrast to the collaborative “fish” trial, participants of the peanut-study received DNA samples that were previously extracted (Table 1). Therefore, the term “samples” mentioned below means DNA extracts for the peanut collaborative trial.

The following material was sent to the participating laboratories:

- 18 (fish) resp. 21 (peanut) encoded samples:
  - fish: 6 materials, 3 aliquots each (Table 1).
  - peanut: 7 materials, 3 aliquots each (Table 1).
- One sample containing the matrix standard (see Sect. 2.4).
- Aliquots of oligonucleotides and probes and master-mixes for the RT-PCR (see Sect. 2.5).
- Collaborative trial fish only: An aliquot of DNA calibration standard containing 1000 copies of cod DNA per 5  $\mu$ l (see Sect. 2.3).

There was no restriction in terms of RT-PCR type system used. The DNA extraction and the RT-PCRs had to be performed according to the procedure described in Sects. 2.2 and 2.5. For the collaborative trial “fish” each encoded sample had to be extracted once. In both studies, each DNA extract had to be analysed in duplicates and each level of the matrix standard (peanut) resp. calibration standard (fish) DNA dilution series in triplicates. The lowest level of the calibration standard/matrix standard DNA dilution series (10 copies (cp)/5  $\mu$ l for fish and “0.5 mg/kg” for peanut) was used as sensitivity control and therefore analysed in 10 replicates.

## 2.9 Qualitative and quantitative evaluation of the inter-laboratory validation

As for the qualitative evaluation, C<sub>q</sub> values < 45 were considered as amplification (= positive result). Additionally, the sample C<sub>q</sub> values were compared with the average C<sub>q</sub> value of the lowest standard (10 copies cod DNA per 5  $\mu$ l and 0.5 mg peanut/kg respectively). If the C<sub>q</sub> value of the sample is at least 1 higher than the C<sub>q</sub> of the lowest standard, the result was considered “negative” (Waiblinger et al. 2011).

For quantitative evaluation of the results, C<sub>q</sub> values were first assigned to concentrations in copy numbers of cod DNA per PCR and mg peanut/kg, respectively, using the DNA calibration standard/matrix standard curve by linear data regression of the calibration series.

Average C<sub>q</sub> values obtained by RT-PCR amplification of the DNA standard dilution levels were plotted against the logarithm of the corresponding DNA concentration (1000, 316, 100, 31.6 and 10 copies per 5  $\mu$ l for fish PCR and 400, 100, 25, 6.25, 1.56 and 0.5 mg/kg for peanut PCR).

For each DNA extraction of the samples including the matrix standards, mean values of the concentrations obtained for the PCR duplicates were calculated.

For fish PCR, DNA concentrations (= copy no. of the target sequence per PCR) were assigned to concentrations in mg/kg using the averaged results of the matrix standard DNA. The participating laboratories were asked to report

results of each PCR as Cq values as well as concentrations. Median, mean and standard deviation were calculated from the average values of concentrations in mg/kg (fish, peanut) and copy numbers/PCR (fish) ( $n = 3$  per concentration level).

In order to be able to compare the trueness of the results of the individual labs, the difference between the individual mean and the median result of all labs was calculated.

Robust mean and robust standard deviation in copy number per 5  $\mu$ l as well in mg/kg were calculated with an algorithm according to ISO 5725-5 (1998).

Before the calculation of the mean and median values, the repeatability standard deviation ( $RSD_r$ ), and reproducibility standard deviation ( $RSD_R$ ), outliers were identified using the Grubbs test (5% significance level) and removed from the dataset. All calculations were performed using the software Valoo 2.4.

### 3 Results and discussion

#### 3.1 Inhouse testing prior to the collaborative trial

##### 3.1.1 Specificity tests

Prior to the collaborative trial, the specificity of the methods as previously reported by Tetzlaff and Maede (2017) and Ladenburger et al. (2018) was confirmed in a pre-study by using DNA that was extracted from materials as mentioned in Sect. 2.

**3.1.1.1 Fish** No amplification was obtained when testing DNA from the following species: Black tiger shrimp (*Penaeus monodon*), Norway lobster (*Nephrops norvegicus*), white pacific shrimp (*Litopenaeus vannamei*), great scallop (*Pecten ssp.*), common mussel (*Mytilus edulis*), squid (*Loligo vulgaris*), pig (*Sus scrofa*), wheat (*Triticum aestivum*) and soy (*Glycine max.*). Very weak amplifications (equal to a cross-reactivity of 0.02% and less) were obtained for northern shrimp (*Pandalus borealis*). However, it cannot be excluded that these weak amplifications were caused by contamination of the material originating from the local market.

**3.1.1.2 Peanut** No amplification was obtained when testing DNA from the following species: almond, white bean, broccoli, celery, hazelnut, blue lupin, white lupin, maize, white mustard, parsley, green pea, pig, pistachio, potato, rice, sesame, soy, tomato, walnut, wheat. Very weak amplifications (equal to a cross-reactivity of less than 0.01%) were obtained for cashew nut, Brazil nut and emmer. However, again it cannot be excluded that these

weak amplifications were caused by contamination of those materials from the local market.

##### 3.1.2 Between cultivar/species variation

Furthermore, complementing the data published in the original paper (Tetzlaff and Maede 2017; Ladenburger et al. 2018), the response of the methods depending on the variation of cultivars or species originally targeted by the methods was verified in a single laboratory. Table S4 (supplementary material) shows the results of response testing in different fish species. For RT-PCR, an up to 57-fold difference of the response of equal DNA amounts of different fish species was obtained.

For confirmation of the sufficiently low variation of the response in peanut PCR depending on cultivar/provenience and processing (Ladenburger et al. 2018), kernels from different peanut cultivars (unroasted) as well as market samples (roasted/unroasted) were analysed using the peanut ring-trial protocol. The overall variation of the response was below 50% (data not shown).

##### 3.1.3 In-house testing of the sensitivity (fish)

Complementing the collaborative “fish” trial, the sensitivity of the method was also verified in a single lab using a dilution series of the DNA calibration standard. Following the procedure described in BVL (2016), the DNA mixture was serially diluted to yield the levels 20, 10, 5, 2, 1 and 0.1 copies/5  $\mu$ l.

Using a web-based tool (Quodata 2019), an  $LOD_{95\%}$  of approximately seven copies was obtained.

#### 3.2 Collaborative trial validation

The reliability of both methods was tested in collaborative trial studies with 13 and 14 participating laboratories, respectively. In both ring-trials, no deviations from the protocol were reported by the participating labs.

##### 3.3 Calibration standard DNA and matrix standard DNA

Table 2 summarizes the results of the calibration series. In addition, for the detection of fish, the results of the matrix standard DNA evaluated by the use of the calibration standard DNA are shown. Performance criteria for standard curves are described e.g. in guidelines for GMO analysis (BVL 2016; ENGL 2015; Broeders et al. 2014). According to these guidelines, the slope of the standard curve should be between  $-3.1$  and  $-3.6$  and  $R^2$  should be  $> 0.98$ . For multiplex PCR used for qualitative analysis, a slope between  $-2.9$  and  $-3.9$  is deemed to be acceptable (Broeders et al. 2014).

**Table 2** a + b: Interlaboratory validation: RT-PCR systems and results of the dilution series of (a) calibration standard DNA (1000–10 copies (cp) cod DNA/PCR) for fish, and (b) matrix calibration DNA (400–1.56 mg/kg) for peanut

| Lab no.                 | RT-PCR-system <sup>a</sup> | Slope  | R <sup>2</sup> | Standard 10 cp <sup>b</sup>     | MS-DNA <sup>c</sup>                          |
|-------------------------|----------------------------|--------|----------------|---------------------------------|----------------------------------------------|
| (a) PCR system “fish”   |                            |        |                |                                 |                                              |
| 1                       | a                          | – 3.38 | 0.990          | 10                              | 1276                                         |
| 2                       | b                          | – 3.49 | 0.986          | 10                              | 929                                          |
| 3                       | c                          | – 3.30 | 0.991          | 10                              | 2340                                         |
| 4                       | a                          | – 2.87 | 0.981          | 10                              | <i>O: 120</i>                                |
| 5                       | b                          | – 3.65 | 0.981          | 10                              | 1780                                         |
| 6                       | d                          | – 4.35 | 0.992          | 6                               | 2521                                         |
| 7                       | e                          | – 3.63 | 0.994          | 10                              | 2038                                         |
| 8                       | f                          | – 3.88 | 0.995          | 9                               | 2309                                         |
| 9                       | g                          | – 3.21 | 0.984          | 10                              | 2873                                         |
| 10                      | f                          | – 3.70 | 0.983          | 10                              | 441                                          |
| 11                      | c                          | – 3.31 | 0.984          | 10                              | 3804                                         |
| 12                      | f                          | – 5.71 | 0.904          | 10                              | 909                                          |
| 13                      | d                          | – 2.78 | 0.997          | 10                              | <i>O: 18103</i>                              |
| Mean                    |                            | – 3.63 | 0.982          | 125 of 130 (= 96.1%)            | 1929 <sup>d</sup> (StDev: 51% <sup>d</sup> ) |
| Lab no.                 | RT-PCR-system <sup>a</sup> | Slope  | R <sup>2</sup> | Standard 0.5 mg/kg <sup>b</sup> |                                              |
| (b) PCR system “peanut” |                            |        |                |                                 |                                              |
| 1                       | e                          | – 3.42 | 0.993          | 10                              |                                              |
| 2                       | b                          | – 3.50 | 0.998          | 10                              |                                              |
| 3                       | d                          | – 3.58 | 0.998          | 10                              |                                              |
| 4                       | b                          | – 3.41 | 0.996          | 10                              |                                              |
| 5                       | d                          | – 3.41 | 1.000          | 10                              |                                              |
| 6                       | h                          | – 3.44 | 0.999          | 10                              |                                              |
| 7                       | b                          | – 3.61 | 0.993          | 10                              |                                              |
| 8                       | d                          | – 3.50 | 0.999          | 10                              |                                              |
| 9                       | a                          | – 3.55 | 0.999          | 10                              |                                              |
| 10                      | b                          | – 3.44 | 0.998          | 10                              |                                              |
| 11                      | c                          | – 3.38 | 0.986          | 10                              |                                              |
| 12                      | a                          | – 3.54 | 0.998          | 10                              |                                              |
| 13                      | f                          | – 3.39 | 0.998          | 10                              |                                              |
| 14                      | g                          | – 3.50 | 0.998          | 10                              |                                              |
| Mean                    |                            | – 3.48 | 0.997          | 140 of 140 (=100%)              |                                              |

Italic: results out of specification for slope (between – 3.1 and – 3.6) and R<sup>2</sup> (> 0.98) (ENGL 2015); specification for multiplex PCR: slope between – 2.9 and – 4.1, R<sup>2</sup> > 0.98 (Broeders et al. 2014)

*O* outlier (Grubbs)

<sup>a</sup>RT-PCR systems used in the collaborative trial: a: Light Cycler 480 (Roche); b: ABI 7500 bzw. ABI 7500 Fast (Applied Biosystems/Life Technologies); c: Aria Mx (Agilent); d: CFX 96 (Biorad); e: Rotorgene Q (QIAGEN, Hilden); f: Rotorgene RS 6000 (Corbett); g: QuantStudio (Thermo Fisher); h: Light Cycler 96 (Roche)

<sup>b</sup>Std 10 cp/Std 0.5 mg/kg = number of positive reactions for standard dilution 10 cp/5 µl and 0.5 mg/kg resp. (n = 3)

<sup>c</sup>MS-DNA = results of matrix standard DNA (cp/5 µl; mean of 2 reactions)

<sup>d</sup>After removal of outliers

### 3.3.1 Fish

Ten out of 13 laboratories reported slopes that at least fulfilled the recommendations for multiplex PCR. For the criterion R<sup>2</sup>, this was the case in 12 out of 13 results.

Results of slope and R<sup>2</sup> of lab no. 12 were far outside the expected range. Therefore, all the results of this lab have not been considered for qualitative and quantitative evaluation. The lowest standard of the dilution series (10 cp cod DNA/PCR) was simultaneously used to estimate the

sensitivity of the respective PCR. For 125 out of 130 reactions (= 96.1%), amplifications were obtained. One single lab reported 4 out of 5 false negative reactions.

Matrix standard DNA was extracted in parallel to the encoded samples from 400 mg/kg sausage material. After the removal of 2 outlying results, a mean copy number/PCR of 1929 with a CV of 51% was obtained.

The results of two laboratories were excluded from the evaluation: One lab reported clearly deviating values of the standard curve (see above). The results of another lab (lab 10) could not be considered due to an obviously inaccurate DNA extraction. The DNA concentrations of the samples determined photometrically were 2- to 10-fold lower compared to the overall mean. In total, the results of 11 laboratories were used for qualitative and quantitative evaluation.

### 3.3.2 Peanut

For both criteria, slope and  $R^2$ , the recommended values for single PCR according to ENGL (2015) were reached by all 14 participating labs. Sensitivity control of 0.5 mg peanut/kg could be amplified in 140 out of 140 reactions (0% false negative rate). One lab voluntarily reported additional results, obtained by the use of a different commercial mastermix for RT-PCR. These results did not differ significantly from the results obtained using the mastermix according to Sect. 2.5 (data not shown).

## 3.4 Qualitative evaluation of samples

The samples were decoded and assigned to six, respectively seven different materials. Per material and lab, six PCR results (three DNA extracts, each analysed in duplicates) were obtained. Table 3 shows the results of the qualitative evaluation of the inter-laboratory validation: All reported amplifications ( $C_t < 45$ ) were considered. In addition, another approach to qualitatively evaluate the results was done (Waiblinger et al. 2011).

### 3.4.1 Fish

With both approaches for evaluation, less than 5% false-negative results were obtained for fish spiked with 50 mg/kg and 10 mg/kg sausage and when spiked with sauce powder at 20 mg/kg. Approximately the same values could be reached when spiked with rice cookies at 10.6 mg/kg (6% and 7.6% false-negatives), whilst shrimp salad spiked with fish species bream (15 mg/kg) showed considerably higher rates of false-negatives.

In summary, ten copies of fish-DNA (cod) per PCR-reaction were detected reliably within the collaborative trial. Expressed as mg fish/kg, incurred amounts of

10–20 mg cod (dried) could reliably be detected by all the labs considered for evaluation. The estimated LOD for the fish species bream (dried) is in the range of 15 mg/kg or slightly above. Calculated as fresh weight, this means an LOD of approximately 50 mg cod or bream/kg.

Note that according to the results of response tests (see Sect. 3.1.2), DNA of other relevant fish species shows a stronger response in the RT-PCR than cod and can therefore be detected with a higher sensitivity. Hence, it can be assumed that the above-mentioned LODs will at least be reached for other relevant fish species.

### 3.4.2 Peanut

For “blank” rice cookie (0 mg peanut/kg), 8 out of 84 reactions were false-positive amplifications with  $C_q$  values  $< 45$ . However, in all cases the other reaction of the PCR duplicate was negative, indicating that the duplicates finally had to be evaluated as “negative”. The  $C_q$  values of the observed amplifications ranged from 38 to 41. Correspondingly, no false positive results were obtained for the blank sample by comparison of the  $C_q$  values with the  $C_q$  value reported for the lowest standard dilution (0.5 mg/kg) (Waiblinger et al. 2011). Evaluating the results of wheat cookie incurred with 0.5 mg/kg (DLA 2017) using this approach, 40 out of 84 reactions were false-negatives (= 48%). This can be explained by the fact that the level of 0.5 mg/kg is equal to the lowest dilution level of the matrix standard DNA. With this approach, all  $C_q$  values of samples at least + 1 higher than the mean  $C_t$  of the lowest standard (0.5 mg/kg) correspond to a negative result. Considering any amplification, in all PCR reactions obtained at the level 0.5 mg/kg in wheat cookie positive results were reported. The results confirm the assumed LOD of 0.5 mg/kg for the detection of peanut.

## 3.5 Quantitative evaluation of samples

Table 4 summarizes the statistical evaluation of all inter-laboratory validation results. Due to the lack of appropriate guidelines and performance criteria related to allergen analysis, guidelines for RT-PCR-based GMO analysis were used for the evaluation of the results (ENGL 2015). Owing to the differing response in RT-PCR amplification of DNA from different species, the method of determination of fish is usually only qualitative. Nevertheless, the results of the collaborative trial were also evaluated quantitatively in order to assess the potential of the method in terms of quantification and of supplementing the qualitative results (LOD) by method precision data.

Data received for the materials incurred at the level of LOD (shrimp salad with 15 mg carp bream/kg and wheat

**Table 3** Inter-laboratory validation, qualitative evaluation

| Material and spike level (mg/kg) <sup>a</sup> | SL 50   | SL 10              | RC 10.6                             | SP 20                             | SS 15                                 | SL 0             |      |
|-----------------------------------------------|---------|--------------------|-------------------------------------|-----------------------------------|---------------------------------------|------------------|------|
| Fish (11 labs considered)                     |         |                    |                                     |                                   |                                       |                  |      |
| Number of samples per lab                     | 3       | 3                  | 3                                   | 3                                 | 3                                     | 3                |      |
| Number of samples total                       | 33      | 33                 | 33                                  | 33                                | 33                                    | 33               |      |
| Number of PCR results total                   | 66      | 66                 | 66                                  | 66                                | 66                                    | 66               |      |
| Number of positive PCR results                | 66      | 65 <sup>b,c</sup>  | 62 <sup>b</sup> , 61 <sup>c</sup>   | 66 <sup>b</sup> , 63 <sup>c</sup> | 58 <sup>b</sup> , 46 <sup>c</sup>     | 0 <sup>b,c</sup> |      |
| False positive results                        | –       | –                  | –                                   | –                                 | –                                     | 0 <sup>a,b</sup> |      |
| False positive results (%)                    | –       | –                  | –                                   | –                                 | –                                     | 0 <sup>b,c</sup> |      |
| False negative results                        | 0       | 1 <sup>b,c</sup>   | 4 <sup>b</sup> , 5 <sup>c</sup>     | 0 <sup>b</sup> , 3 <sup>c</sup>   | 8 <sup>b</sup> , 20 <sup>c</sup>      | –                |      |
| False negative results (%)                    | 0       | 1.5 <sup>b,c</sup> | 6.1 <sup>b</sup> , 7.6 <sup>c</sup> | 0 <sup>b</sup> , 4.5 <sup>c</sup> | 12.1 <sup>b</sup> , 30.3 <sup>c</sup> | –                |      |
| Material and spike level (mg/kg) <sup>a</sup> | RC 20.8 | RC 5.3             | RC 0                                | WC 0.5                            | WC 5                                  | MP 5             | SL 5 |
| Peanut (14 labs considered)                   |         |                    |                                     |                                   |                                       |                  |      |
| Number of samples per lab                     | 3       | 3                  | 3                                   | 3                                 | 3                                     | 3                | 3    |
| Number of samples total                       | 42      | 42                 | 42                                  | 42                                | 42                                    | 42               | 42   |
| Number of PCR results total                   | 84      | 84                 | 84                                  | 84                                | 84                                    | 84               | 84   |
| Number of positive PCR results                | 84      | 84                 | 8 <sup>b</sup>                      | 84                                | 84                                    | 84               | 84   |
| False positive results                        | –       | –                  | 8 <sup>b</sup> , 0 <sup>c</sup>     | –                                 | –                                     | –                | –    |
| False positive results (%)                    | –       | –                  | 9.5 <sup>b</sup> , 0 <sup>c</sup>   | –                                 | –                                     | –                | –    |
| False negative results                        | 0       | 0                  | –                                   | 0 <sup>b</sup> , 40 <sup>c</sup>  | 0                                     | 0                | 0    |
| False negative results (%)                    | 0       | 0                  | –                                   | 0 <sup>b</sup> , 48 <sup>c</sup>  | 0                                     | 0                | 0    |

<sup>a</sup>Abbreviations of the used materials (numbers indicate the amount of spiked allergenic ingredient (dried cod and peanut respectively) in mg/kg: *SL* sausage Lyoner, *RC* rice cookie, *SP* sauce powder, *SS* shrimp salad, *WC* wheat cookie, *MP* milk powder

<sup>b</sup>Amplifications considered with Ct < 45

<sup>c</sup>Evaluation by comparison of the sample Cq value with the average Cq value of the lowest standard. If Cq value of the sample is at least 1 higher than the Cq of the lowest standard dilution, the result is evaluated as “negative” (Waiblinger et al. 2011)

cookie with 0.5 mg peanut/kg, respectively) were not considered for quantitative evaluation.

### 3.5.1 Trueness/recoveries

According to the guidelines for quantitative GMO analysis (ENGL 2015), the deviation of the mean value from the true value or an accepted reference value should not exceed 25%.

**3.5.1.1 Fish** The above-named criterion for trueness was fulfilled for the analysis of both sausage materials incurred with 50 and 10 mg/kg as well as for the sauce powder (20 mg/kg). The recoveries ranged from 89 to 107%, whilst the mean deviation obtained for the 10.6 mg/kg rice cookie material was + 58% and therefore out of the tolerable range.

**3.5.1.2 Peanut** The recoveries obtained from rice cookie materials of 100% and 113%, respectively were in line with the recommendations for the trueness of the results. However, as to the other materials from different matrices, the requirement of this criterion could not be fulfilled. The

maximum deviation of 60% was obtained for the wheat cookie material incurred with 5 mg/kg peanut. Note that for the calibration of all results, we used a dilution series from rice cookie material and the calculated recoveries are referring to % deviations of the results in relation to the rice cookie material (= 100%; Siegel et al. 2013). Material for spiking (peanut), matrix and production process (e.g. heat treatment) was only identical for rice cookie materials. All these parameters were different in wheat cookies and milk powder, but those for sausages spiked with peanut flour were identical with rice cookies.

### 3.5.2 Precision

Moreover, in the quantitative range, the repeatability standard deviation (RSD<sub>r</sub>) should not exceed 25% and the reproducibility standard deviation (RSD<sub>R</sub>) should be below 35%. At the level of the quantification limit, a value below 50% is acceptable (ENGL 2015).

**3.5.2.1 Fish** A repeatability standard deviation < 25% was reached only in the analysis of sausage (50 and 10 mg/kg), when evaluated as copy numbers. The results for sauce

**Table 4** Interlaboratory validation, quantitative evaluation

| Material and spike level (dried cod, mg/kg) <sup>a</sup>                   | SL 50   | SL 10  | RC 10.6 | SP 20 |       |
|----------------------------------------------------------------------------|---------|--------|---------|-------|-------|
| (a) Fish                                                                   |         |        |         |       |       |
| Fish (11 labs considered)                                                  |         |        |         |       |       |
| Number of samples per lab                                                  | 3       | 3      | 3       | 3     |       |
| Number of samples/PCR results total                                        | 33/66   | 33/66  | 33/66   | 33/66 |       |
| Number of samples/PCR results considered <sup>b</sup>                      | 33/66   | 33/66  | 33/66   | 33/66 |       |
| Results in copy numbers (cp) per 5 µl                                      |         |        |         |       |       |
| Mean value (cp/5 µl) <sup>b</sup>                                          | 256.2   | 87.7   | 86.6    | 89.9  |       |
| Median (cp/5 µl) <sup>b</sup>                                              | 254.8   | 68.4   | 77.6    | 75.2  |       |
| Robust mean (algorithm A) (cp/5 µl)                                        | 266.6   | 77.7   | 83.4    | 85.1  |       |
| Standard deviation (cp/5 µl) <sup>b</sup>                                  | 92.6    | 59.0   | 48.6    | 59.1  |       |
| Robust StdDev (algorithm A) (cp/5 µl)                                      | 83.9    | 47.6   | 57.0    | 64.3  |       |
| Repeatability standard deviation RSD <sub>r</sub> (cp/5 µl) <sup>b</sup>   | 56.8    | 15.7   | 36.4    | 35.2  |       |
| RSD <sub>r</sub> (%) <sup>b</sup>                                          | 22.2    | 17.9   | 42.1    | 39.2  |       |
| Reproducibility standard deviation RSD <sub>R</sub> (cp/5 µl) <sup>b</sup> | 94.7    | 60.8   | 49.7    | 60.4  |       |
| RSD <sub>R</sub> (%) <sup>b</sup>                                          | 37.0    | 69.3   | 57.4    | 67.2  |       |
| Results in mg/kg (calculation using matrix control standard DNA)           |         |        |         |       |       |
| Mean value (mg/kg) <sup>b</sup>                                            | 51.5    | 13.2   | 14.2    | 17.3  |       |
| Recovery (%) <sup>b</sup>                                                  | 105     | 107    | 158     | 89.3  |       |
| Median (mg/kg) <sup>b</sup>                                                | 45.7    | 8.4    | 12.0    | 16.0  |       |
| Robust mean (algorithm A) (mg/kg)                                          | 52.5    | 10.7   | 15.8    | 17.9  |       |
| Repeatability standard deviation RSD <sub>r</sub> (mg/kg) <sup>b</sup>     | 14.7    | 3.4    | 4.5     | 5.6   |       |
| RSD <sub>r</sub> (%) <sup>b</sup>                                          | 28.6    | 25.6   | 31.4    | 32.2  |       |
| Reproducibility standard deviation RSD <sub>R</sub> (mg/kg) <sup>b</sup>   | 20.1    | 10.6   | 7.1     | 12.1  |       |
| RSD <sub>R</sub> (%) <sup>b</sup>                                          | 39.1    | 79.7   | 50.1    | 69.9  |       |
| Material and spike level <sup>a</sup>                                      | RC 20.8 | RC 5.3 | WC 5    | MP 5  | SL 5  |
| (b) Peanut                                                                 |         |        |         |       |       |
| Peanut (14 labs considered)                                                |         |        |         |       |       |
| Number of samples per lab                                                  | 3       | 3      | 3       | 3     | 3     |
| Number of samples/PCR results total                                        | 42/84   | 42/84  | 42/84   | 42/84 | 42/84 |
| Number of samples/PCR considered <sup>b</sup>                              | 42/84   | 42/84  | 42/84   | 42/84 | 42/84 |
| Results in mg/kg (calculation using matrix control standard DNA)           |         |        |         |       |       |
| Mean value (mg/kg) <sup>b</sup>                                            | 23.5    | 5.29   | 1.97    | 3.66  | 2.44  |
| Recovery (%) <sup>b</sup>                                                  | 113     | 99.7   | 39.3    | 73.2  | 49.4  |
| Robust mean (algorithm A) (mg/kg)                                          | 23.4    | 5.19   | 1.97    | 3.66  | 2.44  |
| Standard deviation (mg/kg) <sup>b</sup>                                    | 3.35    | 0.95   | 0.38    | 0.54  | 0.39  |
| Robust standard deviation (algorithm A) (mg/kg)                            | 3.66    | 0.78   | 0.32    | 0.58  | 0.38  |
| Repeatability standard deviation RSD <sub>r</sub> (mg/kg) <sup>b</sup>     | 2.74    | 0.78   | 0.31    | 0.47  | 0.29  |
| RSD <sub>r</sub> (%) <sup>b</sup>                                          | 11.6    | 14.7   | 16.0    | 12.8  | 11.9  |
| Reproducibility standard deviation RSD <sub>R</sub> (mg/kg) <sup>b</sup>   | 3.38    | 0.96   | 0.38    | 0.54  | 0.39  |
| RSD <sub>R</sub> (%) <sup>b</sup>                                          | 14.4    | 18.1   | 19.5    | 14.8  | 15.9  |

The robust standard deviation was calculated according to algorithm A without removal of outliers. The recovery was calculated by division of the mean value by the incurred (target) level. RSD<sub>r</sub> (%) and RSD<sub>R</sub> (%) are referring to the mean values of each level

<sup>a</sup>Abbreviations of the used materials (numbers indicate the amount of spiked allergenic ingredient (dried cod and peanut respectively) in mg/kg: *SL* sausage Lyoner, *RC* rice cookie, *SP* sauce powder, *SS* shrimp salad, *WC* wheat cookie, *MP* milk powder

<sup>b</sup>All data of a laboratory were removed, if the slope of standard curve was significantly below − 4.1, or if the mean values of the 3 extractions per lab and sample showed more than 50% deviation from the median of all labs for both the evaluation of copy numbers and of the concentrations in mg/kg

powder, cookie, and generally the results of the evaluation in mg/kg did not fulfil the specification ( $RSD_r = 25.6 - 32.2\%$ ).

$RSD_R$  values were in line with the specification of LOQ ( $< 50\%$ ) for sausage (50 mg/kg). The  $RSD_R$  values of the other materials ranged from approximately 50–80% and thus did not fulfil the requirements of quantification. This could be due to the selection of the spiking levels at the LOD of the method.

**3.5.2.2 Peanut** Precision data of all levels and materials fulfilled the acceptance criteria of quantitative analysis. However, in contrast to former collaborative trials (Siegel et al. 2012; Waiblinger et al. 2014, 2017), the analysis of unknown samples was restricted to ready prepared DNA solutions and did not include the DNA extraction. An increase of  $RSD_r$  and  $RSD_R$  has to be expected if the DNA extraction step is performed individually by the labs.

## 4 Conclusion and further work

Within a single lab validation ahead of collaborative trials, we confirmed previously published data on sensitivity and specificity (Tetzlaff and Maede 2017; Ladenburger et al. 2018). Due to the broad range of species to be detected, the detection method of fish shows a differing quantitative response in RT-PCR amplification depending on the fish species. Therefore, this method cannot be used for quantification of fish, if the fish species in the sample is not known and/or if no species-specific DNA calibrator is available.

In collaborative trial, a LOD of approximately 10–20 mg/kg dried fish (species cod and carp bream) could be confirmed corresponding to approximately 50 mg fresh weight of cod or bream per kg. Trueness and precision data obtained in collaborative trials were comparable to those obtained in former studies for the analysis of other allergens (Siegel et al. 2012; Waiblinger et al. 2014, 2017).

For the detection of peanut, a multicopy target-sequence was used and allowed a very sensitive detection of the allergenic ingredient. Within the collaborative trial, a peanut concentration of 0.5 mg/kg (i.e. approx. 0.1–0.2 mg peanut protein/kg) could be reliably detected in a processed cookie matrix.

In addition, this method revealed acceptable trueness and good precision data and can therefore also be used for quantification of the allergenic ingredient peanut.

Trueness may slightly exceed an estimated target value of  $\pm 50\%$  (DLA 2017; Abbott et al. 2010), if material used for calibration/standard (peanut material, degree of roasting, varieties) or degree of processing of matrix standard differs from the sample.

Both methods will be included into the Official Collection of Methods of Analysis in Germany in 2019 (BVL, Official Collection) as a national standard method. However, even if a standardized method is applied, the use of reference material as standard for quantification remains a prerequisite for uniform and reproducible quantification results. The availability of such materials remains challenging.

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# **Collaborative trial validation of RT-PCR methods for the detection and quantification of the allergenic foods fish and peanut**

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## Supplementary Information

Table S1: RT-PCR primer and probe sequences

*a) Fish [Tetzlaff and Maede 2017]*

| Name                                                                     | Sequence                                                           |
|--------------------------------------------------------------------------|--------------------------------------------------------------------|
| <b>Hoxc 13 gene (nuclear DNA):</b> single copy sequences, 112 and 119 bp |                                                                    |
| CTfish fwd1                                                              | 5'-TTC GCT GGT TCT GCA TCC A-3'                                    |
| CTfish fwd7                                                              | 5'-TTC GCT GGT CCT GCA TCC A-3'                                    |
| CTfish rev4                                                              | 5'-CAG GGC AAT TCC CGC TCA AT-3'                                   |
| CTfish rev6                                                              | 5'-ATT ACC GCT CAG TCC CTC CAT-3'                                  |
| CTfish rev7                                                              | 5'-ATT CCC GCT TAG TCC CTC CAT-3'                                  |
| CTfish1 TMP                                                              | 5' - FAM - ACA CCT TGA TGT ACG TTT ATG AAA AAA GCC CGA - BHQ1 - 3' |
| FAM: 6-Carboxyfluorescein; BHQ 1: Black hole quencher 1                  |                                                                    |

*b) Peanut [Ladenburger et al. 2018]*

| Name                                                                                                              | Sequence                                      |
|-------------------------------------------------------------------------------------------------------------------|-----------------------------------------------|
| <b>Atp6:</b> multicopy sequence from mitochondrial DNA close to the coding region of the ATPase subunit 6, 104 bp |                                               |
| atp6-F                                                                                                            | 5'- CAG GGC ATC CTT AAC TGG AG-3'             |
| atp6-R                                                                                                            | 5'- GGA AAG ACG GGT TGG TGA TA-3'             |
| atp6-P                                                                                                            | 5' - FAM-AAG GCG AAG AAG GGT CAG AT-BHQ1 - 3' |
| FAM: 6-Carboxyfluorescein; BHQ 1: Black hole quencher 1                                                           |                                               |

Table S2: Reaction mixes for RT-PCR

| Reagent                                            | Working concentration* | Final concentration | Volume per reaction (µl) |
|----------------------------------------------------|------------------------|---------------------|--------------------------|
| <b>Fish [Tetzlaff and Maede 2017]</b>              |                        |                     |                          |
| Quantitect Multiplex Mastermix ,<br>QIAGEN, Hilden | 2x                     | 1x                  | 12.5                     |
| Primer CTfish fwd1                                 | 15 µM                  | 0.3 µmol/L          | 0.5                      |
| Primer CTfish fwd7                                 | 15 µM                  | 0.3 µmol/L          | 0.5                      |
| Primer CTfish rev4                                 | 15 µM                  | 0.3 µmol/L          | 0.5                      |
| Primer CTfish rev6                                 | 15 µM                  | 0.3 µmol/L          | 0.5                      |
| Primer CTfish rev7                                 | 15 µM                  | 0.3 µmol/L          | 0.5                      |
| Probe CTfish1 TMP                                  | 5 µM                   | 0.1 µmol/L          | 0.5                      |
| Water                                              |                        |                     | 4.5                      |
| DNA-extract                                        | undiluted              |                     | 5.0                      |
| Total reaction volume each PCR                     |                        |                     | 25                       |
| <b>Peanut [Ladenburger et al. 2018]</b>            |                        |                     |                          |
| Quantitect Multiplex Mastermix ,<br>QIAGEN, Hilden | 2x                     | 1x                  | 12.5                     |
| Primer atp6-F                                      | 10 µM                  | 0.4 µmol/L          | 1.0                      |
| Primer atp6-R                                      | 10 µM                  | 0.4 µmol/L          | 1.0                      |
| Probe atp6-P                                       | 10 µM                  | 0.2 µmol/L          | 0.5                      |
| Water                                              |                        |                     | 5.0                      |
| DNA-extract                                        | undiluted              |                     | 5.0                      |
| Total reaction volume each PCR                     |                        |                     | 25                       |

\*other concentrations of the working solutions may be used; added volumes of water and the solutions have to be adapted.

Table S3: Interlaboratory validation studies – list of participants

| <b>Laboratory</b>                                                             | <b>City</b>     | <b>Country</b> | <b>Collaborative trial fish</b> | <b>Collaborative trial peanut</b> |
|-------------------------------------------------------------------------------|-----------------|----------------|---------------------------------|-----------------------------------|
| AGES Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH     | Vienna          | Austria        | X                               |                                   |
| Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit               | Oberschleißheim | Germany        | X                               |                                   |
| Chemisches und Veterinäruntersuchungsamt (CVUA) Freiburg                      | Freiburg        | Germany        | X                               | X                                 |
| CVUA Karlsruhe                                                                | Karlsruhe       | Germany        | X                               | X                                 |
| CVUA Sigmaringen                                                              | Sigmaringen     | Germany        | X                               |                                   |
| Chemisches Landes- und Staatliches Veterinäruntersuchungsamt Münster          | Münster         | Germany        | X                               | X                                 |
| Congen Biotechnologie GmbH                                                    | Berlin          | Germany        |                                 | X                                 |
| Eurofins Analytik GmbH                                                        | Hamburg         | Germany        |                                 | X                                 |
| IGV GmbH                                                                      | Nuthetal        | Germany        |                                 | X                                 |
| Impetus GmbH & Co. Bioscience KG                                              | Bremerhaven     | Germany        |                                 | X                                 |
| Institut für Hygiene und Umwelt                                               | Hamburg         | Germany        |                                 |                                   |
| Institut für Produktqualität GmbH                                             | Berlin          | Germany        | X                               | X                                 |
| Kantonales Laboratorium Zürich                                                | Zürich          | Switzerland    | X                               | X                                 |
| Landesbetrieb Hessisches Landeslabor (LHL)                                    | Kassel          | Germany        | X                               | X                                 |
| Landeslabor Schleswig-Holstein                                                | Neumünster      | Germany        |                                 |                                   |
| LAVES Niedersachsen, Lebensmittel-und Veterinärinstitut Braunschweig/Hannover | Braunschweig    | Germany        | X                               | X                                 |
| Landesuntersuchungsanstalt für das Gesundheits- und Veterinärwesen Sachsen    | Dresden         | Germany        |                                 | X                                 |
| Landesamt für Verbraucherschutz Sachsen-Anhalt                                | Halle           | Germany        | X                               |                                   |
| Lifeprint GmbH                                                                | Illertissen     | Germany        |                                 | X                                 |
| Max-Rubner-Institut Bundesforschungsinstitut für Ernährung und Lebensmittel   | Kulmbach        | Germany        | X                               | X                                 |

Table S4: In-house validation of fish PCR: Between species variation of Cq values  
20 ng/μl of DNA analysed in PCR. Response-values of selected fish species (see Tetzlaff and Maede 2017).

Calculation of response: From Cq-values, the concentration of ng fish DNA/μl was determined using an external standard curve with the species cod as reference (=100%) for all species. The response values were calculated as the ratio between the concentration of fish DNA of the analysed species and the species cod.

| species                                       | Cq value | Response approx. (%) |
|-----------------------------------------------|----------|----------------------|
| cod ( <i>Gadus morhua</i> )                   | 27.6     | 100                  |
| alaska pollack ( <i>Gadus chalcogrammus</i> ) | 23.6     | 1340                 |
| plaice ( <i>Pleuronectes platessa</i> )       | 23.4     | 1520                 |
| pollack ( <i>Pollachius virens</i> )          | 23.2     | 1780                 |
| haddock ( <i>Melanogrammus aeglefinus</i> )   | 25.2     | 460                  |
| mackerel ( <i>Scomber scombrus</i> )          | 23.7     | 1260                 |
| herring ( <i>Clupea harengus</i> )            | 26.6     | 200                  |
| hake ( <i>Merluccius merluccius</i> )         | 24.1     | 940                  |
| yellowfin tuna ( <i>Thunnus albaceres</i> )   | 21.4     | 5690                 |
| trout ( <i>Salmo trutta</i> )                 | 22.1     | 3570                 |

## References

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