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2D-Gradient in Multiplex PCR for Meat Species Identification

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Abstract

Multiplex PCR assays can be developed to detect meat species for quality control. However, development of a multiplex PCR assay requires more optimization effort than simple single target PCR assays. Gradient PCR technology has enabled time-saving during optimization for the past years. An improved gradient technology called 2D-gradient (two-dimensional) in Mastercycler® X50 allows temperature gradient to be run in two different dimensions (vertically and horizontally) in the same PCR. This allows parallel optimization of two temperature steps when done in combination, thus further reducing optimization time. The results herein show the importance of optimizing both denaturation and annealing temperatures in multiplex PCR, which is achieved reliably using 2D-gradient technology, as well as a strategy that vastly reduces time-to-result and effort spent in optimization.

Introduction

Fraudulent meat scandals are becoming a serious issue in many countries leading to compromised food safety and quality. Due to an increase of such incidences, multiplex PCR has been widely employed as a fast and simple method in the food and beverage industry to authenticate, detect and differentiate between groups of food and beverage or simply to screen the food items for microbial contaminations.

Multiplex PCR involves simultaneous amplification of more than one target gene per reaction and therefore, overcomes limitation of a single-plex PCR. However, the success and reliability of multiplex PCR is highly dependent on several factors, which includes the accessibility of the template DNA, optimal reaction of all primer sets within the mastermix concoction, and a robust PCR system [1, 2]. This article aims to show to the importance of denaturation and annealing temperatures in multiplex PCR optimization and the benefits derived thereafter.

Materials and Methods

Isolation of DNA using DNAzol

Genomic DNA (gDNA) was extracted from raw minced meat of chicken, goat and sheep using DNAzol® (Molecular Research Center, USA) with modifications. 150 mg of meat sample was homogenized in 1 mL of DNAzol in a 2 mL microcentrifuge tube. 10 µL of 20 mg/mL Proteinase K (Invitrogen®, USA) was added into the homogenized sample and incubated overnight (16-18 hours) on an Eppendorf ThermoMixer® C at 25 °C with 500 rpm shaking. After overnight incubation, 300 µL of chloroform was added and mixed by inversion. The mixture was incubated at room temperature for 5 minutes and centrifuged at 12,000 x *g* for 10 minutes at room temperature. The top aqueous layer was transferred to a new tube without disturbing the interphase and lower phase and mixed with 500 µL of ice cold 100% ethanol. The mixture was centrifuged at 12,000 x *g* for 10 minutes after incubating at room temperature for 3 minutes. After removing the supernatant, the gDNA pellet was washed twice by mixing with 1 mL of 75% ethanol and centrifuged at 12,000 x *g* for 10 minutes. The DNA pellet was left to air-dry for 5 minutes after the supernatant was removed. The gDNA precipitate was solubilized in 100 µL of Molecular Biology Grade Water (Nacalai Tesque). The tube was centrifuged at 12,000 x *g* for 1 minute and the supernatant (solubilized DNA) was transferred into a new tube. The concentration of extracted gDNA was determined using Eppendorf BioSpectrometer® kinetic.

Polymerase Chain Reaction

A commercial PCR enzyme kit, i-Taq™ DNA Polymerase (iNtRON Biotechnology, Korea) was used. The optimal PCR master mix consisted of 1X i-Taq buffer, 0.4 mM dNTP mix, 2 units of i-Taq DNA Polymerase, 0.1 µM common forward primer (SIM), 0.07 µM sheep reverse primer, 0.1 µM chicken reverse primer, 0.25 µM goat reverse primer, 12.5 ng sheep gDNA, 12.5 ng chicken gDNA and 50 ng goat gDNA.

A master mix was prepared and 5 µL was aliquoted into each tube. Eppendorf 0.2 mL PCR 8-tube strip with attached caps were used. A 2D-gradient for denaturation and annealing steps was performed on Mastercycler X50s with optimized cycling parameters listed in Table 2. PCR products were visualized with 1.5% agarose (1st Base, Singapore) gel electrophoresis and stained with FloroSafe DNA Stain (1st Base, Singapore).

Table 1: Primer sequences [3]

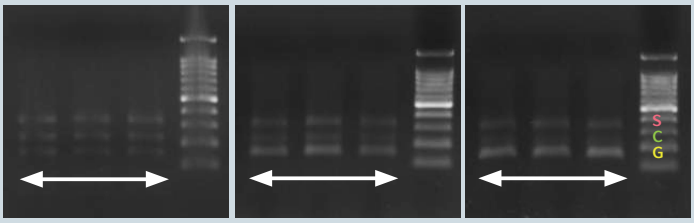
Target species	Forward	Reverse	Amplicon length
Sheep	5′-GACCTCCCAGCTCCATCAAACATCTCATCTTGATGAAA-3′	5′-CTATGAATGCTGTGGCTATTGTCGCA-3′	331 bp
Chicken		5′-AAGATACAGATGAAGAAGAATGAGGCG-3′	227 bp
Goat		5′-CTCGACAAATGTGAGTTACAGAGGGA-3′	157 bp

Table 2: Optimized 2D-Gradient PCR cycling parameter

Header (Eppendorf settings)	Lid	105 °C	
	TSP/ESP	ON	
	Lid auto-off	ON	
	Temperature mode	Safe	
Step	Temperature	Duration	Cycle(s)
Pre-denaturation	92 – 98 °C	2 min	1
Denaturation	92 – 98 °C	15 s	12
Annealing + Extension	50.5 – 59.1 °C + 0.5 °C/ cycle	15 s	
Denaturation	92 – 98 °C	15 s	13
Annealing + Extension	56.5 – 65.1 °C	15 s	
Hold	10 °C	∞	Hold

Results & Discussion

A 2D-gradient and touch-up PCR run (Table 2) was carried out to determine the best denaturing (T_D) and annealing (T_A) temperature combinations that can detect all three meat species without amplification bias and non-specific amplification (Figure 1A). From the results, amplification was successful within the T_D range of 92.1 – 95.8 °C, whereas the T_A of 54.3 °C gives the most balanced amplification yield of all three species within the 92.4-95.8 °C T_D range.



TD	95 °C	93.1 °C	92.4 °C
TA	54.3 °C	54.3 °C	54.3 °C

S - Sheep C - Chicken G - Goat

Figure 1B: Routine multiplex PCR for species identification from meat samples (in triplicates).

Three sets of temperature combination with different T_D were then chosen for validation using standard routine PCR run (T_A and T_D as specified in Figure 1B). Set 1 represents a commonly used denaturation temperature, with Set 2 and Set 3 used as comparison. From the routine run (Figure 1B), it can be seen that T_D of 92.4 °C gave higher yields in comparison to 95 °C at 54.3 °C T_A. This means that the optimal denaturation and annealing temperatures were in practice lower than the manufacturer's recommendation and predictions by primer design software. This shows the importance of optimizing both denaturation and annealing temperatures in establishing a reliable multiplex PCR protocol. The 2D-gradient setting in Mastercycler X50s allows the optimum T_D and T_A to be determined from one run of 25 minutes. In comparison to running similar conditions in a thermal cycler with conventional single dimensional (1D) gradient setting only, one is required to run 8 separate runs, which will take a total run time of 200 minutes. Additionally, separate runs with 1D gradient PCR may require the use of a different batches of master mix, as well as subjected to run-to-run variation. This affects the overall accuracy of results and further increases the difficulty of optimization. Apart from PCR cycling temperatures, primer ratio, magnesium chloride concentration and cycling settings are important parameters to consider for specific amplification of all targets with similar intensity (data unpublished).

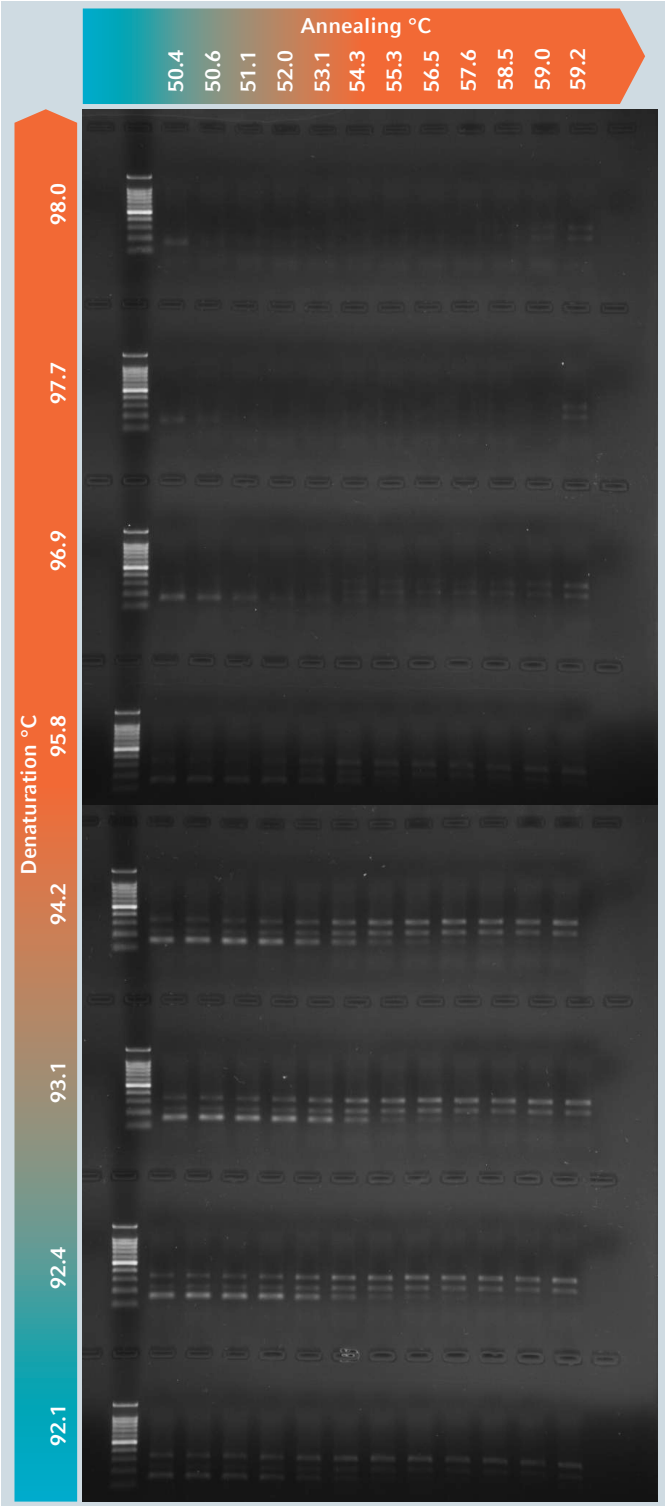


Figure 1A: Species identification from meat samples. Multiplex PCR using 2D-gradient easily identified optimal denaturation and annealing temperatures in a single run. *Sheep 331bp; chicken 227bp; goat 157bp. Denaturation temperature, T_D; annealing temperature, T_A.*

Conclusions

Denaturation temperature plays an important role in PCR optimization as it maximizes the yield of the target sequence and minimizes the presence of non-specific binding. The 2D-gradient feature in Mastercycler X50s reduces the overall runtime as a range of denaturing-annealing temperature combinations can be tested in a single run, vastly simplifying the process of establishing a multiplex PCR system.

Literature

[1] Carlson, C. S. et al. (2013) Using synthetic templates to design an unbiased multiplex PCR assay. Nat. Commun. 4:2680 (doi: 10.1038/ncomms3680)
[2] Markoulatos, P., Siafakas, N., & Moncany, M. (2002). Multiplex polymerase chain reaction: A practical approach. Journal of Clinical Laboratory Analysis, 16(1), 47-51.
[3] Matsunaga, T., Chikuni, K., Tanabe, R., Muroya, S., Shibata, K., Yamada, J., Shinmura, Y. (1999) A quick and simple method for the identification of meat species and meat products by PCR assay. Meat Science, 143-148. (doi.org/10.1016/S0309-1740(98)00112-0)

Ordering information

Description	Order no. international	Order no. North America
Mastercycler® X50s	6311 000.010	6311000010
Eppendorf Thermomixer®	5382000015	5382000023
Eppendorf Biospectrometer® kinetic	6136000002	6136000010
Eppendorf PCR Tubes, 0.2 mL, PCR clean, 8-tube strips	0030124359	951010022

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