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## The detection of commercial duck species in food using a single probe-multiple species-specific primer real-time PCR assay

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**Abstract** A real-time PCR assay for the simultaneous detection of Mallard and Muscovy duck is described. Species-specific primers were designed for Mallard or Muscovy duck using the mitochondrial cytochrome *b* gene sequence. These primer sets were multiplexed with a single duck probe to produce a simple, rapid and robust real-time PCR assay. This assay was shown to be specific for duck compared to a wide range of commercially important meat species and was used for the successful detection of duck meat in complex food matrices. This is the first report of an assay that will detect all species of commercially available duck in commercial products using real-time PCR.

**Keywords** Real-time PCR · Duck · Muscovy · Mallard · Detection

### Introduction

For many years duck meat has been one of the most expensive commercially available meat types, consumption of which was reserved for special occasions. There has, however, been a recent move towards large-scale production of duck using modern husbandry techniques, which has resulted in a greater availability of duck meat and a proliferation of processed products containing duck meat. Additionally, the range of duck species used commercially has been extended from mainly Mallard ducks (*Anas platyrhynchos*) to include the subspecies Gressingham and Allesbury, and the Muscovy ducks (*Cairina moschate*).

There is now a need for a sensitive and robust assay for the detection of all species of commercial duck in heat and pressure-processed products. Heat and pressure pro-

cessing are known to denature protein and fragment DNA, however DNA-based analyses remain the best option for detection since DNA survives heat and pressure processing relatively intact when compared to proteins [1]. There are few reports on the successful detection of duck species in commercial products. Partis et al. [2] used PCR-RFLP for the detection of duck, however they found that pork preferentially amplified over all other species, such that the method was not applicable for mixtures of meats [2]. Cavalo et al. [3] outlined a method using RAPD for duck detection, but found that similar bands were produced by amplification of both duck and pork, making interpretation of the banding patterns subjective [3]. Both of these reports did however demonstrate the utility of a DNA-based approach, and more recent reports have outlined methods based upon species specific PCR systems, which circumvent co-amplification in mixtures [4–6]. Rodriguez et al. [4, 5] used conventional PCR for the detection of Mule duck, a cross between Mallard and Muscovy duck (*Anas platyrhynchos* × *Cairina moschate*), using separate primer sets for two genes: 12S ribosomal RNA and  $\alpha$ -actin genes [4, 5]. Rodriguez et al. [6] went on to develop a real-time PCR assay for the detection of Mule duck. However, this assay was based on Mallard DNA sequence and would not detect wild-type Muscovy duck [6], a duck species now commonly used for human consumption.

We report the development of a real-time PCR assay for the detection of both Mallard and Muscovy duck species. Species-specific primers were designed using the mitochondrial cytochrome *b* gene, which when multiplexed with a generic duck probe, were used for the successful detection of duck meat in complex food matrices which had been heat and pressure processed.

### Materials and methods

Meat and meat product samples

Samples of fresh raw meat from Mallard, Gressingham, Allesbury and Muscovy duck species, pig, deer, pheasant, duck, goose, guinea

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**Table 1** Primer and probe sequences and optimised concentrations, for duck group real-time PCR assay

Species	Oligonucleotide	Sequence (5'–3')	Optimised concentration (nM)
Mallard	Sense primer	TCC TAC CTG TAT AAA GAA ACT TGA AAT	300
	Antisense primer	GGG CTG AAA ATA AGT TGG TAA TT	300
	Probe	FAM-TAG GCT ATG TCC TGC CAT G –MGB	200
Muscovy	Sense primer	TCC TAC CTG TAC AAA GAA ACC TGA AAT	300
	Antisense primer	GGG CTG AGA ACA GGT TGG TAA TT	300

fowl, grouse, pigeon, quail, cow, partridge, sheep, turkey, chicken and commercial products were obtained from local suppliers in the United Kingdom. All samples were stored at 4 °C until DNA extraction.

#### DNA extraction from raw meat

DNA was extracted from raw meat using a modified version of the GenElute Mammalian Genomic DNA Extraction Kit (Sigma). Briefly, 2 g of tissue was finely minced with a scalpel blade, prior to incubation at 65 °C in 8 ml CTAB buffer (0.055 M CTAB, 1.4 M NaCl, 0.1 M Tris and 0.02 M EDTA, pH 8) containing 50 µg/ml proteinase K (Sigma). After 1 h the samples were cooled briefly and 400 µl of the supernatant used to extract the DNA exactly according to the manufacturers instructions. The final elution step was repeated once and the eluates pooled. DNA was stored short term at 4 °C or long term at –20 °C.

#### DNA extraction from complex meat matrices

DNA was extracted from complex meat matrices using a modified version of the Wizard DNA cleanup system (Promega). Briefly, 5 g of each sample was incubated at 65 °C in 10 ml CTAB buffer containing 80 µg/ml proteinase K (Sigma). After 2 h the samples were vortexed for 10 s and centrifuged for 10 min at 4,000×g. One millilitre of the supernatant was combined with 800 µl chloroform, vortexed briefly and centrifuged for 10 min at 16,000×g. Five hundred microlitres of the aqueous phase was combined with 1 ml Wizard DNA resin and incubated for 5 min at room temperature. The DNA was then purified exactly according to the manufacturers instructions and eluted with 100 µl Tris–EDTA buffer.

#### Real-time PCR primer and probe set design

Mallard and Muscovy duck specific primers and probe sets were designed using the mitochondrial cytochrome *b* gene DNA sequence (GenBank Accession numbers AF059081 and LO8385 respectively) using Primer Express software (Applied Biosystems) (Table 1). Primers were designed to mismatch all other commercially important species at the 3' position [7] and were purchased from Sigma Genosys. The probe was labelled with 5' carboxyfluorescein (FAM) and 3' minor groove binding (MGB) fluorescent quencher and was purchased from Applied Biosystems.

#### Real-time PCR reaction set up

Each real-time PCR reaction contained 1 × TaqMan Buffer A, 25 mM MgCl<sub>2</sub> solution, 0.625 Units AmpliTaq Gold DNA Polymerase (Applied Biosystems) and 0.5 µM of each deoxynucleotide triphosphate (Sigma) in 25 µl. Optimised primer and probe concentrations are shown in Table 1. DNA template was diluted 1:4 DNA:water and 5 µl of either DNA or water, as a negative control, was added per reaction. Reactions were assembled in Axygen thin walled 96 well plates with optical caps and run on ABI Prism 7700 or 9700 sequence detection systems with the following thermal

cycling protocol: 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

## Results and discussion

### Primer design

There are two commercially important species of duck: Mallard and Muscovy. These species are of the same family but diverge at the genus level and it is widely considered that all domestic and commercially important duck species, with the exception of the Muscovy, have been bred from the Mallard.

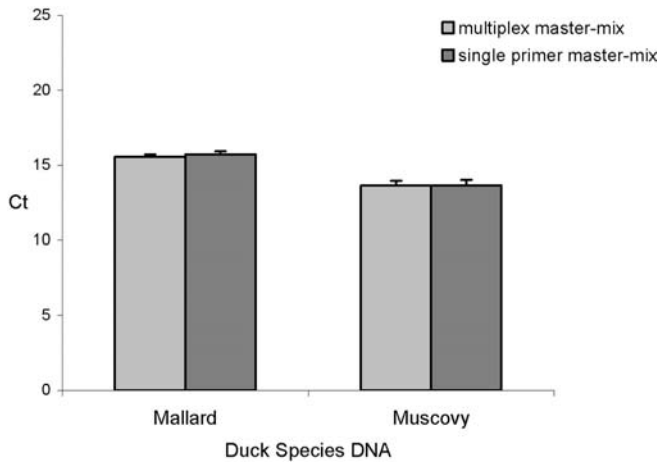
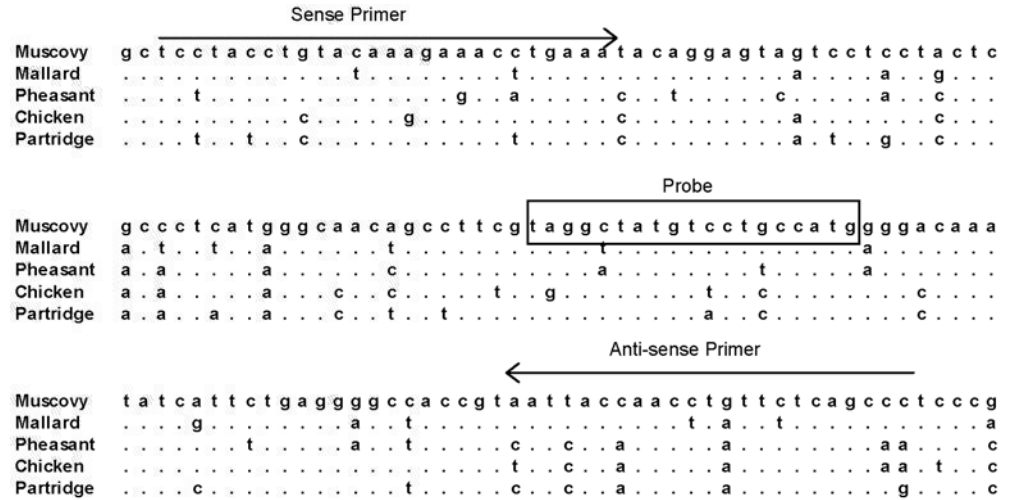
Comparison of the mitochondrial cytochrome *b* gene sequences for these species revealed that although closely related, there is significant sequence heterogeneity between the species (Fig. 1). We designed an assay that would identify both duck species using a probe common to all duck species combined with species-specific primers. Sequence data from the NCBI database for Mallard and Muscovy ducks and other commercially popular species were aligned and areas of mismatch identified. Using Primer Express software, primer sets were designed to have mismatches to all other species at the 3' position of both sense and antisense primers for Mallard and Muscovy ducks. The probe was designed to the Muscovy sequence and had only a single base mismatch for the Mallard sequence (Fig. 1).

### Efficacy and specificity

The aim of the study was to develop an assay which would identify the presence of duck in commercial products. The identification of the type of duck was less important than the detection of duck *per se*. Therefore, the primer sets for Mallard and Muscovy were developed separately and then multiplexed to form a duck group assay.

The Mallard and Muscovy assays were found to be highly sensitive on their matching template (Ct values 14.68 and 13.48 for Mallard and Muscovy DNA template using Muscovy and Mallard assays respectively). After primer limitation and multiplexing, the duck group assay was compared to the single assays for amplification efficiency on either Mallard or Muscovy duck template DNA (Fig. 2). There was no significant difference in the Ct values of the single or multiplexed assays for either

**Fig. 1** Alignment of Muscovy duck, Mallard duck, pheasant, chicken, and partridge mitochondrial cytochrome *b* sequence, showing position of specific Muscovy and Mallard primers and the common probe



**Fig. 2** Effect of multiplexing on sensitivity of duck group real-time PCR assay. The Mallard and Muscovy assays were used as single assays or multiplexed on the same DNA extracts from Mallard and Muscovy duck meat

template. The single assays were found to cross-amplify the alternate template with lower sensitivity (Ct values 28.27 and 30.09 for Muscovy and Mallard DNA template respectively). Cross-amplification at these levels equates to approximately 10,000-fold less amplification efficiency on the alternate template compared to the matching template. However, since there is a common probe for both primer sets, the cross amplification of the alternate template presented no problem for the sensitivity of the assay.

The specificity of the duck group assay was then assessed using DNA from a wide variety of species, including pheasant, chicken, partridge, goose, guinea fowl, pigeon, quail, grouse, turkey, cow, pig, horse, donkey, red deer and sheep, and two varieties of duck originating from the Mallard: Gressingham and Allesbury (Table 2). It was found that there was no cross-amplification of non-duck species (Ct value of 40 equates to no amplification), but good amplification of all types of ducks, demonstrating

**Table 2** Specificity of duck group real-time PCR assay on DNA template from a variety of species

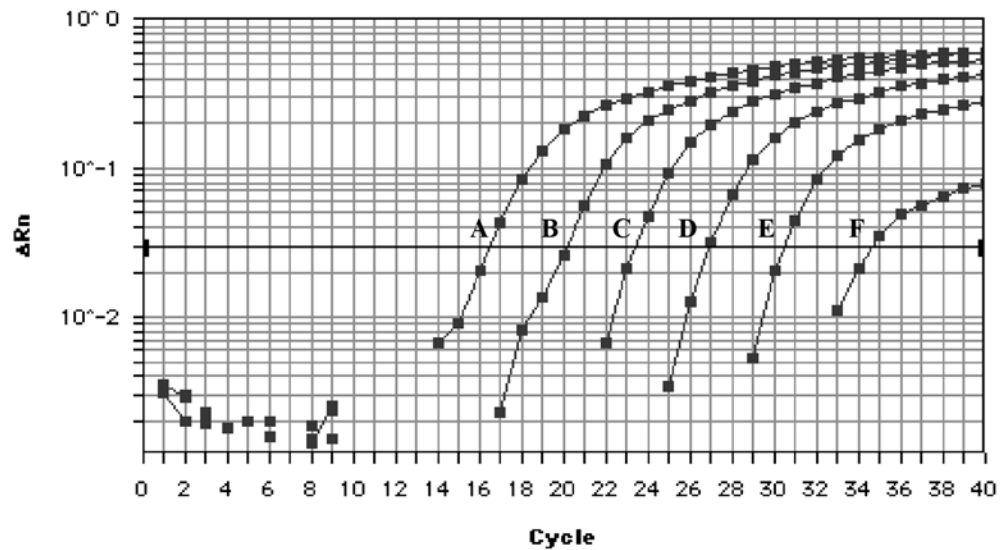
Species	Species scientific name	Average Ct value ± SD
Muscovy duck	<i>Anas platyrhynchos</i>	13.63±0.32
Mallard duck	<i>Cairina moschate</i>	15.54±0.16
Allesbury	<i>Cairina moschate</i>	16.07±0.20
Gressingham	<i>Cairina moschate</i>	15.18±0.10
Turkey	<i>Meleagris gallopavo</i>	40.00±0.00
Chicken	<i>Gallus gallus</i>	40.00±0.00
Pig	<i>Sus scrofa</i>	40.00±0.00
Cow	<i>Bos Taurus</i>	40.00±0.00
Sheep	<i>Ovis aries</i>	40.00±0.00
Goose	<i>Anser anser</i>	40.00±0.00
Pheasant	<i>Phasianus colchicus</i>	40.00±0.00
Partridge	<i>Alectoris rufa</i>	40.00±0.00
Pigeon	<i>Columba livia</i>	40.00±0.00
Guinea fowl	<i>Numida meleagris</i>	40.00±0.00
Grouse	<i>Lagopus lagopus</i>	40.00±0.00
Horse	<i>Equus caballus</i>	40.00±0.00
Donkey	<i>Equus asinus</i>	40.00±0.00
Red deer	<i>Cervus elaphus</i>	40.00±0.00
Quail	<i>Coturnix coturnix</i>	40.00±0.00

complete specificity of the duck group assay for duck DNA.

**Duck group assay efficiency and limit of detection**

Efficiency and limit of detection for the duck group assay were determined using 10-fold dilutions of Mallard duck DNA diluted in water. Primer and probe sets which are working at 100% efficiency will require approximately an additional 3.3 cycles (3.3 Ct) for a 10-fold dilution of template. The limit of detection is the DNA dilution where the Ct values are still reproducibly produced, but beyond which the assay fails. The detection of duck for the duck group assay extended to a 1,000,000-fold dilution of the duck DNA in water (average Ct 33.65) (Fig. 3). Additionally, over this range of dilutions the response was linear, and the slope of the Ct values plotted against the log of the DNA dilution was -3.5, indicating an assay

**Fig. 3** Duck group assay on fold dilution series of duck template (DNA diluted in water, fold dilutions A—10, B—100, C—1,000, D—10,000, E—100,000, F—1,000,000)



**Table 3** Real-time PCR analysis of commercial products using the duck group assay

Product	Declared meat species or animal content	Average Ct value $\pm$ SD
Roasted duck in plum sauce	Roasted duck	12.03 $\pm$ 0.09
Smoked duck slices	Smoked Barbary duck	11.13 $\pm$ 0.09
Duck pate	19% Pork, pork fat, 19% duck liver, milk, 11% chicken liver, duck fat	12.09 $\pm$ 0.07
Whiskers duck cat food (pouch)	Meat and animal derivatives including min 4% duck	30.67 $\pm$ 0.59
Felix duck and heart cat food (tin)	Min 4% duck, min 4% heart, fish and fish derivatives	30.07 $\pm$ 1.81
Sainsburys duck and chicken cat food (tin)	Min 4% duck, min 4% chicken	36.20 $\pm$ 1.48
Friskies duck cat biscuits	Min 4% duck	19.81 $\pm$ 0.14
Game casserole	Raw pheasant, partridge, Mallard duck, venison, pigeon and rabbit	11.35 $\pm$ 0.14
Friskies game, turkey and vegetable cat food (pouch)	Min 4% game, 4% turkey	33.40 $\pm$ 0.97
Whiskers game cat food (pouch)	Meat and animal derivatives including min 4% game	29.87 $\pm$ 0.53
Whiskers poultry cat food (pouch)	Meat and animal derivatives including min 4% poultry	29.99 $\pm$ 0.78

working at 106% efficiency. The limit of detection relates to the detection of 0.0001% (w/w) duck meat in a sample. Although this limit of detection was calculated from dilutions of DNA in water, with detection at these orders of magnitude, the assays could be used as a basis for enforcing accurate product labelling and provide reassurance to consumers concerned about even the smallest levels of contamination of duck meat in products.

#### Application of assay to commercial samples

The duck group assay was then used to test DNA extracted from a range of commercial samples where either duck or game had been included in the ingredients list (Table 3). Duck was successfully detected in all samples. In samples of roasted and smoked duck and of game casserole, the DNA did not show evidence of significant fragmentation by low Ct values. Similarly, a low Ct value was returned

for duck pate which contained not only duck but also pork, pork fat and milk. Higher Ct values were returned for samples which were highly processed or long life: the cat foods. The level of duck in these samples (4%) was only quoted as a minimum since the labelling requirements for pet foods are different from those for human consumption, however we would expect a Ct value of approximately 15 for a sample containing 4% duck. The greater Ct values returned for these samples indicated a high degree of DNA fragmentation. An anomaly for the pet food results was given by the duck biscuits, which returned a relatively low Ct value. This indicated that the duck meat in the biscuits was not as highly processed as in the other pet foods analysed. The presence of duck was therefore detected in all samples analysed, where duck had been listed in the ingredients. Additionally, there was specific detection of duck in complex food matrices, even in the presence of pork, a confounding factor for RFLP and RAPD based assays [2, 3].

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## Conclusions

A real-time PCR assay for the detection of duck has been developed. The optimised assay is specific, highly sensitive and applicable to complex food matrices. This is the first real-time PCR based assay for the detection of commercial duck species to be published and as such, can now be recommended to food control laboratories.

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