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Accurate determination of meat mass fractions using DNA measurements for quantifying meat adulteration by digital PCR

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Summary

The alarming problem of meat adulteration emphasises the demand for accessible analytical approaches for food regulatory agencies to detect and, specially, to measure altered meat fractions. This study proposes a novel cross-species triplex droplet digital Polymerase Chain Reaction (ddPCR) assay to simultaneously identify and quantify the ratios of pork/beef meat fractions from a total DNA content, including processed and autoclaved meat, without requiring a standard, achieving high sensitiviity with a limit of quantification estimated at 0.1% (w/w) and a limit of detection down to 0.01% (w/w). A single copy nuclear gene, *β-actin*, was employed as a target, accompanied with *myostatin* gene as a cross-species target to quantify the meat background. The duplex assay provided a simultaneous quantification of pork and *myostatin*, whereas the triplex assay was able to detect pork, beef and *myostatin* with a decrease of technical error, cost and time.

Keywords: droplet digital PCR, pork/beef adulteration, meat quantification, food mislabelling, DNA measurements, mass fractions

Introduction

Meat adulteration and food fraud have recently become a globally widespread problem. According to the US Federal Meat Inspection Act (FMIA) and the European Parliament under Regulation (EC) No. 178/2002, adulteration of meat products with undeclared meat species is forbidden (Grundy *et al*., 2013). This includes the substitution of higher-valued meats with lower-valued ones during production process, which can lead to an unfair competition (Fajardo *et al*., 2008), and cause health problems such as food allergy (Naaum *et al*., 2018; Temisak *et al*., 2019) and bovine spongiform encephalopathy, a fatal neurodegenerative disease (Sultan *et al*., 2004). Previous studies revealed that over 50% of processed meat products tested were mislabelled (Cawthorn *et al*., 2013; Di Pinto *et al*., 2015; Quinto *et al*., 2016). Therefore, accurate methods for determination of meat species in food products are required.

There are several analytical methods used for identifying meat species, most of which are based on protein and DNA detection. Protein-based methods such as electrophoretic techniques (Montowska and Pospiech 2007), chromatography-mass spectrometry (Grundy *et al*., 2008) and enzyme-linked immunosorbent assay (ELISA) (Chen and Hsieh 2000; Hsieh and Ofori 2014) have been successfully employed for detecting meat species. These techniques, however, showed some disadvantages, such as imprecision, low specificity and sensitivity, as proteins can be denatured by heat, pressure, and when exposed to high metal and salt concentrations. Therefore, the methods that rely on protein measurements may not be suitable for processed-food products, or when quantitative purposes are needed. On the other hand, DNA-based methods are more stable, easy to operate, and widely used with a broad range of detections (Martin *et al*., 2009). Quantitative Real-Time Polymerase Chain Reaction (qPCR) is one of them, and has been used for detecting a wide range of meat species (Köppel *et al*., 2020; Soares *et al*., 2010). This technique is highly sensitive, sequence-specific, amendable to various types of samples and has a vast dynamic range of detection (Nixon *et al*., 2015). However, the need for reference standards to generate a calibration curve is one of the downsides of qPCR (Ren *et al*., 2017). Recently, digital PCR (dPCR) has been adopted for quantifying the amount of nucleic acid targets by partitioning them into several reaction chambers (Whale *et al*., 2016). As a result, each reaction contains one or no copy of the nucleotide sequence of interest, and hence it can be assayed individually and counted without requiring a standard curve as Poisson statistics are applied for corrections (Baker 2012). Compared to qPCR, dPCR is more advantageous in terms of its sensitivity, specificity and precision (Baker 2012; Köppel *et al*., 2019; Manoj 2016; Ren *et al*., 2017; Cao *et al*., 2020, Wang *et al*., 2018).

Many previous studies have shown problems in quantifying meat by trying to convert DNA measurements to weight proportions (Cai *et al*., 2017; Martin *et al*., 2009; Soares *et al*., 2010). One of the key factors in quantifying meat content by DNA measuring methods is to deeply understand the identity of both the target and the background meats, but several assays and calibration curves may be needed to also quantify the background meat. Moreover, the obtained DNA measurements may not reflect the actual quantity (mass fractions) of the target meat if the selected target represents multicopy genes, such as mitochondrial genes; *ND2, ND5 and ATP 6-8* genes (Kesmen *et al*., 2009) *cytochrome b* (Hird *et al*., 2005) or 18S rRNA (Ahmad Nizar *et al*., 2018).In this sense, Ren and colleagues used a *k* constant to transform copy number ratios to mass fractions using a ddPCR method (Ren *et al*., 2017). Using this approach Ren and colleagues reported chicken content in sheep background with LOD 0.1% (w/w) and LOQ 1% (w/w). Koppel *et al.* (2019) also applied the constant factor to convert DNA values to weight proportions in detection pork in beef with LOD 1% (w/w) and LOQ 9% (w/w). However, well-known types of meat were required in order to use the correctly *k* constant, and multiple assays were needed to determine the *k* for each investigated meat species, as meat products in markets may contain a variety of different types of meat, thus resulting in missed quantifications. Other previous works attempted to convert DNA measurement to mass fractions by using the calibration curve (Cai *et al*., 2014; Shehata *et al*., 2017), but the approach discards the advantage of digital PCR method in not requirement of the standard curve.

In order to tackle the previously mentioned challenges, in this study, singleplex, duplex and triplex ddPCR assays were developed and validated for accurately determining the pork mass present in a sample of mixed pork and beef matrices. To achieve the accurate quantification of pork mass-percentage in meat background, a selected single copy nuclear DNA target for species identification, *β-actin*, together with a selected single copy cross-species gene, *myostatin*, for the identification of a broad range of background meat species (mammal and poultry) were used. *Myostatin* gene has been firstly used as an internal target gene by qPCR in reliable detection of mammalian and poultry species by Laube *et al.* (2003). Then Nixon *et al*. (2015) used this gene not only for internal target gene but also for quantitative detection by measuring *myostatin* gene as the total of meat in quantification of the percentage of horse meat using qPCR. Here, for the first time, we have applied the *myostatin* gene with a ddPCR method in both purposes; internal control gene and quantification as pork *β-actin* to total *myostatin* DNA by converting into mass fractions directly without the requirement of a calibration curve. Our findings show that the target and the background DNA meat fractions identified were consistent with our stipulated gravimetric proportions. In addition, our results revealed that cross-species triplex ddPCR was the most effective approach among those tested, and therefore represents a promising technique for the identification and quantification of altered meat fractions, with the advantages of requiring less time and resources to perform than previous methods.

Materials and Methods

Sample Preparation

Fresh pork (*Sus scrofa*), processed foods, and non-target animal species including chicken (*Gallus gallus*), duck (*Anas platyrhynchos*), partridge (*Perdix perdix*), salmon *(Salmo salar)*, and crocodile (*Crocodylus siamensis*) were purchased from supermarkets in Pathum Thani, Thailand while beef (*Bos taurus*) were obtained from Halal Food Markets in Pathum Thani, Thailand. In the control groups, raw pork and beef were separately blended, freeze-dried (CHRIST, Gamma 1-16 LSC, Germany), sieved through 300-µm standard test sieves (Retsch, Fisher Scientific, USA), and stored at -80 ºC until further use. To mimic highly processed foods, raw pork and beef were separately autoclaved for 20 min at 121 ºC and then processed by the same methods for raw meat. For raw meat matrix mixtures, pork meat powders were prepared at 100%, 75%, 50%, 25%, 10%, 1%, 0.1%, 0.01% and 0% (w/w) levels in beef and for autoclave treated meat, 1% and 7% pork in beef (w/w) were prepared by gravimetric balance (Mettler Toledo, XPE26, Switzerland). Eleven independent replicates were prepared for the limit of detection (LOD) and the limit of quantification (LOQ) determination.

DNA extraction

Genomic DNA was extracted and purified from 2 g of samples with the cetyltrimethylammonium bromide (CTAB) method and Genomic-tip 100/G (Qiagen, Hilden, Germany) according to the standard DNA extraction method (Jacchia *et al.*, 2013). The concentrations of each extracted DNA were measured using a Microplate Reader (Tecan Spark, Switzerland). The quality of the extracted DNAs was assessed using 1.5% agarose gel electrophoresis.

Oligonucleotide primers and probes

Primers and probes used in this work have been published elsewhere (Köppel *et al*., 2011; Laube *et al*. 2003). All the primers and probes were synthesised and purified by Macrogen Inc. (Seoul, Korea). The nucleotide sequences of the primers and probes, and PCR product sizes are presented in Table 1.

Quantitative real-time PCR

The qPCR reactions were performed in 20 µL reaction comprising 1x TaqMan Universal PCR mastermix (Applied Biosystems, USA), with optimal concentrations of forward and reverse primers, 200 nM of probe, approximately 30 ng of DNA template and water. The thermo-cycling of qPCR (ABI 7500 Real-Time PCR) was performed at 95 ºC, 10 min for enzyme activation, followed by 45 cycles of 15 s at 95 ºC of denaturation and 1 min at 58 ºC (optimal temperature) for annealing and extension. qPCR data was analysed by 7500 Software v2.3 (ABI 7500 Real-Time PCR). Primer and probe concentrations were optimised according to

suggestion in the manufacturer's instructions (TaqMan Universal PCR mastermix, Applied Biosystems, USA).

Droplet digital PCR reactions and data analysis

To perform ddPCR, the prepared Mastermix for ddPCR contained 1XddPCR supermix probes without dUTP (Bio-Rad, USA), 300 nM (optimal concentrations) of each primer, 200 nM of probe, 5U of *Hind*III, DNA template (approximately 1,000 copies/µL of sample) and water to make up 20 µL of its total volume. The combinations of the assays were pork/*myo* and pork/beef/*myo* assays for duplex and triplex experiments, respectively. Only in triplex reaction, beef probe concentration was reduced to 100 nM. The PCR reactions were prepared in a 96-well plate (Bio-Rad, CA, USA). The plate was placed on an AutoDG (Bio-Rad, USA) for droplet generation. After generating the droplets, the collected droplets plate was sealed using pierceable foil heat seal and the PX1 PCR plate sealer (Bio-Rad, CA, USA) at 180 ℃ for 5 s. The PCR cycle of ddPCR was performed at 95 ºC for 10 min for enzyme activation, 40 cycles of 30 s at 94 ºC for denaturation, 1 min of 58 ºC (optimal temperature) for annealing and extension, and one cycle at 98 ºC, 10 min for enzyme deactivation in a thermal cycler (T100, Bio-Rad, Pleasanton, CA, USA) with the temperature ramp rate of 2 ºC/s. To obtain the optimal annealing temperature for ddPCR assays, the gradient temperature between 55-64 ºC was used. In this study, the optimal annealing temperature was in the range between 55 ℃ to 60 ℃ which could separate among the populations of the target and negative droplets to minimize the number of partitions with intermediate fluorescence amplitude (Figure S2), and there was not much difference of the copies at the optimal range temperature as seeing in the supplementary Table S2, therefore, a midpoint of 58 ℃ from this range was selected taking also into consideration of the specificity result of this temperature. When the PCR was completed, the droplets were read using a QX200 Droplet Reader (Bio-Rad, CA, USA). The data was finally analysed by QuantaSoft software (v1.7.4.0917, Bio-Rad, CA, USA). The software measured the gene copy number by discriminating the positive droplets (target amplified) from negative droplets (no target amplified) by applying a fluorescence amplitude threshold and correcting by Poisson statistics. In the data analysis, a 1-D plot was used for a single target, while a 2-D plot was applied for two and three targets. The multiplex target data was analysed following Kosir *et al.* (2017) methodology, by setting up a manual threshold and using the QuantaSoft software (v1.7.4.0917, Bio-Rad, CA, USA). The Digital MIQE guidelines were followed (Table S1).

Results and discussion

Method validation

In this study, *β-actin* gene was selected as a species-specific target gene for pork and beef identification (Köppel *et al.*, 2011). *β-actin* gene is found as a single copy in pork and beef genomes. Therefore, for

quantitative measurements the use of *β-actin* gene is more suitable than multiple copy number genes such as mitochondrial DNA and 18s rRNA genes, as they can vary among tissue types and animal species (Barakat *et al*., 2014). In order to transform the ratio of DNA copy numbers to mass proportions, a constant number factor has been employed (Ren *et al*., 2017). However, when this factor was applied to an unknown meat mixture, the method had to be monitored for accuracy, since the constant factor depends on the type of the meat (Ren *et al*., 2017). There have been previous attempts to use reference gene for cross-species detection such as Ahmad Nizar and colleagues which used 18S rRNA as the target gene for cross species of eukaryotes in crocodile meat detection with conventional PCR (Ahmad Nizar *et al.*, 2018). Laube *et al*. (2003) and Nixon *et al.* (2015) used *myostatin* gene with qPCR. As m*yostatin* gene is a single copy nuclear gene and is highly conserved amongst mammals and poultry which normally cover the most types of meat, this gene was found to be a suitable target for quantitative meat purpose (Nixon *et al*., 2015). Therefore, in this study, *myostatin* gene was selected as a cross-species target for measuring the total amount of meat DNA content using the ddPCR method with the advantage of not requiring a standard curve.

The nucleotide sequences for primers and probes used for ddPCR in this study (Table 1) were obtained from previous works using qPCR (Köppel *et al*., 2011; Laube *et al*., 2003). The assays have been already tested for the specificity by using qPCR (Köppel *et al*., 2011; Laube *et al*., 2003; Nixon *et al*., 2015). In this study, the specificity of the assays was also verified by performing qPCR with isolated DNA from pork (*Sus scrofa),* beef (*Bos taurus*), chicken (*Gallus gallus*), duck (*Anas platyrhynchos*), partridge (*Perdix perdix*), salmon *(Salmo salar),* crocodile (*Crocodylus siamensis*) and human *(Homo sapiens)* (Promega, USA), while water was used as NTC. The qPCR result indicated that pork and beef assays were specific to their target species (Figure S1A and S1B). The *myo* assay showed positive detection with DNAs from mammals and poultry, whereas it did not indicate positive detections for DNAs from salmon *(Salmo salar)* and crocodile (*Crocodylus siamensis*) (Figure S1C). This indicated that the *myostatin* was a potential cross-species gene for assaying mammal and poultry DNAs.

In this study it was identified that the optimal concentrations of the forward and reverse primers were 300 nM, while the optimal concentrations of the probes were 200 nM for all the assays. In the triplex assays, the optimal probe concentration for the beef assay was 100 nM (Table 1), and the optimal annealing temperature for ddPCR was approximately 58 ^{o}C (Figure S2), in which the signal obtained from positive droplets was clearly separated from negative droplets. In this study, the PCR efficiency of pork and beef assays was 94.93% ± 3.50% (Figure S3 A and E) and 96.38% ± 1.54% (Figure S3 B and F), respectively, while the *myo* assay with pork DNA was 97.23% ± 5.16% (Figure S3 C and G) and the *myo* assay with beef DNA was 101.58% ± 4.34% (Figure S3 D and H).

Singlexplex, duplex and triplex assays

In the development of ddPCR assays for pork and beef DNA detection and quantification, three ddPCR assays, singleplex, duplex and triplex, were tested for cross reactivity and compared. In the singleplex assay, pork, beef and *myo* assays were independently performed. The pork assay showed positive detection when tested with the samples containing pork DNA (Figure 1A), whereas no signal was detected with the samples containing only pure beef DNA (Figure 1B). On the other hand, the beef assay showed a positive signal for the beef DNA samples (Figure 1C), but undetectable results for the samples with pork DNA only (Figure 1D). As expected, the *myo* assay for *myostatin* gene showed a positive detection for pork and beef DNA samples (Figure 1E and F).

Since the singleplex assay is time-consuming and cost-ineffective (Whale *et al*., 2016), duplex and triplex ddPCR assays were performed for the quantification of pork and beef to improve such issues. In the duplex assay, two genes, pork or beef *β-actin* and *myostatin*, were simultaneously detected. Figure 2 shows the results of ddPCR for pork-*myo* and beef-*myo* assays. Using a pork-*myo* duplex assay to detect the samples containing 100% purified pork DNA, it was able to detect pork *β-actin* and *myostatin* at the same time (Figure 2A). In contrast, when a pork-*myo* duplex assay was performed with 100% purified beef DNA, only *myostatin* was detected (Figure 2B). In the same way, the beef-*myo* duplex assay was tested for its usability. Results showed that only the *myostatin* gene was detected in the 100% pork DNA samples (Figure 2C). Positive signals from both beef *β-actin* and *myostatin* can be observed when 100% beef DNA was used (Figure 2D).

In the triplex ddPCR assay, the limitation of ddPCR is that there are only two channels for two fluorescent dyes; therefore, to simultaneously detect multiple targets, varying the concentration of probes labelled with the same fluorescent dyes should be done (Whale *et al*., 2016). The probe concentration for the pork assay (200 nM-FAM-probe) was twice as high as the probe for the beef assay (100 nM-FMA-probe), while *myostatin* was labelled with a different fluorescence dye (Hex-labelled). The triplex assay was also tested with 100% pork DNA and showed a positive detection for pork *β-actin* and *myostatin* (Figure 3A). When performing the triplex ddPCR with 100% beef DNA, positive signals were seen for beef *β-actin* and *myostatin* (Figure 3C). To show the possible results in order to determine three independent targets test of the triplex assay, the mixture of 50% pork and 50% beef DNAs was used as a template for ddPCR. Figure 3B and D illustrate that positive droplets were found for pork *β-actin*, beef *β-actin* and *myostatin* even in one PCR reaction, indicating that the triplex assay was able to measure three target genes at a time.

The ratio consistency of *β-actin* and *myostatin* genes

Since the key aim of our research was to investigate the potential of the selected single copy gene to quantify the mass fractions of pork in meat products, it was important to confirm the consistency of 1:1 ratio of pork

or beef *β-actin* species-specific target gene to a cross-species target (*myostatin*) gene. We used duplex ddPCR to confirm the consistency of the ratio between *β-actin* species-specific target and *myostatin* genes. If both genes were a single copy in the genome, the *β-actin* and *myostatin* ratio should be close to one irrespective if the concentrations of the genes changed by the serial-dilutions. The results showed that the ratio of pork *β-actin*/*myostatin* (Figure 4A) and beef *β-actin*/*myostatin* (Figure 4B) were near or equal to 1 as predicted, although the concentrations of *β-actin* and *myostatin* genes were varied. Since pork *β-actin*, beef *β-actin* and *myostatin* were proved to be a single copy gene in pork or beef genome, then the pork, beef and *myo* assays were further used in the DNA quantification approach.

Testing the developed ddPCR assays with isolated DNA from mixed meat matrices

In this section, the copy number ratio between a species-specific target gene and a cross-species gene was used to determine the mass fraction of pork in mixed pork and beef matrices. Prior to independent triplicate DNA template extractions, various ratios of mixed pork and beef matrices were gravimetrically prepared as follows: 100%, 75%, 50%, 25%, 10%, 1%, 0.1%, 0.01% and 0% (w/w) pork in beef. The extracted DNA samples from mixed matrices were then investigated through either singleplex, duplex or triplex ddPCR assays. In order to quantify meat proportions, the percent ratio of *β-actin* copy number/*myostatin* copy number was calculated. The ratios of *β-actin*/*myostatin* were then plotted on the y-axis against the percent weight of meat matrices on the x-axis to show whether or not the fraction from the copy numbers of *β-actin*/*myostatin* gene was related to the percentage of mass fractions (Figure 5). The result showed that there were no statistically significant differences among the assays to quantify the proportion of pork DNA in the mixed meat ($P > 0.05$) (Figure 5A). It was found that the relation between the percentage of gene copy ratio and the mass fraction was linear for all the three ddPCR assays for pork target (Figure 5A) and vice versa for beef mass in meat mixture when using the triplex assay (Figure 5B). The correlation coefficients (R^2) for pork singleplex, duplex and triplex assays were 0.9988, 0.9996 and 0.9996, respectively whereas it was 0.9946 for the beef triplex assay. The coefficient of variation (CV) of the given result increased with a poor level of precision when the percentage of pork content decreased (Figure 6A). The same increasing trend of bias with a poor trueness was also observed with the decreasing percentage of pork content (Figure 6B). The CV and bias from singleplex ddPCR at 0.01% pork in beef were higher than those of the duplex and triplex assays. This might be due to the subsampling error associated with the low concentrations of the target molecules. This type of error was also presented when using qPCR (Köppel *et al*., 2020; Soares *et al*., 2010; Taylor *et al*., 2019). Simultaneous PCR reactions in duplex and triplex assays could possibly reduce technical errors, reagents, and time requirement (Köppel *et al*., 2020; Whale *et al*., 2016). In addition, the performance of the *myo* assay has been shown not only to be suitable for quantifying the total amount of meat, but also for confirming the quality of the extracted nucleic acid for a

reliable exclusion of false-negative detections (Laube *et al*., 2003; Nixon *et al*., 2015). The internal control plasmid DNA has been previously used for quality control by Shehata *et al.* (2017). However, this control has to be aware of the cross reactivity and the competition with target sequences. Moreover, adding an internal control plasmid may risk losing the plasmid DNA during extraction processes, resulting in not being representative as a real internal control (Shehata *et al*., 2017). The *myostatin* could be an alternative real internal control to ensure reliability, normalised variabilities and safeguard against false negatives. However, one needs to be careful when using a DNA based method for quantification purposes, as a big difference in the genome sizes of meat species may affect the accuracy of ratio quantifications; for example, a difference in the genome size between pork (2,800 Mb) and chicken (200-5 Mb) (Burt 2005; Groenen *et al.*, 2012). Further study needs to be carried out to investigate the impact of the genome size in DNA relative quantification.

In order to determine the LOD and LOQ, DNA from 10 independent replicates for each ratio of pork in beef matrices (10%, 1%, 0.1%, 0.01% and 0% (w/w) pork in beef) were isolated. LOD was defined as the lowest percentage of pork content that could be reliably detected. Although the proportion of pork in beef was decreased to 0.01%, the developed ddPCR assays can still estimate pork containing in samples. With the limit of gravimetric balance, the LOD of all developed pork detection assays by ddPCR was ≤ 0.01% (Table 2). Mostly previous works have been based on qualitative detection where the LOD was reported as ng or the percentage of mass fractions. In pork detection, our ddPCR approach resulted in one order of magnitude lower LOD than the reported with a conventional PCR (Soares *et al*., 2010) and qPCR (Laube *et al*., 2003). Although, our methods showed capability to detect pork even in 0.01% pork contamination (with 95% confidence), the detection of pork with the lower concentration may still be required for the cultural or religion issue which is very strict in pork adulteration. By using the gravimetric method, the pork content could not go down less than 0.01%, therefore, pure pork DNA was also used to determine the sensitivity of pork detection. The results showed that our methods can detect pork DNA down to 2-5 copies/µL with 100% detection rate (data not shown). This result was in agreement with other publications for the LOD of ddPCR (Carolina *et al*., 2020; Hindson *et al*., 2011; Rungkamoltip *et al*., 2021; Vishnuraj *et al*., 2021).

LOQ was defined as the lowest percentage of pork content that was precisely quantified with confidence (<25%CV) (Cai *et al*., 2017; Deprez *et al*., 2016; Košir *et al*., 2017). Moreover, according to Guidelines for the Validation of Analytical Methods for Nucleic Acid Sequence-Based Analysis of Food, Feed, Cosmetics and Veterinary Products (FDA, 2020), the accepted criteria of trueness should be ±25% levels, our findings suggest that our approaches with 0.1% LOQ can quantify the percentage of pork in beef background with good precision levels (CV was not higher than 25%) and accuracy. However, it should be

noted that when the triplex assays were applied to quantify 0.1% of pork content as the trueness was over the criteria by approximately 3%. Therefore, 0.1% pork contamination in beef was the LOQ for singleplex, duplex and triplex ddPCR assays (Table 2), that was lower than reported by Koppel *et al.* (2019) but one order of magnitude higher than reported by Shehata *et al.* (2017) and Floren *et al.* (2015) with the determination of mixing pure DNA. However, in this study, the limitation of weighing the sample matrix by a gravimetric method did not allow us to weight the pork matrix less than 0.01% contamination.

Analysis of processed foods

As the triplex ddPCR assay has the potential to reduce cost and time with high accuracy and reliability as shown this study, the triplex ddPCR assay was used to evaluate nine commercial processed food products and four autoclave treated meat samples (Table 3). The results demonstrated that six samples were identified as pork in the products and five products showed positive signals of beef, while only one sample (shrimp) did not detect the *myostatin* gene as expected (Table 3). Other works also found the qPCR methods have the capability for detecting traces of meat species in processed food under various processing conditions (Ali *et al*., 2012; Barakat *et al*., 2014; Che Man *et al*., 2012; Naaum *et al*., 2018; Yusop *et al*., 2012). For quantitative detection, eight of the nine samples showed a successful quantification with the percentage of pork or beef close to the declared percentage on their labels for commercial meat products, or to the true value of the weight proportions for mimicking processed foods (Table 3). Interestingly, there was one product, pork sausage A, showing the detection result of the percentage of pork content by only 30.40%± 1.78%, which was approximately 3-times lower than the declaration on its label (87%). With our method, other commercial meat products with various types of processed food showed the pork content closely to the label, only one product, pork sausage A, resulted in a significantly lower percentage than the declaration on the label. This could be from intentional or unintentional mixing of other meat content by manufacturer, as well as unexpected effects during the food processing. Our method showed the potential to quantify the pork content in the processed food and this could help the regulators to control food adulteration or mislabelling in commercial meat products.

When comparing the autoclaving to other cooking methods such as boil, grill or stream, the extracted DNA from the autoclave treated meat samples was clearly more fragmented (approximately 80-500 bp) than the ones obtained through other cooking methods (Figure S4). Although, degraded DNA was observed, the DNA fragments sizes were still in a range of the target template with the same DNA degradation pattern among the meat through the samples (Figure S4). Consequently, these might not affect the relative quantification in the highly processed foods by the ddPCR assays. Moreover, in the ddPCR system, a restriction enzyme is recommended for digesting the input genomic DNA, as the viscosity of the template can interfere in the partition samples (Yukl *et al*., 2014). Hence, DNA fragments from highly processed

foods may be appropriate for ddPCR systems without the need for a treatment with a restriction enzyme. This work details the development of triplex ddPCR assays that can be employed to accurately determine not only qualitative, but also quantitative data on pork, beef and *myostatin* fractions simultaneously in processed meat samples without a standardisation curve, henceforth wiping out various steps from the investigation and lessening time and expenses. Therefore, we believe that the ddPCR assays developed in this study are suitable and could be fruitful for investigating the mislabelling of meat content inside the food industry.

Conclusions

In this study, droplet digital Polymerase Chain Reaction (ddPCR) assays were developed to accurately measure ratios of pork DNA in a total amount of meat DNA in a range of gravimetrically prepared matrices of pork and beef. The single copy *β-actin* nuclear gene was employed as a target, and the cross-species *myostatin* gene was selected for the identification of different meat backgrounds, such as mammal and poultry meat. All of the proposed ddPCR methods allowed us to quantify pork additions in beef with a limit of quantification (LOQ) estimated at 0.1% (w/w) and a limit of detection (LOD) down to 0.01% (w/w). The singleplex assays provided more biases than the other two assays when performing with a low concentration of target species. The duplex assays provided a simultaneous quantification of pork and *myostatin*, whereas the triplex assay was able to detect pork, beef and *myostatin* simultaneously with a significant decrease of technical error, cost and running time, thus representing a promise ddPCR technique for the proposed objectives. ddPCR triplex assays were also tested with commercial processed foods and showed the ability to determine not only the presence of a particular pork or beef mass but also the measures of each mass fractions simultaneously without demanding any previous calibration assays. Hence, the outcomes depicted in this work proof that our novel cross-species triplex ddPCR assays have the capacity to both identify and quantify adulterated meat fractions from DNA content, including in processed market meats, with high sensitivity and exactness. Moreover, the extent of the detected DNA copies can be directly related to each mass divisions without the need for a calibration curve or a constant number, thus reducing time and costs. Hence, these novel ddPCR triplex approaches have great potential to be utilised as a standard method in the simultaneous determination of multiple-species meat fractions to aid regulatory agencies in controlling meat adulteration and promoting overall food safety.

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Ethical Guidelines Statement

Ethics approval was not required for this research as it does not involve human or animal subjects.

Data Availability

The data that supports the findings of this study are available in the supplementary material of this article.

Statement of Conflict of Interests

The authors of this manuscript certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest (such as personal or professional relationships, affiliations, knowledge, or beliefs) in the subject matter or materials discussed in this manuscript.

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List of legends

Figure 1. Singleplex ddPCR assays for identification of pork and beef gDNAs. The representatives for each assay were taken and presented as follows: (A) pork DNA with pork assay, (B) beef DNA with pork assay, (C) pork DNA with beef assay, (D) beef DNA with beef assay, (E) pork DNA with the *myo* assay and (F) beef DNA with *myo* assay. NTC is no-template control (ddH_2O). Black dots indicate negative droplets, blue dots are positive droplets for *β-actin* genes, and green dots are positive droplets for *myostatin* genes.

Figure 2. The 2D plots for duplex ddPCR assays for quantifying pork and beef genomic DNA. Each figure is (A) pork-*myo* duplex with 100% (w/w) pork DNA, (B) pork-*myo* duplex with 100% beef DNA, (C) beef-*myo* duplex with 100% pork DNA, and (D) beef-*myo* duplex with 100% beef DNA. Black dot populations are negative droplets, blue dot populations are positive droplets for pork or beef *β-actin*, green dot populations are positive droplets for *myostatin*, whereas orange dot populations are positive droplets for *β-actin* and *myostatin*.

Figure 3. The 2D plots for triplex ddPCR assays for quantifying pork and beef genomic DNA. Each figure is triplex assays tested with (A) 100% (w/w) pork DNA, (B) 50% pork DNA and 50% beef DNA, highlighting pork *β-actin* positive detection, (C) 100% beef DNA, and (D) 50% pork DNA and 50% beef DNA, highlighting beef *β-actin* positive detection. Black dot populations are negative droplets, blue dot populations are positive droplets for pork or beef *β-actin*, green dot populations are positive droplets for *myostatin*, and orange dot populations are positive droplets for *β-actin* and *myostatin*.

Figure 4. The ratio of *β-actin* and *myostatin* genes determined by duplex ddPCR assays. A tenfold dilution series (10,000 – 1 copies/µL of sample) of purified pork (A) or beef (B) DNA was assessed by duplex ddPCR (pork/*myo* and beef/*myo* assays) for absolute pork *β-actin* and *myostatin* or beef *β-actin* and *myostatin* concentrations (the left y-axis). The black bars represent the amount of *β-actin* gene as a species-specific target whereas the amount of *myostatin* gene is represented by the grey bars. The concentrations of pork or beef DNA are on the x-axis. The ratio (cp/cp) of *β-actin* gene to *myostatin* gene is represented by the dotted line with orange dots (the right y-axis). The error bars are the standard deviation obtained from three independent experiments (n=3).

Figure 5. Linear regression between the percentage of ddPCR output ratio (cp/cp) and the percentage of expected pork adulteration with beef by gravimetric balance method (w/w). A: % pork in a beef background that was measured by singleplex (black), duplex (blue) and triplex (orange) ddPCR assays (y-axis) compared with assigned value (x-axis). B: For triplex ddPCR assay for beef quantification in pork background.

Figure 6. Accuracy of singleplex (blue), duplex (orange) and triplex (grey) ddPCR assays. A: The percentage of coefficient of variation (%CV) and B: the percent of bias (%bias) expressed precision and trueness, respectively.

Table 1. Primers and probes used in this study

Table 2. Accuracy (%CV and % bias represent precision and trueness, respectively) of the developed ddPCR assays in measuring pork and beef meat.

Table 3. Determination of pork or beef meat percentage in highly processed foods (autoclaved meat) and commercial processed foods (products from Thailand) by triplex ddPCR assay. Three biological replicates and three technical replicates were presented.

Figure S1. Amplification plots of qPCR for specificity test of primers and probes to DNA extracted from different animal species. Pork (A), beef (B) and myo (C) assays were challenged with DNAs extracted from chicken, pork, beef, duck, partridge, salmon, crocodile and human DNA. NTC is no-DNA-template control.

Figure S2. Optimisation of annealing temperature of primers and probes by ddPCR system. The PCR reactions were observed using a thermal gradient PCR ranging from 55 to 64 ^{o}C. The figure shows the discrimination of droplet populations, black: negative droplets, blue: positive droplets with *β-actin* gene targets in pork (A) and beef (B) DNA by pork and beef assays, respectively. Green: positive droplets with *myostatin* gene target in pork (C) and beef (D) DNA by myo assay.

Figure S3. The amplification plots for each assay. The amplification plots of qPCR for 10-fold dilution series from 10^5 to 10 copies/µL of pork DNA by the pork assay (A), beef DNA by the beef assay (B), pork DNA by the myo assay (C) and beef DNA by the myo assay (D). The E-H graphs show the linear regression line derived from A-D graphs; pork DNA by the pork assay (E), beef DNA by the beef assay (F), pork DNA by the myo assay (G), and beef DNA by the myo assay (H). Three replicates were performed for each assay. PCR efficiency (PCR EF) can be calculated from Efficiency = ($10^{-1/slope}$-1), where the slope can be obtained from the linear regression equation. Pure pork and beef genomic DNAs were used while PCR efficiencies were derived from three independent experiments.

Figure S4. Example of 1.5 % agarose gel electrophoresis of extracted DNA derived from proceeded foods. M: marker, AP: autoclaved 100% pork, AB: autoclaved 100% beef, Ps-A: pork sausage A, Ps-B: pork sausage B, Pb: pork ball, Bb: beef ball, and Bbur: beef burger.

Table S1. Digital MIQE checklist