# Comparison of specificity and sensitivity of biochip and real-time polymerase chain reaction for pork in food

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**Summary**
Adulteration and food fraud are serious global problems. Analytical methods regarding animal species should represent a comprehensive tool for identification and possible quantification of the components. The aim of the study was to confirm usability and sensitivity of the MEAT 5.0 LCD-Array Kit (version 5.0, Chipron, Berlin, Germany) for forensic analysis of food, which simultaneously detects 24 kinds of animals. We verified its reliability for identification of pork mixed with meat of other 15 animal species. Mixtures of animal species meat were tested in the raw state and after heat treatment. For comparison, real-time polymerase chain reaction innuDETECT Pork Assay (Analytic Jena, Berlin, Germany) with quantitative potential was used. A standard pork DNA curve was established and the detection limit was determined. MEAT 5.0 LCD-Array Kit was found to have a high degree of robustness, probe specificity and repeatability, with a detection limit down to 0.5 % (w/w) but did not allow quantification. We also used both test methods for analysis of commercial products. By these analyses, we identified the presence of pork DNA in 14 products for which this fact was not reported on the product label.

**Keywords**
meat; pork; biochip; detection; real-time polymerase chain reaction

Following European Union labelling regulations [1], meat products should be accurately labelled regarding their species content. Control organs have the responsibility to promote transparency and proper description of food products according to legislation. The introductory statement to Regulation (EU) No 1169/2011 of the European Parliament and Council [1], on the provision of food information to consumers, states that, in order to achieve a high level of health protection for consumers and to guarantee their right to information, it should be ensured that consumers are appropriately informed about the food they consume. Also, hygiene of materials and articles intended to come into contact with food is very important for public health [2]. The largest portion of meat consumed is chicken and beef. Producers falsify especially expensive products or products that are produced in large volumes due to higher profits [3]. A prominent case of food fraud was the horse meat scandal uncovered in Europe in 2013, when undeclared horse meat was substituted for beef in ready meals distributed through supermarkets [4, 5]. This large-scale fraud made it clear that, along with local frauds, consumers can be exposed to organized food crime [6]. Because these regulations underpinning mandatory label information are often neglected, control mechanisms using robust analytical tests are needed to ensure adherence to regulations and, eventually, to enforce punitive measures [4, 7]. In connection with the growing number of cases of food fraud, various analytical methods were developed to ensure correct identification [8]. One of the most specific methods for detecting food adulteration is polymerase chain reaction (PCR), which is generally able to produce qualitative results for the species identified, while real-time PCR (RT-PCR) has been demonstrated to be a useful tool for determination of minimal amounts of

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different species, even in complex foodstuffs [9, 10]. The increasing extent of meat adulteration is the reason for need of the effective methods for meat products authentication. DNA-based PCR is a well-suited alternative for this purpose. Furthermore, the method facilitates quantification of animal DNA in meat products based on the correlation between target copy number and cycle numbers in quantitative PCR [11]. In fact, almost every animal species can be genetically characterized to a high degree of certainty [4, 12]. The analysis of DNA connected with PCR presents a fast, sensitive and highly specific alternative compared to protein-based methods [13]. Mitochondrial genes are widely used for species identification because they present high variability among different species and low variability among individuals of the same species [4, 14]. A new technique called DNA microarray has been increasingly used in food safety studies. The MEAT 5.0 LCD-Array Kit (Chipron, Berlin, Germany) was designed to detect both domestic and wild animal species for human consumption [4]. It has also opened new challenges for food analysis of adulteration of meat and meat products. DNA microarrays make it possible to display the whole genome on a chip and to express the interaction of thousands of genes with each other simultaneously [15]. In previous evaluation studies, it has been demonstrated that DNA microarray technique is efficient and reliable for meat identification [4, 15 – 19]. The MEAT 5.0 LCD-Array Kit allows the analysis of 115 – 125 base pair (bp) fragments of the 16S rRNA mitochondrial gene belonging to 24 vertebrate species (MEAT 5.0 Manual, version 1-1-2014). Beltramo et al. [4] carried out a validation process for the Low Cost and Density (LCD) Array (MEAT 5.0 version) kit designed to detect both domestic and wild species for human consumption. Real-time PCR can be used as a multiplex reaction and simultaneous detection of several species was possible similarly to biochips. However, biochip methods are more appropriate for diagnostic laboratories, while PCR methods are more flexible, thus more useful for research purposes [15, 20]. Therefore, the DNA microarray method is widely preferred for understanding mechanisms in food safety studies [21]. In recent years, the literature relating to DNA microarrays focused on the detection of adulteration in foods, meat and meat products [22].

This study presents the confidence results of a process to identify animal species with the MEAT 5.0 LCD-Array Kit for species identification in food. The study aimed to identify pork in a meat mixture. For comparison, we used the innuDETECT Pork Assay detection tool (Analytic Jena, Berlin, Germany), a real-time PCR method, which allows quantification of DNA.

## MATERIALS AND METHODS

### Meat species

Meat mixtures were prepared using muscle tissue from pig (*Sus scrofa*) and 15 other animal species: cattle (*Bos taurus*, *Bos bison*), sheep (*Ovis aries*), equines (*Equus caballus*, *E. asinus*), goat (*Capra hircus*), hare (*Lepus europaeus*), rabbit (*Oryctolagus cuniculus*), roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*), fallow deer (*Dama dama*), chicken (*Gallus gallus*), turkey (*Meleagris gallopavo)*, goose (*Anser* sp.), mallard duck (*Anas platyrhynchos*), Muscovy duck (*Cairina moschata*) and pheasant (*Phasianus* sp.).

Meat samples were obtained from a grocery store and meat from pheasant, fallow deer, red deer, roe deer and hare were obtained from the regional hunting association. Meat samples were cut into small pieces using a sterile scalpel, placed into sterile tubes and stored in intact packaging. All samples were stored at –18 °C.

### Mixtures

Mixtures of meats were prepared using Blender 8008 (Waring Commercial, Torrington, Connecticut, USA) to a final weight of 100 g. To prevent any cross-contamination, each mixture was processed separately using a different blender container. Two sets of samples were made, one set was analysed in the raw state and the other set after the heat treatment (100 °C, 90 min). The grinding was accomplished by passing through a small hand grinder and blender. Mixing was done in five cycles for three minutes. In the meantime, the mixing vessel was placed in the refrigerator at 8 °C for 10 min. The mixtures of meat were immediately stored at –18 °C until DNA extraction. Binary mixtures designed to verify the quantification potential of the compared methods were prepared by dilution of DNA in the laboratory.

Commercial food samples were collected from local markets in the Slovakia and coded appropriately. We focused on a wide spectrum and variety of products, including traditional specialities. The product description and country of origin were recorded. The food products included 16 samples of frankfurters, 15 sausages, 75 samples of meat pastes, 14 hams, 15 salami samples, 5 samples of protein additives, 25 minced meat samples, 15 samples of luncheon meat, 6 burger patties and 7 mixtures of spices with protein additives.

### DNA extraction and PCR amplification

DNA was extracted from the prepared mixtures of meat with admixtures and meat products using Maxwell 16 Tissue DNA Purification Kit (Promega, Madison, Wisconsin, USA) and Maxwell 16 system (Promega), respectively, following the manufacturer's instructions. DNA was extracted from 50 mg of sample. The amount of DNA in each sample was quantified using a Quantus fluorimeter (Promega, Madison, Wisconsin, USA). All DNA extracts used for both analyses had the same overall concentration. DNA solutions were stored at –18 °C until further use. PCR runs were performed in a TOptical Gradient 96 thermocycler (Biometra, Göttingen, Germany). Each PCR reaction contained 25 $\mu$l of an amplification mixture consisting of 5 $\mu$l of diluted DNA sample, 12.5 $\mu$l of 2 × PCR Master Mix (included Taq Polymerase (EC 2.7.7.7), dNTPs, buffer and $MgCl_2$), 1.5 $\mu$l of MEAT Primer Mix and 6 $\mu$l of PCR-grade water. MEAT PCR primers were supplied in the MEAT 5.0 LCD-Array Kit. The thermal process was set to one cycle at 95 °C for 5 min for initiation of denaturation. The thermocycling process consisted of 35 cycles including a denaturation step for 30 s at 94 °C, an annealing step for 45 s at 57 °C and an elongation step for 45 s at 72 °C. A final step ended the PCR program and took 2 min at 72 °C.

### Agarose gel electrophoresis

Analysis of PCR amplicons was performed using agarose gel electrophoresis with a Consort Maxi Series EV243 (Cleaver Scientific, Rugby, United Kingdom). Agarose gel (2%) was prepared by dissolving the appropriate quantities of agarose (SERVA Electrophoresis, Heidelberg, Germany) in 1× Tris-Acetate-EDTA buffer (Focus Bioscience, Queensland, Australia) (pH 8.0) in a ProLine SM117 microwave oven (ProLine, Schiphol, Netherlands). Ethidium bromide GelRed 10.000× (Biotium, Fremont, California, USA) stock solution was added directly to molten agarose at a concentration of 1 $\mu g \cdot ml^{-1}$, before pouring the gel. The electrophoretic samples were mixed with 5× Green GoTaq Flexi Buffer (Promega) before loading into the gel. After electrophoresis, DNA fragments in the agarose gel were visualized with an EB-20 UV transilluminator (Ultra Lum, Claremont, California, USA).

### LCD array hybridization and detection

The MEAT 5.0 LCD-Array Kit is composed of eight chips, with 25 species-specific capture probes that are fixed to each chip. The LCD array system can detect cattle, buffalo, pig, sheep, goat, horse, donkey, rabbit, hare, chicken, turkey, goose and two duck varieties in food samples. The probes are spotted as duplicates in an 8 × 8 scheme. The PCR products were identified on the LCD array following the manufacturer's instructions (Chipron). Three different spots on the chip are marked as hybridization control points (Hyb-Control) to detect a positive reaction and are located in the upper-left, upper-right and lower-right corners, respectively. Biotinylated amplicons were hybridized at 35 °C to the probes on the array and then linked to a conjugate. A dark precipitate provided in the test kit, after adding peroxidase substrate, was visualized by a PF3650u LCD-array scanner (PacificImage Electronics, Torrance, California, USA) using SlideReader V12 software (Chipron). The default detection cut-off threshold was a pixel value of 2000 (MEAT 5.0 Manual, version 1-1-2014).

### Real-time PCR

Samples analysed by MEAT 5.0 LCD-Array Kit were verified by real-time PCR using the innuDETECT Pork Assay. DNA previously isolated using a Maxwell 16 Tissue DNA Purification Kit (Promega) and Maxwell 16 system (Promega) stored at –18 °C was used. The procedure given for the innuDETECT Pork Assay was followed. Positive and negative controls were run. All solutions and materials in the assay were dissolved before use, 20 $\mu$l of PCR Master Mix including 10 $\mu$l of 2× Master Mix, 3 $\mu$l primer/probe mix, 1 $\mu$l internal control and 1 $\mu$l PCR-grade water was pipetted into each tube, and 5 $\mu$l of previously extracted DNA was added into each. The tubes were closed tightly and the reaction took place in LightCycler 2.0 (Roche Diagnostics, Mannheim, Germany) using a thermal programme consisting of a period at 95 °C for 120 s, then 35 cycles each containing a period at 95 °C for 10 s and a period at 62 °C for 45 s. Results were processed by LightCycler 2.0 Software 4.1 (Roche Diagnostics). Experiments were replicated three times and reactions were replicated twice per experiment.

### Verifying and comparison of analytical tools

We focused on verifying the species specificity, sensitivity, stability and repeatability of the biochip to identify the animal species in the mixture with pork meat (Tab. 1). Comparison of the results obtained from the innuDETECT Pork Assay and MEAT 5.0 LCD-Array Kit for pork in mixtures with beef and chicken was performed as described above. We prepared two variants of the mixture with 18 different ratios of pork addition (Tab. 2, Tab. 3).

### Sensitivity and quantification ability of the methods

Pork DNA concentrations were determined using a standard curve. The sensitivity and linearity of the detection system used in this study were determined by using serial dilutions of pork genomic DNA extracted from pork meat, starting with 100 ng·$\mu$l$^{-1}$ of DNA. The cycle threshold (*Ct*) values were plotted against the logarithms of DNA concentrations to create a standard curve for pork DNA. Linearity was observed for pork DNA over six orders of magnitude (100–0.001 ng·$\mu$l$^{-1}$). Determination of the detection limit for pork DNA was carried out using real-time PCR. The minimum quantities of DNA detected are listed in Tab. 4 and described as the limit of detection (*LOD*). The detectable *Ct* values from the reaction were compared with the signal strength measured for the detection of pork meat using the MEAT 5.0 LCD-Array Kit.

**Tab. 1.** Composition of mixtures to determine probe specificity and sensitivity of the LCD array.

| Admixture | Major component [%] | Minor component 1 [%] | Minor component 2 [%] |
|---|---|---|---|
| | Pork | Goat | Sheep |
| A1 | 98.0 | 1.0 | 1.0 |
| A2 | 99.0 | 0.5 | 0.5 |
| A3 | 99.8 | 0.1 | 0.1 |
| | Pork | Beef | Equines |
| B1 | 98.0 | 1.0 | 1.0 |
| B2 | 99.0 | 0.5 | 0.5 |
| B3 | 99.8 | 0.1 | 0.1 |
| | Pork | Hare | Rabbit |
| C1 | 98.0 | 1.0 | 1.0 |
| C2 | 99.0 | 0.5 | 0.5 |
| C3 | 99.8 | 1.0 | 1.0 |
| | Pork | Red deer | Fallow deer |
| D1 | 98.0 | 1.0 | 1.0 |
| D2 | 99.0 | 0.5 | 0.5 |
| D3 | 99.8 | 0.1 | 0.1 |
| | Pork | Turkey | Pheasant |
| E1 | 98.0 | 1.0 | 1.0 |
| E2 | 99.0 | 0.5 | 0.5 |
| E3 | 99.8 | 0.1 | 0.1 |
| | Pork | Chicken | Goose |
| F1 | 98.0 | 1.0 | 1.0 |
| F2 | 99.0 | 0.5 | 0.5 |
| F3 | 99.8 | 1.0 | 1.0 |
| | Pork | Mallard duck | Muscovy duck |
| G1 | 98.0 | 1.0 | 1.0 |
| G2 | 99.0 | 0.5 | 0.5 |
| G3 | 99.8 | 0.1 | 0.1 |
| | Pork | Roe deer | Fallow deer |
| H1 | 98.0 | 1.0 | 1.0 |
| H2 | 99.0 | 0.5 | 0.5 |
| H3 | 99.8 | 0.1 | 0.1 |

Composition is expressed in weight percent.

## RESULTS AND DISCUSSION

### DNA extraction yield

Isolations were performed from the femoral muscle of selected animal species. The concentration of DNA ranged from 5.9 ng·$\mu$l$^{-1}$ to 30.0 ng·$\mu$l$^{-1}$ for both raw meats and raw mass mixtures. For heat-treated meats and mass mixtures, it was 0.9–21.0 ng·$\mu$l$^{-1}$. To evaluate the specificity of the method and to verify the absence of potential cross-reactivity, pure samples from individual meat species were tested first.

Comparison of the concentrations of the extracted DNA showed lower values for the samples after heat treatment, which was similar to the study of Beltramo et al. [4]. Those authors worked with DNA concentrations ranging from 6.6 ng·$\mu$l$^{-1}$ to 25.0 ng·$\mu$l$^{-1}$ for the raw meat mixtures that contained portions of contaminant species, and from 0.6–1.3 ng·$\mu$l$^{-1}$ for the cooked mixtures. According to the real-time PCR efficiency and correlation coefficient, only some commercial kits produce DNA of sufficient quality for real-time PCR [23]. It is known that heat treatment, which is often used in the production of processed foods, can cause DNA degradation with lower yields [15, 17, 24].

Extraction of DNA from meat supplements containing various amounts of contaminating species required several additional steps to be included in the protocol, which could have had an impact on the extraction efficiency. Using MEAT 5.0 LCD-Array Kit, the various DNA extracts did not affect the sensitivity or repeatability of the method.

### Specificity and sensitivity of DNA biochip

In the first part, we focused on the specificity of MEAT 5.0 LCD-Array Kit at pork identification. Species specificity trials were conducted on 16 species combined in mixtures of three species together, pork being used in each mixture as the basic ingredient of the mixture. The kit manufacturer declares a detection limit of $< 0.5\%$ (w/w) depending on the sample's processing level (MEAT 5.0 Manual). We tested samples with 0.1% (w/w) contamination to determine if the kit is actually able to detect the low-level contamination that may be important to consumers [25] or to those holding a religious belief that prohibits the consumption of certain species [26]. To deter-

**Tab. 2.** Results of LCD array and real–time PCR for mixtures of pork and beef.

| No. | Composition [%] | | MEAT 5.0 LCD–Array Kit | | innuDETECT Pork Assay | |
|---|---|---|---|---|---|---|
| | Beef | Pork | Beef | Pork | Beef | Pork |
| 1 | 0.0 | 100.0 | – | + | – | + |
| 2 | 100.0 | 0.0 | + | – | + | – |
| 3 | 10.0 | 90.0 | + | + | + | + |
| 4 | 20.0 | 80.0 | + | + | + | + |
| 5 | 30.0 | 70.0 | + | + | + | + |
| 6 | 40.0 | 60.0 | + | + | + | + |
| 7 | 50.0 | 50.0 | + | + | + | + |
| 8 | 55.0 | 45.0 | + | + | + | + |
| 9 | 60.0 | 40.0 | + | + | + | + |
| 10 | 65.0 | 35.0 | + | + | + | + |
| 11 | 70.0 | 30.0 | + | + | + | + |
| 12 | 75.0 | 25.0 | + | + | + | + |
| 13 | 80.0 | 20.0 | + | + | + | + |
| 14 | 85.0 | 15.0 | + | + | + | + |
| 15 | 90.0 | 10.0 | + | + | + | + |
| 16 | 95.0 | 5.0 | + | + | + | + |
| 17 | 99.0 | 1.0 | + | + | + | + |
| 18 | 99.5 | 0.5 | + | + | + | + |
| 19 | 99.9 | 0.1 | + | – | + | – |

(+) – analysis confirmed the presence of the component, (–) – presence of the component was not confirmed by analysis.

**Tab. 3.** Results of LCD array and real-time PCR for mixtures of pork and chicken meat.

| No. | Composition [%] | | MEAT 5.0 LCD-Array Kit | | innuDETECT Pork Assay | |
|---|---|---|---|---|---|---|
| | Chicken | Pork | Chicken | Pork | Chicken | Pork |
| 1 | 100.0 | 0.0 | + | – | + | – |
| 2 | 0.0 | 100.0 | – | + | – | + |
| 3 | 10.0 | 90.0 | + | + | + | + |
| 4 | 20.0 | 80.0 | + | + | + | + |
| 5 | 30.0 | 70.0 | + | + | + | + |
| 6 | 40.0 | 60.0 | + | + | + | + |
| 7 | 50.0 | 50.0 | + | + | + | + |
| 8 | 55.0 | 45.0 | + | + | + | + |
| 9 | 60.0 | 40.0 | + | + | + | + |
| 10 | 65.0 | 35.0 | + | + | + | + |
| 11 | 70.0 | 30.0 | + | + | + | + |
| 12 | 75.0 | 25.0 | + | + | + | + |
| 13 | 80.0 | 20.0 | + | + | + | + |
| 14 | 85.0 | 15.0 | + | + | + | + |
| 15 | 90.0 | 10.0 | + | + | + | + |
| 16 | 95.0 | 5.0 | + | + | + | + |
| 17 | 99.0 | 1.0 | + | + | + | + |
| 18 | 99.5 | 0.5 | + | + | + | + |
| 19 | 99.9 | 0.1 | + | – | + | – |

(+) – analysis confirmed the presence of the component, (–) – presence of the component was not confirmed by analysis.

mine the potential cross-reactivity or competitiveness of the individual probes, mixtures of pork containing two animal species in the same ratio (Tab. 1, mixtures A1 to H3) were tested in the raw state and after heat treatment. These reactions were replicated twice. In general, there was 100% specificity for all samples tested, without false positives or false negatives. All species present in the additions provided hybridization signals on their respective species-specific capture probes in the meat, and the species were successfully identified by their specific probes. The exceptions were red deer and reindeer, which cross-reacted. However, this feature was mentioned in the user's guide.

**Tab. 4.** Specificity and sensitivity (detection limit) for porcine DNA detected by innuDETECT Pork Assay.

| Binary meat mixtures | | Concentration [ng·$\mu$l$^{-1}$] | Logarithm of concentration | *Ct* value | Signal intensities (pixel value 2000) | |
|---|---|---|---|---|---|---|
| Maj [%] | Min [%] | | | | Replicate 1 | Replicate 2 |
| **Beef + pork** | | | | | | |
| 90.0 | 10.0 | 0.771 | –0.113 | 22.79 | 45678 | 41651 |
| 95.0 | 5.0 | 0.175 | –0.757 | 25.01 | 38501 | 43876 |
| 99.0 | 1.0 | 0.115 | –0.939 | 25.64 | 8906 | 11543 |
| 99.5 | 0.5 | 0.025 | –1.590 | 27.90 | 3219 | 2088 |
| 99.9 | 0.1 | ND | ND | ND | ND | ND |
| **Chicken + pork** | | | | | | |
| 90.0 | 10.0 | 5.062 | 0.704 | 19.97 | 51623 | 34562 |
| 95.0 | 5.0 | 0.911 | –0.041 | 22.54 | 12874 | 6719 |
| 99.0 | 1.0 | 0.036 | –1.449 | 27.40 | 11764 | 13007 |
| 99.5 | 0.5 | 0.001 | –2.870 | 32.30 | 2098 | 3215 |
| 99.9 | 0.1 | ND | ND | ND | ND | ND |

Maj – major component (beef or chicken meat), Min – minor component (pork), ND – not detected.

Similar results were obtained by Iwobi et al. [18] and Cottenet et al. [17], using two different versions of the MEAT 5.0 LCD-Array Kit and analysing each species separately. The cross-reaction between red deer and reindeer was observed at the analysis of raw meat. Thermal treatment eliminated the occurrence of the cross-reaction.

Raw and heated meat and mixtures were analysed at three different concentrations, each analysis being repeated twice. All animal species were correctly identified down to the detection limit of 0.5% (w/w) in each meat sample. In samples containing 0.1% (w/w) adulterant species, the LCD array detected all the DNA but was less repeatable because there was no comparable result for the two replicates that were performed in each analysis. For comparison with other authors, we evaluated the repeatability of the method by computing Cohen's kappa (κ) based on data obtained from three different concentrations of meat admixture (1%, 0.5%, 0.1% (w/w)). A κ value of $> 0.63$ was the average of the results for all animal species analysed. This value was considered to be the threshold for a good level of accuracy. Cohen's kappa was also calculated for the results for meat supplements containing only 1% or 0.5% (w/w) adulterants. In this case, $\kappa = 1$ due to the high rate of repeatability of the method for the estimated allowances. The results were similar to the findings of Beltramo et al. [4]. No apparent difference was observed between crude and heat-treated mixtures, indicating that heat treatment did not affect the accuracy of the LCD array (Tab. 5).

In meat supplements, the MEAT 5.0 LCD-Array Kit sensitivity was 0.5% (w/w) for detection of the contaminating species present in the samples, both raw and cooked, although the amount of DNA extracted from the raw samples was higher. The results coincided with the findings of Beltramo et al. [4] who found a high degree of repeatability for the method even when adding to milk with an *LOD* of 0.1% (w/w) for the contaminants present, although yields of DNA extracted from the milk matrix were lower than for meat samples.

Studies by Iwobi et al. on the MEAT 1.6 LCD Array determined *LOD* of 0.1% (w/w) in meat samples [18]. This *LOD* value was determined in a single analysis for each meat mixture at different levels of contamination. In the validation process described in this study, each mixture was analysed twice to better evaluate the sensitivity of the method and its repeatability. For both replicates, the addition of 0.1% (w/w) meat in meat mixtures was not found. Detection was from 0.5% and 1% (w/w) in the mixtures. This worse *LOD* value for meat samples could be caused either by lower sensitivity of the probe or by difficulties in obtaining a perfectly homogenous sample.

The 1% (w/w) rate set by the 2013/99/EU Commission Recommendation of 19 February 2013 [27] in a coordinated control plan with a view to establish the prevalence of fraudulent practices in the marketing of certain foods, as a level to differentiate between cross-contamination/counterfeiting and trace levels from accidental contamination, is considered an acceptable limit for determining counterfeiting [17]. Based on testing conducted to determine the ability of the method to obtain results from DNA obtained from samples exposed to various treatments, no difference in performance was observed, indicating that the kit could also be used to test cooked or sterilized foods.

#### Tests effectivity for pork detection in meat mixtures

To compare the efficiency of the two approaches to identify pork, we prepared two variants of binary mixtures with a specific proportion of pork as a main component of the study and compared the results from analyses (Tab. 2, Tab. 3). Bovine and chicken meat were added in various percentage ratios. In the first combination of a binary mixture of beef and pork, the tests were able to detect pork at the addition greater or equal to 0.5% (w/w). Upon addition of 0.1% (w/w) pork to beef, innuDETECT Pork Assay was not successful in identifying pork in multiple repetitions. The second variant, a combination of pork with chicken meat, consisting of 18 different concentrations of pork, showed that these two methods were able to detect pork addition greater or equal to 0.5% (w/w). The addition of 0.1% (w/w) of pork was not detected by either MEAT 5.0 LCD-Array Kit or innuDETECT Pork Assay kit. The addition of 1% and 0.5% (w/w) had to be analysed in multiple repetitions as we experienced difficulties with this combination. The results of the two approaches were identical and repeatable only for higher concentrations of meat addition. Results of the analysis of binary mixtures were identical before and after the heat treatment. Similar results were reported by Al-Kahtani et al. [28]. However, those authors did not examine the addition of 0.5% and 0.1% (w/w) of pork.

#### Sensitivity of the detection system

The sensitivity of the innuDETECT Pork Assay system was determined using serial dilutions of pork meat genomic DNA extracted from pork meat, starting with 100 $ng \cdot \mu l^{-1}$ of DNA. In assessing the sensitivity and quantification potential of the method, we constructed a standard curve,

**Tab. 5.** Species detected by MEAT 5.0 LCD-Array Kit in ternary mixtures.

| | Before heat treatment | | | | | | After heat treatment | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Repetition 1 | | | Repetition 2 | | | Repetition 1 | | | Repetition 2 | | |
| | Maj | Min1 | Min2 | Maj | Min1 | Min2 | Maj | Min1 | Min2 | Maj | Min1 | Min2 |
| **Pork + mutton + goat meat** | | | | | | | | | | | | |
| A1 | + | + | + | + | + | + | + | + | + | + | + | + |
| A2 | + | + | + | + | + | + | + | + | + | + | + | + |
| A3 | + | – | – | + | + | – | + | + | + | + | + | + |
| **Pork + beef + equine meat** | | | | | | | | | | | | |
| B1 | + | + | + | + | + | + | + | + | + | + | + | + |
| B2 | + | + | + | + | + | + | + | + | + | + | + | + |
| B3 | + | + | + | + | + | – | + | + | + | + | + | + |
| **Pork + hare + rabbit meat** | | | | | | | | | | | | |
| C1 | + | + | + | + | + | + | + | + | + | + | + | + |
| C2 | + | + | + | + | + | + | + | + | + | + | + | + |
| C3 | + | – | – | + | + | + | + | + | + | + | + | + |
| **Pork + red deer meat + fallow deer meat** | | | | | | | | | | | | |
| D1 | + | +* | + | + | + | + | + | + | + | + | + | + |
| D2 | + | +* | + | + | + | + | + | + | + | + | + | + |
| D3 | + | –* | + | + | – | – | + | + | + | + | + | – |
| **Pork + turkey meat + pheasant meat** | | | | | | | | | | | | |
| E1 | + | + | + | + | + | + | + | + | + | + | + | + |
| E2 | + | + | + | + | + | + | + | + | + | + | + | + |
| E3 | + | + | + | + | + | + | + | + | + | + | + | + |
| **Pork + chicken meat+ goose meat** | | | | | | | | | | | | |
| F1 | + | + | + | + | + | + | + | + | + | + | + | + |
| F2 | + | + | + | + | + | + | + | + | + | + | + | + |
| F3 | + | + | + | + | + | – | + | + | + | + | + | + |
| **Pork + mallard duck meat + muscovy duck meat** | | | | | | | | | | | | |
| G1 | + | + | + | + | + | + | + | + | + | + | + | + |
| G2 | + | + | + | + | + | + | + | + | + | + | + | + |
| G3 | + | + | – | + | – | + | + | + | + | + | + | – |
| **Pork + roe deer meat+ fallow deer meat** | | | | | | | | | | | | |
| H1 | + | + | + | + | + | + | + | + | + | + | + | + |
| H2 | + | + | + | + | + | + | + | + | + | + | + | + |
| H3 | + | + | – | + | + | – | + | + | + | + | + | + |

Composition of mixtures is given in Tab. 1.
Maj – major component (pork), Min1 – minor component 1, Min2 – minor component 2.
(+) – analysis confirmed the presence of the component, (–) – presence of the component was not confirmed by analysis, (*) – weak cross reactivity of the capture probe for reindeer with pure red deer and vice versa.

through which we determined the concentration of pork DNA in binary pork mixtures. *Ct* values were plotted against the DNA concentration logarithms to generate a standard curve for pork DNA. Linearity was observed for pork DNA over six orders of magnitude. The correlation between *Ct* values and the logarithmic DNA concentration of the pork template showed a regression coefficient of 0.9952, indicating a linear relationship between *Ct* values and DNA concentrations in the range of 100–0.001 ng·$\mu$l$^{-1}$ of porcine DNA. Target DNA at a concentration as low as 0.001 ng·$\mu$l$^{-1}$ could even be observed, indicating that real-time PCR *LOD* for pure porcine DNA was 0.001 ng·$\mu$l$^{-1}$. Tanabe et al. [29, 30] concluded that the *LOD* values for porcine DNA using real-time PCR and conventional PCR were 10 fg·$\mu$l$^{-1}$ and 1 pg.$\mu$l$^{-1}$, respectively. These contentions agree with those of Kesmen et al. [31], who found that real-time PCR could detect chicken and turkey DNA in a concentration of 0.0001 ng·$\mu$l$^{-1}$.

In the study, we attempted to estimate the concentration of DNA at the bottom of the 18 stages of concentration ranging from the addition of 10%, 5%, 1%, 0.5% and 0.1% (w/w) pork to beef and chicken. In the pig and beef mixtures, we

were able to detect: 10% (w/w) addition of pork with a *Ct* value of 22.79, 5% (w/w) addition at a *Ct* value of 25.01, 1% (w/w) with a *Ct* value of 25.64, and 0.5% (w/w) with a *Ct* value of 27.90. Addition of 0.1% (w/w) pork meat to beef was not detected. In the pork and chicken meat mixtures, a *Ct* value of 19.97 was found for addition of 10% (w/w) of pork, a *Ct* value of 22.54 for 5% (w/w) addition; a *Ct* value of 27.4 for 1% (w/w) addition, a *Ct* value of 32.3 for 0.5% (w/w) addition and addition of 0.1% (w/w) pork to chicken meat was not detected, as shown in Tab. 4. Similar results were reported by Al-Kahtani et al. [28] who detected pork DNA in meat mixtures using both conventional PCR and real-time PCR. They analysed 30 meat mixtures containing beef, chicken and other meats with a varying content of pork (0%, 1%, 5%, 10% and 20% (w/w)). They used pork DNA standard curves and *Ct* values for quantification. The analysis detected pork at 1–20% (w/w) in all meat mixtures with only one exception, 1% (w/w) pork in chicken, where 1% (w/w) pork was not detected. Beef mixtures tested positive for pork using real-time PCR and had *Ct* values of 19.09, 22.78, 24.26 and 23.90 for beef meat containing 20%, 10%, 5% and 1% (w/w) pork, respectively. Those authors did not analyse admixtures at low levels (0.5% and 0.1% (w/w)). The results of this study demonstrate reliability of the assay for detecting pork at different levels. In the case of pork and beef combinations, *LOD* value for porcine DNA in meat mixtures was 0.025 $ng \cdot \mu l^{-1}$ and in the second variant of pork/chicken mixtures, *LOD* value was 0.001 $ng \cdot \mu l^{-1}$.

The recorded *Ct* values were compared to the gained signal strength, which we recorded with MEAT 5.0 LCD-Array Kit. No linear relationship was found between the values measured. The quantification potential of innuDETECT Pork Assay was confirmed (Tab. 4).

**Food samples from the market**

Within the monitored set of 193 samples taken from commercial food networks, several products were identified that contained animal species other than those indicated on the product label (Tab. 6). For 14 products, we noticed undeclared pork and we tried to quantify it. Samples from shops and supermarkets were also analysed using the LCD array. The results presented in this article as an example of practical application showed that the LCD array can detect species present in various types of food even in the presence of various food ingredients. Two products that were positive for the presence of porcine DNA identified by MEAT 5.0 LCD-Array Kit were not confirmed by innuDETECT Pork Assay.

The kits worked well also on matrices other than muscle tissues, such as sausages and ham, which contain other types of tissue such as fatty tissue or connective tissue. Samples in which non-labelled pork meat was recorded were further examined to determine the concentration of DNA in the product. The concentration of porcine DNA in samples positive for pork contamination was estimated using a standard porcine DNA curve, the results are listed in Tab. 7. Ulca et al. [32], in a survey of 42 samples of Turkish meat products, found four samples positive for porcine DNA. Ali et al. [33] reported chicken nuggets containing pork. Demirhan et al. [34] reported that two out of 11 retail products from Germany were found to contain pork gelatine with *Ct* values of 30 and 43. They also tested 32 samples from Turkey, and one of those products (cake covered with gelatine) was positive with a *Ct* value of 36.3. Sahilah et al. [35]

**Tab. 6.** Undeclared species detected in food samples from the market analysed by the LCD array kit.

| Product description | *n* | Incorrectly labelled [%] | Contaminated by porcine DNA [%] |
|---|---|---|---|
| Protein additive | 5 | 40 | 0 |
| Mixture of spices with protein additive | 7 | 28 | 14 |
| Frankfurter | 16 | 31 | 0 |
| Raw sausage | 15 | 40 | 0 |
| Minced meat – mixtures for meat production | 25 | 16 | 12 |
| Meat paste | 75 | 22 | 8 |
| Ham | 14 | 78 | 21 |
| Salami | 15 | 60 | 6 |
| Burger patty | 6 | 16 | 0 |
| Luncheon meat | 15 | 20 | 0 |
| Total | 193 | 31 | 7 |

**Tab. 7.** Results of analysis of samples contaminated with porcine DNA.

| Sample | Concentration [ng·$\mu$l$^{-1}$] | Logarithm of concentration | *Ct* value | Signal intensities (pixel value 2000) | |
|---|---|---|---|---|---|
| | | | | Replicate 1 | Replicate 2 |
| 1 | 0.040 | –1.397 | 27.22 | 31675 | 36754 |
| 2 | 0.118 | –0.928 | 25.60 | 47191 | 32789 |
| 3 | 1.230 | 0.090 | 22.09 | 51906 | 53812 |
| 4 | ND | ND | ND | 3204 | 3001 |
| 5 | 0.005 | –2.301 | 30.34 | 2098 | 4563 |
| 6 | 1.706 | 0.232 | 21.60 | 44860 | 50142 |
| 7 | 0.726 | –0.139 | 22.88 | 33571 | 50676 |
| 8 | 0.003 | –2.513 | 31.07 | 3491 | 4561 |
| 9 | 0.062 | –1.206 | 26.56 | 10391 | 31602 |
| 10 | 1.041 | 0.017 | 22.34 | 56371 | 31760 |
| 11 | 0.309 | –0.510 | 24.16 | 50417 | 43712 |
| 12 | ND | ND | ND | 2045 | 3112 |
| 13 | 0.006 | –2.255 | 30.18 | 4461 | 5820 |
| 14 | 0.099 | –1.003 | 25.86 | 23654 | 39410 |

ND – not detected.

found that 37.2 % of the pharmaceutical capsules they tested contained porcine DNA, *Ct* values ranging from 21.40 to 31.07. Kesmen et al. [36] reported that more than 30 samples of cooked and raw meat mixtures were detected with *LOD* as low as 0.0001 ng. Such small amounts of porcine DNA in commercial food products may result from cross-contamination in the production line rather than from deliberate adulteration of food products with pork [28]. There are no acceptable levels of pork contamination specified by any regulatory authorities for Halal foods. Ulca et al. [32] suggested 0.1% (w/w) as an appropriate limit .

## CONCLUSIONS

MEAT 5.0 LCD-Array Kit identifies 24 meat species in eight samples simultaneously. It enables detection of the usual meat species used in the food industry in Europe. The present evaluation showed that this kit is able to detect admixtures at levels as low as 0.5% (w/w) in raw meat and cooked meat. The capture probe providing pork identification, which contains multiple sequences to detect polymorphisms of individual nucleotides in the target region, is specific as cross-reactivity was not detected. The Real-time PCR-based innuDETECT Pork Assay, which is already long used by laboratories for foods authentication, can identify raw and cooked meat. When combining pork and beef, we managed to identify 0.5% (w/w) addition of pork meat. In a binary mixture of chicken and pork meat, we were able to identify pork meat from the addition of 0.5% (w/w). A high degree of sensitivity was demonstrated for this method at identification of food containing one animal species. The present study can be used as a reference for any food or forensic laboratory. This method is fast, specific, sensitive, easy to use and fit for purpose for meat-testing laboratories. The study also highlights the importance of control, as there was a high number of animal species detected but not declared on the product label.

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