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ORIGINAL PAPER

# Tetraplex real-time PCR with TaqMan probes for discriminatory detection of cat, rabbit, rat and squirrel DNA in food products

**Mohammad Nasir Uddin Ahamad[1] · M. A. Motalib Hossain[1] · Syed Muhammad Kamal Uddin[1] · Sharmin Sultana[1] · Nina Naquiah Ahmad Nizar[1] · Sharmin Quazi Bonny[1] · Mohd Rafie Johan[1] · Md. Eaqub Ali[1,2]**

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## Abstract

Cat, rabbit, rat, and squirrel species are very sensitive in food products because most of them are potential carriers of zoonotic diseases and rejected in most religions and cultures. Since cats and rats are abundant in most parts of the world and their meats do not carry any value in legal markets, these meats could be considered as potential adulterants in halal, kosher, and other food markets. Rabbit and squirrel meats are also susceptible to adulteration. Therefore, both health and economic interests in rat, rabbit, cat and squirrel species are significant. In this work, a novel tetraplex real-time PCR assay with TaqMan probes was described to discriminate and identify all four species (cat, rabbit, rat, and squirrel) in a single assay platform. Species-specific primers and probes were developed against ATP6, and cytochrome b genes to amplify 108, 123, 161 and 176 bp DNA fragments from rat, rabbit, squirrel and cat meat products under various states. A 141-bp internal amplification control (IAC) of 18S rRNA was used to avoid any false-negative results. Specificity was evaluated against 22 species but no cross-reactivity was found. Efficiency of PCR assay as well as target quantification were determined based on a standard curve that was generated using tenfold serially diluted mixed DNA extract (1:1:1:1) from squirrel, rat, rabbit and cat species. The assay was valid under pure, processed and admixed states with 10–0.1% (w/w) adulterant from each species. The limit of detection was 0.1% under admixed samples and 0.003 ng DNA under pure states from each species. Analyses of 18 model burgers (9 chicken and 9 beef) and 18 frankfurters (9 chicken and 9 beef) revealed 91–122% target recovery at 0.1–10% adulteration. Finally, 72 commercial burgers (36 chicken and 36 beef) and 72 frankfurters (36 chicken and 36 beef) were screened but no target species was detected except IAC.

**Keywords** Tetraplex real-time PCR · Internal amplification control · TaqMan probes · PCR efficiency · Limit of detection

## Introduction

Authenticity issues in food products are intricately linked to public health, religious and cultural issues as well as fair-trade economic practices. Recently, rat meat was chemically modified to change physical appearances and sold as lamb [1]. This greatly aggravated the concerns and worries of general public and also health professionals because rats are carriers of several infectious and deadly diseases, such as listeriosis, yersiniosis, pasturellosis, meilioidosis and plague diseases [2]. The close cousins of rats are squirrels that are also a carrier of Creutzfeldt–Jakob disease (CJD) syndrome (neurodegenerative disease) [3] and Lyme disease [4]. On the other hand, rabbit and cat carcasses are almost identical to look at and so cats were sold as rabbits in several instances [5]. Recently, cat meat was sold as mutton in China [6] and India [7]. The issue is a grave concern because rabbit meat is a blooming industry but cat meat does not have any apparent market prices as cat consumption is a taboo in most of the societies and cultures [8]. While rabbit meat is appreciated because of its huge contents of essential minerals [9], and proteins but low content of saturated fats and cholesterol [10], cat meat is a carrier of hepatitis, severe acute respiratory syndromes, anthrax and some other deadly diseases [11]. Therefore, both health and economic interests in rat, rabbit, cat and squirrel species are huge and so

✉ M. A. Motalib Hossain
motalib_123@yahoo.com

[1] Nanotechnology and Catalysis Research Center, Institute of Graduate Studies, University of Malaya, 50603 Kuala Lumpur, Malaysia

[2] Centre for Research in Biotechnology for Agriculture (CEBAR), University of Malaya, 50603 Kuala Lumpur, Malaysia

there is a demand for a reliable, low cost and easily applicable authentication technique that could both detect and quantify any ingredients from these species under any matrices.

For meat and meat product authentication, recently, DNA-based PCR techniques have been evolved as the method of choice because protein- and lipid-based biomarkers are easily modified and so cannot offer so much reliability as is offered by robustly stable short-length polymorphic DNA sequences [12–15]. Several DNA-based methods such as species-specific PCR [16, 17], PCR-restriction fragment length polymorphism (PCR-RFLP) [18, 19] multiplex PCR [20, 21], randomly amplified polymorphic DNA (RAPD) [22], real-time PCR [23], and DNA barcoding systems [24] have been documented. However, among these methods, only real-time PCR approaches offer quantification, automation and high throughput [25, 26]. For the species targeted in this paper, a real-time PCR assay has been proposed which could detect red and gray squirrels [27]. On the other hand, several conventional PCR assays are available for rat [28, 29] and rabbit specification [30–32]. However, the main problems with these methods are that they are based on a single species target and long DNA amplicon (243–672 bp) that often breaks down under extensive food processing atmospheres, making them unreliable and quite expensive for forensic investigations [33]. To overcome the problems, recently, we have documented a short amplicon length (108–176 bp) multiplex PCR assay for rabbit, rat and squirrel detection in a conventional platform [5, 34]. However, this assay did not include cat species that is frequently adulterated in rabbit meat [5]. In this regard, multiplex real-time PCR (qPCR) assay with TaqMan probes are greatly advantageous because it provides data with high sensitivity and specificity at real time and ensures additional security through primers and specialized probes whose sequences are required to be perfectly matched with the selective sites of the target analytes for successful identification [35].

Recently, TaqMan-based multiplex qPCR assays have been reported for cattle, buffalo and pig [25], duck, pig and chicken [26], and deer [36], as well as pork, beef, turkey, sheep and chicken species [37]. However, such a featureful assay has not been reported for the cat, rabbit, rat and squirrel species. To address this knowledge gap, we documented here a short amplicon length (108–176 bp) tetraplex qPCR assay with TaqMan probes for the simultaneous detection of their DNA in processed food products for the first time. The method was validated with regard to specificity, limit of detection (LOD), accuracy, repeatability and robustness.

## Materials and methods

### Sample collection

Specimens of fresh muscle tissues were obtained from rabbit (*Oryctolagus cuniculus*), squirrel (*Calloscious notatus*), rat (*Rattus rattus*), cat (*Felis catus*), chicken *(Gallus gallus*), beef (*Bos taurus*), buffalo (*Bubalus bubalis*), sheep (*Ovis aries*), goat (*Capra hiscus*), pig (*Sus scrofa*), duck (*Anas platyrhynchos*), pigeon (*Columba livia*), crocodile (*Crocodylus niloticus*), amboina box turtle (*Cuora amboinensis*), Chinese edible frog (*Hoplobatrachus rugulosus*), deer (*Cervus nippon yesoensis*), dog (*Canis lupus familiaris*), tuna (*Thunnus orientalis*), salmon (*Salmo salar*) and plant species, namely, wheat (*Triticum aestivum*), cucumber (*Cucumis sativus*), onion (*Allium cepa*), and chili (*Capsicum annuum*). Commercially available meat, fish and plant samples were collected from local wet (Pudu Raya) and super markets (Aeon, Tesco and Giant) at Kuala Lumpur on three different days to increase the genetic diversity of the collected samples. Deer (*Cervus nippon*) meat was procured in triplicates from the Faculty of Veterinary Sciences at the University of Putra Malaysia, located at Serdang in Selangor. Stray dog *(Canis lupus familiaris)*, cat (*Felis catus*) and rat (*Rattus rattus*) muscle tissues were donated by Kuala Lumpur City Hall (KLCH/DBKL) at Air Panas in Kuala Lumpur [38]. Monkey (*Macaca fascicularis sp.)* meat was a gift from the Department of Wildlife and National Park Malaysia (DWNPM/PERHILITAN) at Cheras in Kuala Lumpur. The commercial burger and frankfurter of chicken and beef origins of four different brands were purchased from Malaysian outlets. All samples were transported under ice-chilled conditions and were cut into the smallest possible pieces with surgical blades prior to storage at − 20 °C until further uses.

### Preparation of admixtures and model meat products (burger and frankfurter)

Tertiary admixture (100 g) was prepared by mixing minced squirrel, rat, rabbit and cat at a ratio of 1:1:1:1 and was homogenized by blending. To prepare burgers and frankfurters, raw meat samples of squirrel, rat, rabbit and cat were minced and blended separately. Model burger and frankfurter of beef and chicken were made in the laboratory following Asing et al. [12] (Table 1). Certain amounts of beef and chicken were mixed with a balanced amount of squirrel, rat, rabbit and cat meat to make 10%, 1% and 0.1% adulteration for each target species.

### DNA extraction

Total DNA was extracted from 30 mg of muscle tissue of each meat and fish species as well as their mixed meat

**Table 1** Formulation of model burger and frankfurter

| Ingredients | Burger (≥ 100 g/peach) | | Frankfurter (≥ 80 g peach) | |
|---|---|---|---|---|
| | Beef | Chicken | Beef | Chicken |
| Minced meat | 70[a] g | 70[a] g | 55[a] g | 55[a] g |
| Soy protein | 10 g | 10 g | 10 | 10 |
| Starch/breadcrumb | 8 g | 8 g | 7 | 7 |
| Chopped onion[b] | 4 g | 4 g | 2 | 2 |
| Chopped ginger[b] | 0.4 g | 0.4 g | 0.2 | 0.2 |
| Cumin powder[b] | 1 g | 1 g | 1 | 1 |
| Garlic powder[b] | 1 g | 1 g | 0.5 | 0.5 |
| Black pepper[b] | 0.3 g | 0.3 g | 0.2 | 0.2 |
| Tomato paste | 2.5 g | 2.5 g | 2 | 2 |
| Butter | 2.5 g | 2.5 g | 2.5 | 2.5 |
| Egg | 1 g | 1 g | – | – |
| Salt | SA | SA | SA | SA |
| Others[c] | SA | SA | SA | SA |

*SA* suitable amounts

[a]10%, 1%, 0.1% of squirrel, rat, rabbit and cat meat were mixed with a balance amount of minced chicken and beef to make ≥ 100 and 80 g specimen of each burger and frankfurter, respectively

[b]Amounts are in approximate values and some items were taken in teaspoon measurements

[c]Enhancers and flavoring agents

products using a Yeastern Genomic DNA Mini Kit (Yeastern Biotech Co., Ltd. Taipei, Taiwan) [39]. Plant DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN GmgH, Hilden, Germany) [40]. DNA from commercial burger and frankfurter was extracted using NucleoSpin Food DNA kit (MACHEREY–NAGEL, GmbH & Co., Duren, Germany) [41]. The purity and concentration of all extracted DNA were determined using a UV–Vis spectrophotometer (Biochrom Libra S70, Biochrom Ltd, Cambridge, UK) based on absorbance at A260/A280 and the ratios were calculated [42–44]. All extracted DNAs were kept at − 20 °C until further uses.

## Primer and probe design

The oligonucleotide primers and probes used in the present study were designed targeting mitochondrial cytb and ATP6 gene of squirrel, rat, rabbit and cat (Table 2). The 5′ and 3′ ends of each probe for squirrel, rat, rabbit and cat were labeled with ROX and TAO/3IAbRQSp; HEX and ZEN/3IABkFQ; Cy5 and TAO/3IAbRQSp; and TAMRA and TAO/3IAbRQSp, respectively. Eukaryotic 18S rRNA-specific primers and TaqMan probe (Table 2) were used as internal amplification control (IAC) [35]. The IAC probe was labeled with FAM at the 5′ end and ZAN/IOWA BLACK FQ at the 3′ end (Table 2). The designed primers

**Table 2** Sequences and concentrations of primers and probes used in this study

| Species | Target gene | Sequence (5′–3′) | $T_m$ value | Amplicon size (bp) | Final concentration (nM) | Reference |
|---|---|---|---|---|---|---|
| Squirrel | Cytb | Forward: TCCGACCTCTAAGCCAATG | 58.8 °C | 161 | 500 | This study |
| | | Reverse: ACTAACAGCTGGCATAAATAGAAGG | 59.3 °C | | 500 | |
| | | Probe: ROXN/GCCTGTAGA/TAO/ATACCCCTTTAT CACAATCGG/3IAbRQ | 69.3 °C | | 150 | |
| Rabbit | Cytb | Forward: TCCGATACCTCCACGCTAAC | 60.1 °C | 123 | 250 | This study |
| | | Reverse: GGAGGATGATGCCAATGTTTC. | 61.6 °C | | 250 | |
| | | Probe: Cy5/GTAGGCCGC/TAO/GGAATCTACTATGGA TCATAC/3IAbRQ | 69.4 °C | | 100 | |
| Rat | ATP6 | Forward: CATCATCAGAACGCCTTATTAGC | 60.1 °C | 108 | 250 | This study |
| | | Reverse: AGGTTCGTCCTTTTGGTGTATG | 60.3 °C | | 250 | |
| | | Probe: HEX/CGCCTCCAC/ZEN/ACATTTCAACAC TGACTAAT/3IABkFQ | 69.6 °C | | 100 | |
| Cat | Cytb | Forward: GGAATAATGTTTCGACCACTAAGC | 60.3 °C | 172 | 250 | This study |
| | | Reverse: TGCCTGAGATGGGTATTAGGAT | 59.8 °C | | 250 | |
| | | Probe: TAMRA/TCTGACTCCT/TAO/AGTAGCGGA TCTCCTAACCC/3IAbRQ | 69.1 °C | | 100 | |
| Internal amplification control (IAC) | 18srRNA | Forward: GGTAGTGACGAAAAATAACAATAC AGGAC | – | 141 | 150 | Ali et al. 2012 |
| | | Reverse: ATACGCTATTGGAGCTGGAATTACC | | | 150 | |
| | | Probe: FAM-AAGTGGACTCATTCCAATTACAGG GCCT- ZEN/IOWA BLACK FQ | | | 100 | |

and probes were supplied by Integrated DNA technologies (IDT), Singapore.

### Conditions applied for tetraplex real-time PCR

Tetraplex real-time PCR assay of squirrel, rat, rabbit, cat and IAC was carried out using a Quant Studio 12 K flex real-time PCR system in a 20 µL reaction volume, comprising Prime time Gene Expression Master 2× Mix (IDT, Singapore), primer and probes, total DNA template of each target species and nuclease-free water. In the total volume of reaction mixture, the concentrations of each species DNA template and master mix were 30 ng and 1×, respectively. The primer and probe information is given in Table 2. The amplification was performed using initial denaturation step at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 20 s, and annealing and extension at 58 °C for 60 s.

### Specificity test

The specificity of the developed real-time tetraplex PCR assay was tested against the DNA templates of 22 non-target species including chicken, cow, goat, pig, pigeon, sheep, duck, buffalo, crocodile, turtle, donkey, deer, monkey, dog, turkey, Chinese frog, tuna, salmon, wheat, cucumber, onion and chili.

### Limit of detection

To determine the limit of detection (LOD), the tetraplex qPCR assay was calibrated with a serially diluted DNA extract from a mixture of equal amounts of squirrel, rat, rabbit, and cat meat. A mixture was prepared with extracted DNA from the four target species (squirrel, rat, rabbit, and cat) consisting of 30 ng/µL of each species. After that, the DNA mixture was tenfold serially diluted [26] and the concentrations of the diluted DNA samples were 3, 0.3, 0.03, 0.003 ng/µL. In this assay, 1 µL of DNA mixture from each diluted sample was added to the 20 µL reaction mixture. As a result, the final volume of each reaction mixture contained the same amounts of DNA from all target species and the serially diluted five reaction mixtures contained the 30, 3, 0.3, 0.03, 0.003 ng of DNA, respectively. The tetraplex qPCR of each dilution was assayed in five replicates.

### Generation of standard curve

Standard curve was constructed to determine qPCR efficiency and quantify PCR targets. To generate the standard curve of the tetraplex qPCR system for squirrel, rat, rabbit and cat, the DNA was extracted from the admixture (1:1:1:1) of squirrel, rat, rabbit and cat. The concentration of the extracted DNA was made 120 ng/µL consisting of 30 ng/µL DNA of each species. Then the admixture of DNA was tenfold serially diluted with nuclease-free water to make the concentration of DNA of each species of 3, 0.3, 0.03, 0.003 ng/µL. This resulted in mixtures containing 100–0.001% of DNA from each species. So, 1 µL of each diluted DNA was added to 20 µL of reaction mixture. After performing the tetraplex qPCR assay, the $C_t$ values of each target species were plotted against the logarithmic concentration of DNA from each species [26, 45]. Subsequently, the standard curve was built up and the efficiency of the assay was calculated based on the slope of the curve according to the equation [36] as stated below:

$$E\,(\%) = \left[10^{(-1/\text{slope})} - 1\right] x\,100.$$

The acceptance range of qPCR efficiency was between 90 and 110% that corresponded to a regression slope between −3.1 and −3.6 and an $R^2$ value of ≥ 0.98 [45].

### Sensitivity and validity

To evaluate the sensitivity and suitability of the tetraplex qPCR assay for food product analysis, two different types of model meat products, namely burger and frankfurter of beef and chicken origins were prepared in laboratory. Beef and chicken products were deliberately adulterated with 10, 1 and 0.1% (w/w) of squirrel, rat, rabbit and cat meat. The DNA was extracted from adulterated meat products and concentration was adjusted to 30 ng/µL with nuclease-free deionized water. The sensitivity and validity of the qPCR assay were determined based on respective $C_t$ values according to the formula described in the generation of standard curve section.

## Results and discussion

### Assessment of DNA quality

Total genomic DNA was extracted from pure, admixed and commercial burger and frankfurter products at raw and post-processed states. Absorbance value at 260 nm and absorbance ratio at A260/A280 was used to measure concentration and purity of the extracted DNA, respectively. The concentration of extracted DNA was 110–245 ng/µL for animal and fish muscle tissues, 85–123 ng/µL for plant species and 20–45 ng/µL for meat products. The poor concentration of DNA in meat products was attributed to the complex nature of the food matrices that are composed of many ingredients. However, the absorbance ratio of all DNA samples at A260/A280 was 1.8–2.0 which reflected that good quality DNA was extracted from all specimens [13, 26].

## Development of tetraplex qPCR model

The species-specific primers and probes were carefully evaluated for mismatch and melting temperature ($T_m$) because in a multiplex PCR system multiple primers and probes interact with several templates at the same or very closely related temperature [26]. In this assay, squirrel-, rat-, rabbit- and cat-specific primers and probes had very closely spaced $T_m$ (59 ± 1 and 69 ± 1 °C) that ensured proper annealing of the primers and probes with their respective templates at a selective PCR condition [26]. The $T_m$ values of the developed primers were 58.8–60.3 °C and so all the primers were annealed at 58 °C while $T_m$s of the probes (68.5–70.70 °C) were 8–10 °C higher than that of the primers (Table 2). The higher $T_m$ of the probes allowed preferential probes' annealing before binding of the primers and it was the prerequisite for TaqMan chemistry [46]. These allowed the discrimination of four different amplicons in the same reaction tube through four different fluorescent reporter dyes tagged with the probes (Table 2). Initially, simplex qPCR system for each target species was optimized and then sequentially duplex, triplex and finally tetraplex qPCR was optimized. The $C_t$ values of the tetraplex qPCR assay were 17.16 ± 0.05, 17.62 ± 0.07, 17.14 ± 0.04 and 16.92 ± 0.04 and they were very close to the corresponding $C_t$ values of simplex qPCR for squirrel ($C_t$ = 17.06 ± 0.05), rat ($C_t$ = 17.46 ± 0.06), rabbit ($C_t$ = 17.1 ± 0.03) and cat ($C_t$ = 16.86 ± 0.06), respectively. Thus, the findings of the simplex and multiplex qPCR systems were very consistent, mutually validating each other.

## Specificity evaluation of the tetraplex qPCR system

The species specificity of the tetraplex qPCR system was critically evaluated because it is the foundation pillar of any PCR system. Following optimization, specificity was tested by cross-challenging the primers and probes against 22 non-target species on three different days in triplicates. To avoid false-negative detection, IAC was used as universal internal target to ensure that good quality DNA templates were present in all tubes (Fig. 1). Conversely, a blank or negative template control was made with everything but the template was replaced with equal volume of nuclease-free deionized water to eliminate the chances of any false-positive amplification. The obtained amplification profile clearly demonstrated that the species-specific amplification curves and background fluorescence were realized only for the relevant species in a 40 cycle PCR, confirming that no cross-amplifications took place in the tetraplex qPCR system (Fig. 1). While the amplification signal ($C_t$ values) of the tetraplex qPCR assay for squirrel, rat, rabbit and cat were 17.143 ± 0.04, 17.48 ± 0.03,17.196 ± 0.05 and 16.8 ± 0.09, respectively, only IAC signal ($C_t$ = 15.24–18.78) was obtained for the other 22 non-target species (Table 3).

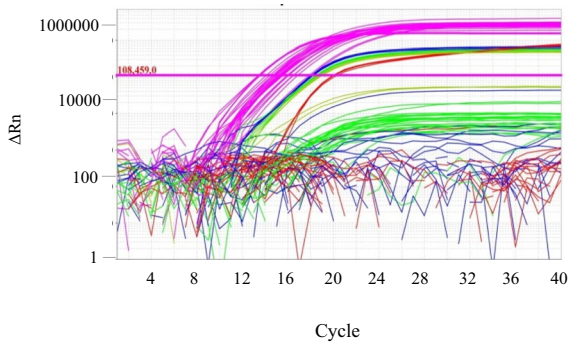


**Fig. 1** Amplification plot of the tetraplex qPCR for squirrel (red), rat (lime), rabbit (blue) and cat (bright green) species along with IAC (pink) against 22 species (below the threshold cycle)

## Limit of detection

The limit of detection (LOD) of an assay determines the minimum amount of target analytes that could be detected in adulterated food stuff. So, it was ascertained by analyzing serially diluted DNA extracts mixed into equal proportion in a fixed amount of genomic DNA and subsequent PCR amplification. In this case, tenfold serially diluted mixed genomic DNA was used and consequently the concentration of the diluted DNA sample of each target species was 30, 3, 0.3, 0.03, 0.003 ng/µL. The amplification plots reflected detectable $C_t$ from all concentrations, starting from 30 ng to 0.003 ng of DNA, suggesting the assay could detect and quantify minimum 0.003 ng of target DNA in a 20 µL reaction mixture. The resulting $C_t$ values and the corresponding relative standard deviations (RSD) for all diluted DNA templates are listed in Table 4. It was found that the assay could detect and quantify the 0.003 ng/µL of DNA from each target species under mixed states with RSD values 0.07–0.56. Recently, Hossain et al. [25] reported an LOD of 0.003 ng/µL in a tetraplex qPCR assay for beef, buffalo and pork. On the other hand, Cheng et al. [26] detected 0.15 ng/µL DNA from blood curd samples of duck, pig and chicken. Similarly, 0.32 ng of DNA was determined by Koppel et al. [47] from boiled and raw sausages as well as fresh meat of beef, pork, chicken and turkey. These studies clearly revealed that LOD may vary from species to species and depends on many factors such as degree of decomposition, sample age, processing conditions and background matrices.

## Target detection and qPCR efficiency

To determine the efficiency of the developed qPCR assay, four different standard curves were constructed for squirrel, rat, rabbit and cat by plotting the $C_t$ values obtained from the tenfold serially diluted DNA (30, 3, 0.3, 0.03, 0.003 ng/µL) against the logarithmic concentration of

**Table 3** Specificity/cross-reactivity test of multiplex qPCR and endogenous system

| Animal species tested | Multiplex real-time PCR system | | IAC PCR system | |
|---|---|---|---|---|
| | Increase of fluorescence signal | Mean $C_t$ value | Increase of fluorescence signal | Mean $C_t$ value |
| Squirrel | + | 17.14 ± 0.04 | + | 15.44 ± 0.13 |
| Rat | + | 17.48 ± 0.03 | + | 16.11 ± 0.09 |
| Rabbit | + | 17.19 ± 0.05 | + | 16.87 ± 0.1 |
| Cat | + | 16.8 ± 0.09 | + | 15.79 ± 0.12 |
| Chicken | − | − | + | 15.24 ± 0.06 |
| Goat | − | − | + | 16.58 ± 0.13 |
| Cow | − | − | + | 18.7 ± 0.16 |
| Buffalo | − | − | + | 16.21 ± 0.09 |
| Sheep | − | − | + | 15.52 ± 0.15 |
| Pigeon | − | − | + | 17.42 ± 0.14 |
| Duck | − | − | + | 15.84 ± 0.13 |
| Pig | − | − | + | 16.22 ± 0.13 |
| Quail | − | − | + | 15.71 ± 0.11 |
| Turkey | − | − | + | 15.36 ± 0.14 |
| Monkey | − | − | + | 16.24 ± 0.11 |
| Donkey | − | − | + | 15.48 ± 0.16 |
| Salmon | − | − | + | 18.78 ± 0.16 |
| Tuna | − | − | + | 17.6 ± 0.06 |
| Frog | − | − | + | 16.24 ± 0.12 |
| Crocodile | − | − | + | 16.63 ± 0.08 |
| Turtle | − | − | + | 16.18 ± 0.07 |
| Deer | − | − | + | 15.59 ± 0.08 |
| Dog | − | − | + | 15.9 ± 0.11 |
| Onion | − | − | + | 17.24 ± 0.06 |
| Chili | − | − | + | 17.48 ± 0.13 |
| Wheat | − | − | + | 17.79 ± 0.13 |
| Cucumber | − | − | + | 16.68 ± 0.14 |

+, positive PCR result ($C_t$ value < 40); −, no increase of the fluorescence signal within 40 cycles

DNA (Fig. 2). A good linear regression was found for all of the standard curves as reflected by the respective regression coefficient ($R^2$), 0.9996, 0.9987, 0.9992 and 0.9988 for squirrel, rat, rabbit and cat, respectively. The corresponding slopes of each standard curve were − 3.1162, − 3.1671, − 3.174 and − 3.184, respectively. Thus, the calculated PCR efficiency was 109.36%, 106.87%, 106.53% and 106.06% for squirrel, rat, rabbit and cat, respectively. The obtained regression coefficient, corresponding slope and PCR efficiency were within the recommended values as described in published reports [35]. Previously Cheng et al. [26] found an mqPCR efficiency of 104.38, 91.75 and 97.46% for chicken, duck and pig species, respectively. On the other hand, Iwobi et al. [45] realized 101.1% and 91.6% efficiency for beef- and pork-specific multiplex qPCR system, respectively. Recently, Hossain et al. [25] got 108.73%, 107.82% and 94.68% efficiency for cow, buffalo, and pig, respectively. Thus, the developed method contained sufficient merit for the discriminatory detection of target species as the efficiency and $R^2$ values of generated standard curves were found within recommended limits.

## Sensitivity and validity of the tetraplex qPCR assay under commercial matrices

To evaluate the sensitivity of the tetraplex qPCR assay under complex matrices, two types of model meat products were made following Asing et al. [12]. The chicken and beef burger and frankfurter were spiked with 10%, 1% and 0.1% of squirrel, rat, rabbit and cat meat. The tetraplex qPCR assay was performed using the extracted DNA from adulterated meat products (beef and chicken burgers and frankfurters). For all the four target species the $C_t$ values obtained for the lowest detectable quantity (0.1%) were 24.441 ± 0.266 to 25.83 ± 0.341 (Table 5) while that for IAC was constantly maintained mean between 14.534 ± 0.13 and 15.35 ± 0.19

**Table 4** $C_t$ value of each target species obtained from the amplification plot with a tenfold serially diluted DNA of each target species for the determination of LOD

| DNA concentration (ng) | Squirrel | | | | Rat | | | | Rabbit | | | | Cat | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $C_t$ value | Mean $C_t$ value | SD | RSD | $C_t$ value | Mean $C_t$ value | SD | RSD | $C_t$ value | Mean $C_t$ value | SD | RSD | $C_t$ value | Mean $C_t$ value | SD | RSD |
| 30 | 17.113 | 17.081 | 0.064 | 0.38 | 17.69 | 17.59 | 0.09 | 0.52 | 17.055 | 17.067 | 0.03 | 0.18 | 16.956 | 16.939 | 0.053 | 0.32 |
| | 17.007 | | | | 17.513 | | | | 17.045 | | | | 16.88 | | | |
| | 17.125 | | | | 17.567 | | | | 17.102 | | | | 16.983 | | | |
| 3 | 20.459 | 20.466 | 0.026 | 0.13 | 20.808 | 20.751 | 0.116 | 0.56 | 20.322 | 20.414 | 0.085 | 0.42 | 20.163 | 20.181 | 0.113 | 0.56 |
| | 20.496 | | | | 20.618 | | | | 20.492 | | | | 20.078 | | | |
| | 20.445 | | | | 20.829 | | | | 20.428 | | | | 20.302 | | | |
| 0.3 | 23.434 | 23.457 | 0.024 | 0.1 | 24.179 | 24.188 | 0.017 | 0.07 | 23.715 | 23.723 | 0.011 | 0.05 | 23.552 | 23.562 | 0.033 | 0.14 |
| | 23.455 | | | | 24.177 | | | | 23.719 | | | | 23.535 | | | |
| | 23.482 | | | | 24.209 | | | | 23.736 | | | | 23.6 | | | |
| 0.03 | 26.417 | 26.46 | 0.037 | 0.14 | 27.264 | 27.309 | 0.083 | 0.31 | 26.683 | 26.714 | 0.044 | 0.17 | 26.766 | 26.729 | 0.048 | 0.18 |
| | 26.483 | | | | 27.258 | | | | 26.695 | | | | 26.675 | | | |
| | 26.48 | | | | 27.406 | | | | 26.766 | | | | 26.747 | | | |
| 0.003 | 29.563 | 29.618 | 0.059 | 0.2 | 30.124 | 30.1 | 0.047 | 0.16 | 29.742 | 29.74 | 0.126 | 0.42 | 29.658 | 29.538 | 0.137 | 0.46 |
| | 29.681 | | | | 30.131 | | | | 29.865 | | | | 29.465 | | | |
| | 29.611 | | | | 30.046 | | | | 29.613 | | | | 29.393 | | | |

*SD* standard deviation, *RSD* relative standard deviation

**Table 5** Mean $C_t$ values and inter-day RSD of different model meat products

| Products | Spike level (%) | Species | Mean $C_t$ value | | | SD | RSD (%) |
|---|---|---|---|---|---|---|---|
| | | | Day 1 | Day 2 | Day 3 | | |
| Chicken burger | 10 | Squirrel | 18.437 | 18.614 | 18.782 | 0.172 | 0.93 |
| | | Rat | 19.141 | 19.275 | 19.026 | 0.124 | 0.65 |
| | | Rabbit | 18.747 | 18.698 | 18.631 | 0.058 | 0.31 |
| | | Cat | 18.632 | 18.5 | 18.589 | 0.067 | 0.36 |
| | 1 | Squirrel | 21.715 | 21.637 | 21.841 | 0.102 | 0.47 |
| | | Rat | 22.383 | 22.589 | 22.145 | 0.222 | 0.99 |
| | | Rabbit | 21.73 | 21.814 | 21.767 | 0.042 | 0.19 |
| | | Cat | 21.72 | 21.952 | 21.714 | 0.135 | 0.62 |
| | 0.1 | Squirrel | 24.936 | 24.795 | 24.647 | 0.144 | 0.58 |
| | | Rat | 25.537 | 25.159 | 25.492 | 0.206 | 0.81 |
| | | Rabbit | 24.946 | 25.204 | 25.175 | 0.141 | 0.56 |
| | | Cat | 25.138 | 24.912 | 25.016 | 0.133 | 0.45 |
| Chicken frankfurter | 10 | Squirrel | 18.639 | 18.821 | 18.487 | 0.167 | 0.9 |
| | | Rat | 19.044 | 19.129 | 19.178 | 0.067 | 0.35 |
| | | Rabbit | 18.671 | 18.784 | 18.553 | 0.115 | 0.62 |
| | | Cat | 18.606 | 18.418 | 18.624 | 0.144 | 0.62 |
| | 1 | Squirrel | 21.748 | 21.967 | 21.693 | 0.144 | 0.66 |
| | | Rat | 22.129 | 22.583 | 22.315 | 0.228 | 1.02 |
| | | Rabbit | 21.706 | 21.752 | 21.983 | 0.148 | 0.68 |
| | | Cat | 21.857 | 21.946 | 21.592 | 0.184 | 0.84 |
| | 0.1 | Squirrel | 24.975 | 24.645 | 24.512 | 0.238 | 0.96 |
| | | Rat | 25.608 | 25.73 | 25.046 | 0.364 | 1.43 |
| | | Rabbit | 24.876 | 24.695 | 24.783 | 0.09 | 0.37 |
| | | Cat | 24.869 | 24.513 | 24.988 | 0.247 | 1 |
| Beef burger | 10 | Squirrel | 18.627 | 18.784 | 18.393 | 0.196 | 1.06 |
| | | Rat | 19.331 | 19.174 | 19.014 | 0.158 | 0.83 |
| | | Rabbit | 18.485 | 18.63 | 18.498 | 0.08 | 0.43 |
| | | Cat | 18.458 | 18.557 | 18.519 | 0.049 | 0.27 |
| | 1 | Squirrel | 21.762 | 21.295 | 21.932 | 0.329 | 1.52 |
| | | Rat | 22.182 | 22.198 | 22.216 | 0.017 | 0.08 |
| | | Rabbit | 21.73 | 21.585 | 21.618 | 0.307 | 1.4 |
| | | Cat | 21.756 | 21.642 | 21.759 | 0.075 | 0.35 |
| | 0.1 | Squirrel | 24.813 | 24.958 | 24.441 | 0.266 | 1.08 |
| | | Rat | 25.148 | 25.83 | 25.52 | 0.341 | 1.34 |
| | | Rabbit | 24.998 | 24.817 | 24.793 | 0.112 | 0.45 |
| | | Cat | 24.673 | 24.975 | 24.751 | 0.156 | 0.63 |
| Beef frankfurter | 10 | Squirrel | 18.686 | 18.493 | 18.932 | 0.22 | 1.18 |
| | | Rat | 19.149 | 19.387 | 19.152 | 0.136 | 0.71 |
| | | Rabbit | 18.74 | 18.661 | 18.485 | 0.13 | 0.7 |
| | | Cat | 18.514 | 18.482 | 18.414 | 0.051 | 0.28 |
| | 1 | Squirrel | 21.874 | 21.632 | 21.325 | 0.275 | 1.27 |
| | | Rat | 22.359 | 22.198 | 22.269 | 0.08 | 0.36 |
| | | Rabbit | 21.549 | 21.746 | 21.619 | 0.099 | 0.46 |
| | | Cat | 21.98 | 21.462 | 21.518 | 0.284 | 1.31 |
| | 0.1 | Squirrel | 24.764 | 25.01 | 24.912 | 0.123 | 0.5 |
| | | Rat | 25.791 | 25.371 | 25.448 | 0.223 | 0.88 |
| | | Rabbit | 24.812 | 24.549 | 24.992 | 0.222 | 0.9 |
| | | Cat | 24.751 | 24.938 | 24.563 | 0.187 | 0.76 |

*SD* standard deviation, *RSD* relative standard deviation

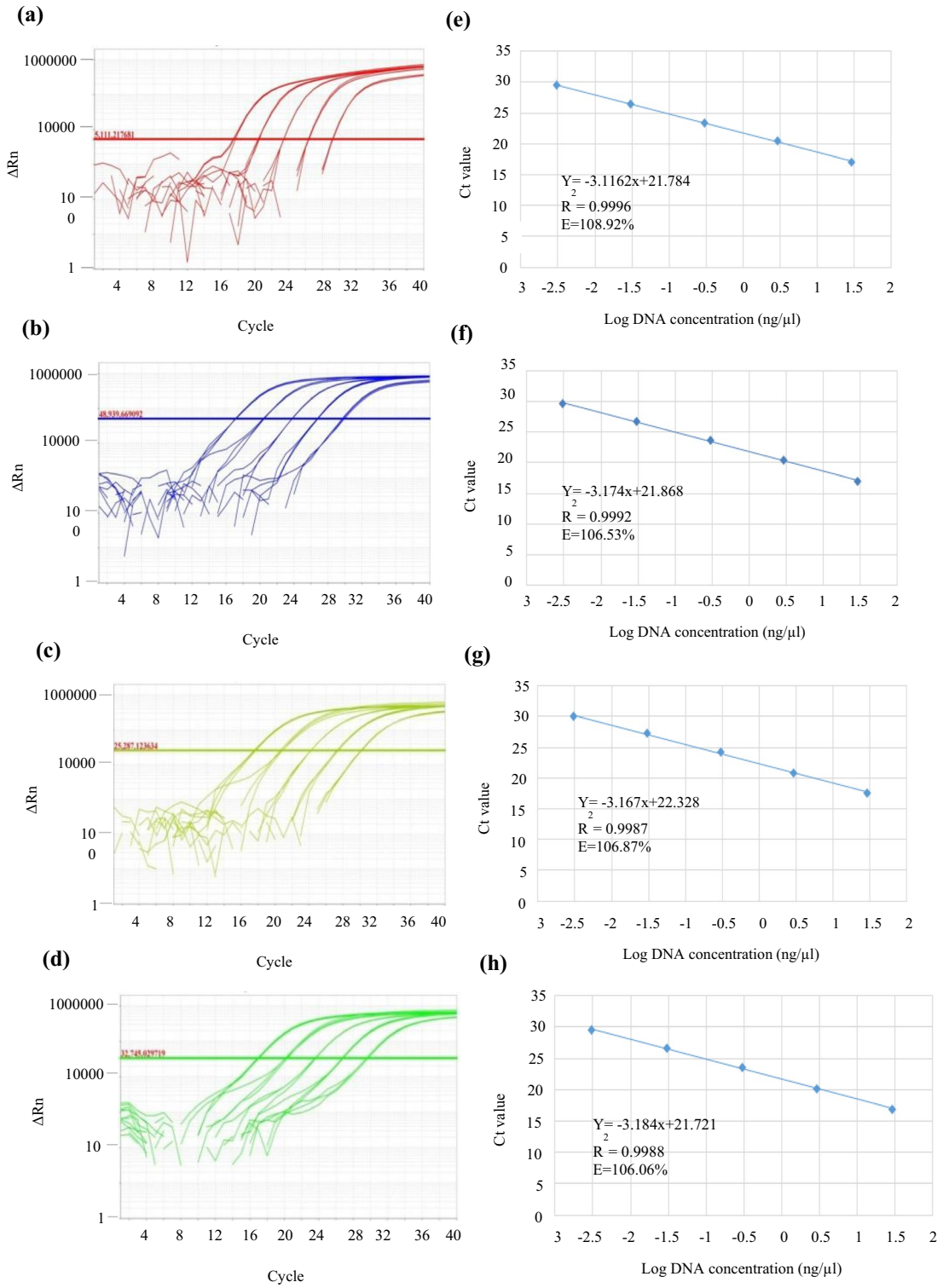


**Fig. 2** Amplification plots (**a**–**d**) and standard curves (**e**–**h**) of the tetraplex qPCR obtained from tenfold serially diluted mixed DNA of four target species. Shown are amplification plots and standard curves for squirrel (**a** and **e**), rabbit (**b** and **f**), rat (**c** and **g**) and cat (**d** and **h**), respectively

for all levels of adulteration, indicating that any variation in adulteration level could not significantly change the endogenous target as all adulterants were from eukaryotic origins.

## Commercial burger and frankfurter analysis

The commercial food products such as burger, meatball,

frankfurter, hotdog, and nugget are very popular all over the world. These types of products are highly processed that results in total or partial annihilation or modification of morphological features and other physical properties. Therefore, manufacturers can easily mix an unexpected and lower priced meat in the final products for profit making purposes. To prevent or monitor these types of undesirable incidences, the accurate screening of commercial meat products can play a great role and build public confidence on health, religious, social and cultural perspectives. In this work, two types of commonly used commercial products, namely burger and frankfurter were evaluated using the developed tetraplex qPCR system. A total of 72 burgers (36 beef and 36 chickens) and 72 frankfurters (36 beef and 36 chickens) were purchased from different Malaysian outlets and were tested (Table 6). The experimental results revealed that no target species (cat, rabbit, rat, and squirrel) were present in the burger and frankfurter products but only IAC was amplified, reflecting the 100% accuracy of the qPCR assay. The meat of cat, rat, rabbit and squirrel species was not mixed with commercial burger and frankfurter, this might be due to the fact that halal products are under strict surveillance in Malaysia by Government agencies. Conversely, all model products were positively detected showing the efficiency of the assay (Table 6).

**Table 6** Screening of model and commercial meat products using the developed tetraplex qPCR assay

| Sample | Spiked level of target Species (%) | Detected species | | | | PCR accuracy (%) |
|---|---|---|---|---|---|---|
| | | Squirrel | Rat | Rabbit | Cat | |
| Chicken burger | 10 | 3/3 | 3/3 | 3/3 | 3/3 | 100 |
| | 1 | 3/3 | 3/3 | 3/3 | 3/3 | 100 |
| | 0.1 | 3/3 | 3/3 | 3/3 | 3/3 | 100 |
| Chicken frankfurter | 10 | 3/3 | 3/3 | 3/3 | 3/3 | 100 |
| | 1 | 3/3 | 3/3 | 3/3 | 3/3 | 100 |
| | 0.1 | 3/3 | 3/3 | 3/3 | 3/3 | 100 |
| Beef burger | 10 | 3/3 | 3/3 | 3/3 | 3/3 | 100 |
| | 1 | 3/3 | 3/3 | 3/3 | 3/3 | 100 |
| | 0.1 | 3/3 | 3/3 | 3/3 | 3/3 | 100 |
| Beef frankfurter | 10 | 3/3 | 3/3 | 3/3 | 3/3 | 100 |
| | 1 | 3/3 | 3/3 | 3/3 | 3/3 | 100 |
| | 0.1 | 3/3 | 3/3 | 3/3 | 3/3 | 100 |
| Commercial chicken burger | | | | | | |
| Ramly | | 0/9 | 0/9 | 0/9 | 0/9 | 100 |
| Tesco | | 0/9 | 0/9 | 0/9 | 0/9 | 100 |
| Ayamus | | 0/9 | 0/9 | 0/9 | 0/9 | 100 |
| Prima | | 0/9 | 0/9 | 0/9 | 0/9 | 100 |
| Commercial chicken frankfurter | | | | | | |
| Ramly | | 0/9 | 0/9 | 0/9 | 0/9 | 100 |
| Tesco | | 0/9 | 0/9 | 0/9 | 0/9 | 100 |
| Ayamus | | 0/9 | 0/9 | 0/9 | 0/9 | 100 |
| Prima | | 0/9 | 0/9 | 0/9 | 0/9 | 100 |
| Commercial beef burger | | | | | | |
| Ramly | | 0/9 | 0/9 | 0/9 | 0/9 | 100 |
| Tesco | | 0/9 | 0/9 | 0/9 | 0/9 | 100 |
| Ayamus | | 0/9 | 0/9 | 0/9 | 0/9 | 100 |
| Prima | | 0/9 | 0/9 | 0/9 | 0/9 | 100 |
| Commercial beef frankfurter | | | | | | |
| Ramly | | 0/9 | 0/9 | 0/9 | 0/9 | 100 |
| Tesco | | 0/9 | 0/9 | 0/9 | 0/9 | 100 |
| Ayamus | | 0/9 | 0/9 | 0/9 | 0/9 | 100 |
| Prima | | 0/9 | 0/9 | 0/9 | 0/9 | 100 |

The numerator and denominator of each fraction denote the number of positive detection and total number of samples analyzed using the multiplex real-time PCR assay

## Conclusion

The developed tetraplex qPCR system was greatly promising in detecting cat, rabbit, rat and squirrel in a single assay platform even under any matrices and processing treatments given its species-specific primers, probes and short-length amplicon. The designed primers and probes bound only with complementary DNA at specific sites, offering a double-checking point for a well-protected secured target detection. The short amplicon size also increased the stability of the qPCR assay since short-length amplicon offers more stability over longer one for thermodynamic reasons. Additionally, the use of IAC effectively eliminated any false-negative results, enhancing the assay reliability. Above all, the designed primers, probes, shorter size of amplicon and IAC targets provided extraordinary specificity, stability and reliability of the developed tetraplex qPCR system. This assay detected 0.003 ng of DNA of the target species in pure and 0.1% of DNA in admixed states. Furthermore, it demonstrated high correlation coefficient ($R^2 = 0.999$) between the actual values and reference values for 10–0.1% admixtures of the target species in burger and frankfurter formulations. The assay was also evaluated for the screening of commercial meat products. Overall the developed tetraplex qPCR assay for cat, rabbit, rat and squirrel was robust, specific, sensitive and cost effective for monitoring the target species in any forensic detection.

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## Compliance with ethical standards

**Conflict of interest** All authors declare that they have contributed to this article and they do not have any conflict of interest to publish it in journal.

**Compliance with ethics requirements** Ethical clearance of Ref. no.: NANOCAT/23/07/2013/A(R) was obtained from the Institutional Animal Care and Use Committee, University of Malaya (UM IACUC), and all experiments were conducted following the national and institutional guidelines while handling animal meats used in this study.

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