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DEVELOPMENT OF MULTIPLEX PCR ASSAY FOR MEAT PRODUCTS AUTHENTICATION: TARGETING DOUBLE GENE

M. A. Motalib Hossain
Nanotechnology and Catalysis Research Centre (NANOCAT), University of Malaya, 50603 Kuala Lumpur, Malaysia
motalib_123@yahoo.com

Syed Muhammad Kamal Uddin
Nanotechnology and Catalysis Research Centre (NANOCAT), University of Malaya, 50603 Kuala Lumpur, Malaysia
syedmdkamaluddin@gmail.com

Sharmin Quazi Bonny
Nanotechnology and Catalysis Research Centre (NANOCAT), University of Malaya, 50603 Kuala Lumpur, Malaysia
bonny.sharming@gmail.com

Mohd Rafie Johan
Nanotechnology and Catalysis Research Centre (NANOCAT), University of Malaya, 50603 Kuala Lumpur, Malaysia
mrafiej@um.edu.my

Md. Eaqub Ali
Nanotechnology and Catalysis Research Centre (NANOCAT), University of Malaya, 50603 Kuala Lumpur, Malaysia
eaqubali@gmail.com

Abstract

Authentication of the species origins of animal-originated food products is a rapidly growing field because of its direct relevance to public health as well as people's religious and cultural

traditions. Current polymerase chain reaction assay (PCR) based methods to authenticate the animal materials in food chain are based on mainly single gene targets which are generally longer in length and thus breakdown during food processing treatments. Consequently, there is a chance of a false negative result. For the first time, here we targeted double gene sites in short-amplicon length multiplex PCR (mPCR) for confirmed detection and differentiation of bovine, buffalo and porcine materials in food chain. Multiple targets detection in single assay saves analytical cost and time. The design of primer sets for mPCR assay is more complex and complicated because all biomarkers are annealed to their respective targets under a single set of PCR conditions. Inaccurately designed primers might prompt less amplification or formation of primer-dimer and/or non-specific products. Here we approached the techniques to design biomarkers for the development of double gene targeted mPCR assay. Mitochondrial cytochrome b (cytb) and NADH dehydrogenase subunit 5 (ND5) genes were targeted to design six different biomarkers, two for each of cow (121 and 106 bp), buffalo (90 and 138 bp) and pig (73 and 146 bp). The in-silico specificity of the developed primers was checked against three targets and 28 non-target species. Complete sequence matching was found only with target species, and 3−18 nucleotides (12.5−80%) mismatches were found with other species. The pairwise distance was also computed using the neighbour-joining method; the lowest and highest distances were observed between 0.144 and 1.993. These indicated adequate genetic distances among the studied species, eliminating the probability of any cross-target detection and thus facilitated the target detection through mPCR assay.

Keywords

Double Gene Targeted, Multiplex PCR, Food Authentication, Primer Design, Short Amplicon

1. Introduction

In recent years, the detection of meat species in processed and unprocessed food has gained increasing attention due to perspectives of public health, religious faith and fair trade (Bottero & Dalmasso, 2011). Some recent occurrences of food fraud made consumers extremely concerned and worried about food labelling: police seized about 20 tons fake beef which were made up by chemically treated pork meat in China (Ali, Razzak, & Hamid, 2014), rat, fox and mink meat was sold as lamb in China, pig was detected in meat pies and pasties in UK despite certified as Halal, and in Europe, horse meat was found in the beef food product (Hossain et al., 2016). Moreover, consumption of beef, pork and poultry was dramatically reduced in Europe due to bovine spongiform encephalopathy (ESB) and swine influenza, the polychlorinated biphenyl (PCB) (dioxin contamination) and avian influenza (Bottero &

Dalmasso, 2011). However, identification and differentiation of species in food products, especially in processed ones, where different ingredients discrimination is more difficult, is an important issue to verify compliance with labelling requirements.

Now a days, various techniques have been introduced for the detection of meat species namely, protein based (Ayaz, Ayaz & Erol, 2006), lipid based (Szabo, Febel, Sugar & Romvari 2007) and DNA based (Hossain et al., 2019; Safdar and Junejo, 2016) assays. However, utilization of the protein- and lipid-based techniques has been dropped since protein-based biomarkers can be effectively denatured and the amount and types of lipids can be significantly modified through the processing treatments (Ali et al., 2014). In contrast, DNA based techniques are more advantageous due to conserved structure and high heat stability of DNA as compared to protein. Moreover, among the DNA based assays, PCR has gained increasing attention as reliable and rapid investigation scheme due to accuracy and higher sensitivity (Aida, Man, Wong, Raha & Son, 2005; KN, Daniel & Varghese, 2017; Nair & Jayachandran, 2017; Vaidya et al., 2015). Recently, the multiplex PCR (mPCR) methods, where multiple targets of DNA are amplified simultaneously in a single reaction tube, are highly promising because they reduce both time and cost (Hossain et al., 2017). To develop double genes targeted mPCR, the design of species-specific primers is a crucial step because here all primers must amplify their respective targets in a single set of PCR condition (reaction components and volume, and cycling parameters). In designing primers, special precautions should be taken regarding primers specificity and Tm (melting temperature) to avoid mis-priming and formation of primer dimers in mPCR, as compared to conventional PCR. Here, we proposed the step by step development of double genes targeted mPCR assay for the detection and differentiation of beef, buffalo and pork simultaneously in a single assay platform.

2. Development of Multiplex PCR

Multiplex PCR is a wide-ranging molecular biology technique by which multiple primers are used to amplify more than one targets in a single PCR experiment. This technique often require extensive optimization for successful result because some non-specific products may interfere with the amplification of specific products. Consequently, various factors should be considered to design primers for mPCR, particularly when targeting two genes of each species, and hence it is indeed a challenging work.

3. Primer Design for Multiplex PCR

Proper design of primer is a vital step/factor for efficient and successful PCR amplification. Increased efficiency and maximum specificity of PCR depend on optimal primer sequence and adequate primer concentration (He, Marjamäki, Soini, Mertsola, & Viljanen, 1994). An inaccurately designed primer may yield no or little product due to formation of primer-dimer and/or non-specific amplification (Abd-Elsalam, 2003). Design of mPCR primer sets is more complex as all primers are amplified to their respective targets under a single set of PCR conditions. Moreover, specificity and Tm are more important factors in multiplex system over conventional PCR (Ali et al., 2014). In addition, PCR products length (amplicon size) should also be taken into account during the design of primers. The size of the amplicons depends on the resolution capability of the detection system used so that the generated PCR products can distinguish easily from one another. There are many factors involved for acting an oligonucleotide as a PCR primer, such as i) the association and dissociation kinetics at the temperature of annealing and extension of primer-template duplexes; ii) the location of mismatched nucleotides and their duplex stability and iii) the efficiency of polymerase for recognition and extension of mismatched duplex (Abd-Elsalam, 2003).

To design the primers, initially the whole genomic sequences of the target species are retrieved from NCBI database (http://www.ncbi.nlm.nih.gov/) and are aligned using Molecular Evolutionary Genetics Analysis version 5 (MEGA5) alignment tool (Tamura et al., 2011) for identifying the inter-species hyper-variable and intra-species conserved regions. Then publicly available primer designing software is used to generate the desired primers. There are many free online softwares available for designing primers namely Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi), PrimerSelect (http://www.dnastar.com/t-primerselect.aspx) PrimerQuest Tool (http://sg.idtdna.com/ primerquest/ home/index), Primer Designer (http://genamics.com/expression/primer.htm). The following criteria and guidelines should be considered for the design of species specific primers to amplify specific target sequence.

3.1 Primer Length

The length of primer plays an important role for the specificity as well as annealing time and temperature of the target template. These parameters are crucial for successful PCR amplification (Wu, Ugozzoli, Pal, Qian, & Wallace, 1991). Too long primers may decrease the efficiency of template DNA binding at normal annealing temperature due to the chance to form secondary structure whereas, too short primers may be responsible for low specificity and non-

specific amplification (Abd-Elsalam, 2003). The ideal primer length is 18-28 nucleotides, usually good activity is obtained with 20-24 nucleotides containing primers (Ali et al., 2014).

3.2 GC content

One of the most important characteristics of DNA is GC content which refers the annealing strength. To get good PCR product, a reasonable GC content should be maintained. Tm and annealing temperature (Ta) fully depend on the percentage (%) of GC content (Rychlik, Spencer, & Rhoads, 1990). The ideal GC content is 40-60%. One should avoid 3 or more G's or C's at the 3′-end because it has adverse effect to the primer specificity (Ali et al., 2014). Primer should not have long polyG or polyC stretches which result in non-specific annealing.

3.3 Melting and Annealing Temperature

Tm is an important parameter of primer, since it plays a vital role for primer annealing. Primers with Tm of 55-65^0 C work best in most amplification. Tm of both forward and reverse primers should be similar as they are annealed simultaneously. Moreover, mPCR efficiency is effected by a little difference of Tm between the primer sets. Since in the mPCR, all targets are amplified in a single reaction mixture, all primers should have very close Tm. The acceptable Tm variation is 3-5^0 C between the primers but to get good result $\leq$ 2 Tm variation is preferable. Significantly lower Tm of a primer than the PCR annealing temperature (Ta) may cause failure to anneal and extend, while significantly higher Tm may lead to mis-hybridization and can extend at an incorrect location along the DNA sequence (Ali et al., 2014). The approximate Tm value of the primer can be calculated by using the formula (generally valid for oligos in the 18–30 base range) of Wallace et al. (1979) Tm (°C) = 2 x (nA+nT) + 4 x (nG + nC), where, nA, nT, nG and nC are the number of respective nucleotides in the primer.

3.4. 3'-End Specificity

For design of primer to achieve a successful PCR experiment, primer 3′-end is important because during the extension step, DNA polymerase starts to attach nucleotides from the 3′-end of a primer. Since, complete annealing of the primer 3′-end is mandatory and incomplete binding at the 3′-end refers to lower PCR or often no PCR products (Yuryev, 2007). Primers should have mismatch with non-target species at 3′-end, as it prohibits the PCR amplification (Ali et al., 2014). It is well known that for the control of mis-priming, the 3′-end position of the primer plays an important role (Kwok et al., 1990).

3.5 Primer-Primer Interactions

Primer should have a minimum of intermolecular or intramolecular homology that can promote to the formation of either primer dimerization or hairpins (Figure 1a & b). Primer with nucleotide sequences that would allow anneal one primer to other primer(s) result in primer-

dimer formation (Figure 1a), particularly when 3′-ends of the primers anneal to each other. Inter primer homology in the middle position of two primers may also interfere with hybridization. Primer with a self-homology region results in “sanp back” or able to form partially double stranded structures, hairpin (Figure 1b), which will interfere with annealing to the template. To overcome the formation of hairpin, it is recommended that intra-primer homologies of 3 bp or more should be avoided (Abd-Elsalam, 2003).

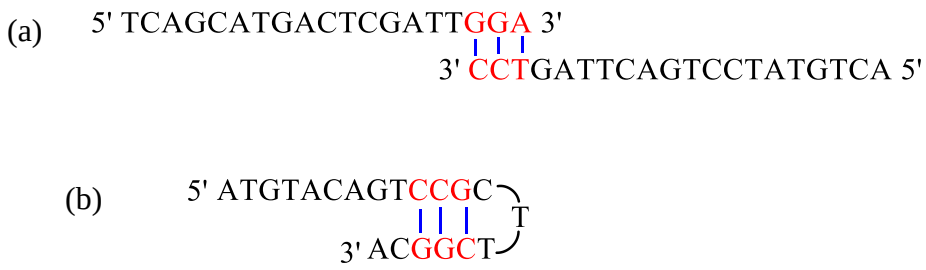

Figure 1: *Primer-Primer Interactions (a) Primer-Dimer; (b) Hairpin*

3.6 Specificity

Primer specificity is checked in three different ways. At first, primers are aligned by using online Basic Local Alignment Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to screen the identical and distant species. Secondly, to determine the total number of mismatch between target and non-target species, the primers are multiple sequence aligned with some common species using an alignment tool such as ClustalW (http://www.genome.jp/tools/clustalw/) or MEGA5. Finally, each primer is assayed in PCR experiment with template DNA of all non-target species to confirm the specificity. Here we showed multiple sequence alignment of the primer of beef cytb gene (Table 1).

Table 1: *The Mismatch Comparison of the Beef Cytb-Specific Primer Pair against Non-Target Species*

Species	forward primer																			Mis-	Reversed primer																	Mis-
Beef Primer (cytb)	C	G	G	C	A	C	A	A	A	T	T	T	A	G	T	C	G	A	A	T	C	A	A	A	T	T	G	C	C	A	T	A	G	T	C	C	A	match
Cow (V00654.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
Bufallo (NC 006295.	T	.	.	T	.	.	.	G	.	C	.	G	.	.	T	.	.	G	.	7	.	G	C	.	.	.	.	C	.	.	.	.	A	.	.	.	.	.	.	4
Goat (KM244714.1)	T	.	.	.	.	.	.	.	.	C	C	.	.	.	.	.	.	.	.	3	.	.	C	.	.	.	C	C	.	C	.	.	.	.	.	.	.	.	.	4
Sheep (KR868678.1)	T	.	.	.	.	.	.	.	.	C	C	.	.	.	.	.	.	.	.	3	.	G	C	.	.	.	C	C	.	C	.	.	.	.	.	.	.	T	.	6
Deer (NC 006973.1)	T	.	.	.	.	.	.	.	.	C	C	.	.	.	.	.	.	.	.	3	.	G	C	.	.	.	.	C	.	.	.	.	T	.	.	.	.	A	.	6
Donkey (KP825304.1)	.	.	.	T	.	.	T	.	C	G	C	.	C	.	.	.	.	.	.	6	.	.	C	.	.	.	C	C	.	G	.	T	A	.	.	C	.	.	.	7
Horse (KU575247.1)	.	.	.	T	.	.	T	.	C	C	C	.	C	.	.	.	.	G	.	7	.	.	C	.	.	.	C	C	.	G	.	T	A	G	.	C	.	.	A	9
Pig (AF034253.1)	.	.	.	A	.	.	.	G	.	C	C	.	C	.	.	A	.	.	.	6	T	.	C	C	.	.	C	C	.	C	.	.	A	G	C	C	.	.	A	11
Cat (NC 001700.1)	.	.	.	G	.	.	T	G	.	A	C	.	.	.	.	A	.	.	.	6	.	T	C	.	.	.	C	T	.	A	.	.	A	G	G	.	.	.	A	9
Dog (KJ522809.1)	.	.	.	A	.	.	T	G	.	C	.	.	.	.	.	A	.	.	.	5	.	G	C	.	.	.	T	C	.	A	.	.	A	.	.	.	.	.	A	7
Rabbit (NC 001913.1	.	.	.	A	.	.	.	.	C	C	.	.	.	.	.	T	.	.	.	4	T	G	C	.	A	.	T	T	.	A	.	T	.	T	.	.	A	.	T	11
Monkey (FJ906803.1)	.	.	.	A	.	.	T	.	.	C	C	.	T	.	.	.	C	.	.	6	.	.	.	C	.	.	C	C	.	C	.	.	A	G	.	C	.	.	A	8
Duck (KJ778676.1)	.	.	.	A	C	A	G	.	C	C	C	.	G	.	.	A	.	.	.	9	.	G	C	.	.	G	.	.	.	C	A	.	.	C	.	.	.	.	.	6
Pigeon (KF926376.1)	T	.	.	.	C	A	.	.	C	C	C	.	C	.	.	T	.	.	.	8	.	G	C	.	.	G	C	C	.	C	A	.	T	.	.	C	A	.	.	10
Salmon (AF133701.1)	A	.	.	A	G	G	C	G	C	C	C	.	T	.	.	A	C	.	.	12	T	G	C	.	.	.	T	G	C	C	A	.	A	G	.	.	C	.	.	11
Cod (NC 002081.1)	A	.	.	T	G	A	T	G	C	C	.	.	.	.	.	T	C	.	.	10	T	G	C	T	.	.	T	T	.	.	A	.	A	.	.	.	C	.	.	9
Tuna (KM588080.1)	T	.	.	A	.	.	T	.	C	.	C	.	C	.	.	T	.	.	.	7	.	G	C	.	.	.	T	.	.	G	A	.	A	.	.	T	C	.	T	.	9
Wheat (GU985444.1)	A	.	.	A	G	A	T	.	C	C	A	.	.	.	.	G	A	C	T	.	12	A	G	.	.	.	G	C	G	C	C	A	G	T	C	.	T	C	.	T	.	14
Onion (NC 030100.1)	A	.	.	A	G	A	T	.	C	C	A	.	.	.	.	G	A	C	T	.	12	A	G	.	.	.	G	C	G	C	C	A	G	T	C	.	T	C	.	T	.	14
Pepper (KJ865410.1)	A	.	.	A	G	A	T	.	C	C	A	.	.	.	.	G	A	C	T	.	12	A	G	.	.	.	G	C	G	C	C	A	G	T	C	.	T	C	.	T	.	14

4. Optimization of Multiplex PCR

Optimization is the essential and final step for successful PCR products. The following components have to be optimized:

4.1 Sample Volume and Reaction Tubes

Most PCR assays are performed at the 25 µl - 50 µl reaction volumes in 0.2 mL micro centrifuge tubes. However, PCR protocols with 10 µl reaction are also successful but >100 µl volumes are usually not recommended.

4.2 Buffer

The cation of buffer neutralizes the negatively charged phosphate group of DNA template which decreases the electrorepulsive forces between the DNA strands. Consequently, primer can come into contact with DNA strand easily that facilitates annealing between them. Thus, appropriate buffer concentration is important in successful PCR amplification. Usually, recommended final buffer concentration is1x.

4.3 Magnesium Chloride Concentration

For the success in PCR amplification, concentration of Magnesium is critical. Mg^{2+} is said to be a cofactor of the polymerase enzyme because it forms soluble complexes with deoxynucleoside triphosphates (dNTPs) to prepare a recognizable substrate for Polymerase. Therefore, Magnesium may affect DNA polymerase activity and fidelity, specificity of PCR, denaturation temperatures of both template and PCR product DNA strand, annealing of primer and formation of primer dimer. Excess magnesium leads to nonspecific amplification due to nonspecific primer annealing, while inadequate magnesium results in decreased yield of expected amplified product. Thus, for optimum activity, polymerase enzyme requires sufficient free magnesium in addition to that bound with dNTP and template DNA (Markoulatos, Siafakas, & Moncany, 2002). The recommended magnesium concentration used in PCR is between 0.5 to 5 mM.

4.5 Deoxynucleoside Triphosphates (dNTPs) Concentration

Concentration of dNTPs may affect the specificity, fidelity and yield of a PCR amplification, because concentration of free magnesium is affected by the amount of dNTPs, since Mg^{2+} bind with dNTPs. The recommended concentration of each dNTP (dATP, dCTP, dGTP and dTTP) is 20-200 µM. DNA polymerase fidelity reduces due to imbalance in the amount of four dNTPs (Kunz & Kohalmi, 1991), whereas, excess dNTPs may result in inhibition of amplification due to increase error rate of polymerase (Kramer & Coen, 2001).

4.6 Annealing Temperature

The optimal annealing temperature has to be determined experimentally. Initially, an annealing temperature should be 5°C below the estimated Tm. The optimal annealing temperature may differ from calculated Tm even when using primer pairs containing a similar Tm value.

5. Conclusion

Multiplex PCR is a comprehensive molecular biology technique for the detection of multiple species simultaneously in a single PCR experiment. Here we described systematically the critical steps of primer design and optimization of the mPCR for effective and successful amplification of target species in a single assay platform.

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