American Journal of

Biochemistry and Molecular Biology

ISSN 2150-4210

Academic
Journals Inc.

www.academicjournals.com

OPEN ACCESS American Journal of Biochemistry and Molecular Biology

ISSN 2150-4210
DOI: 10.3923/ajbmb.2023.1.4

Editorial
High Annealing Specificity of Mitochondrial DNA Primers Towards ±0.1°C Temperature Differences

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Citation: Parthipan, S., S.A. Mualif, M.Y.A. Aziz, A.R. Ishak, N.A. Suaidi, M.Y. Aziz and S.M. Ishar, 2023. High annealing specificity of mitochondrial DNA primers towards ±0.1°C temperature differences. Am. J. Biochem. Mol. Biol., 13: 1-4.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

Mitochondrial DNA is widely used in population studies and forensic human identification through matrilineal lineage. Therefore, the development of mitochondrial DNA typing methodologies has been an interest among researchers recently. Optimization of Polymerase Chain Reaction (PCR) forms the basic platform in DNA typing methodologies that requires optimum annealing temperature along with setting the proper concentration of ions and PCR components for high product yield. Nevertheless, usually, the concentration of ions and volume of PCR components could be followed from the manufacturer's protocol yet the determination of optimal annealing temperature for best yield is cumbersome. Many scholars suggested running gradient PCR 3-5°C below the melting temperature of primers to determine the optimum annealing temperature. In this study, we implemented the same concept but noted high specificity of mtDNA primers even between ±0.1°C differences in annealing temperature during PCR optimization which could widely influence the amplification of the target region[1].

The mtDNA target regions chosen in this study contain SNPs for the development of allele-specific PCR among the Malay population[2-4]. This stage is classified as first-round PCR and is focused on this study[5]. The selected SNPs are located in both the control and coding regions to increase the discrimination power. The primers were designed using NCBI primer blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The length of the target region was set from 250-450 bp to assist in forensic cases involving degraded samples. The parameter for primers was left in default settings except the Tm was set at 40-65°C.

For optimization purposes, only one human buccal swab sample was collected using FTA card. The 1.2 mm DNA containing FTA card was punched out and then purified using FTA purification reagent and TE buffer. The disc was left to dry completely. The DNA containing disc was added with PCR components such as ddH_2O (18.75 μL), 10x Taq buffer (2.5 μL), dNTP (0.5 μL), 10 μM forward primer (1 μL), 10 μM reverse primer (1μL) and Taq DNA polymerase (0.25 μL). The PCR amplification was carried out in T100 Thermocycler at pre-denaturation (94°C at 5 min), complete denaturation (94°C at the 30 sec), annealing (55°C at 30 sec), extension (72°C at 1 min) and final elongation (72°C at 7 min). The PCR amplification was run for 30 cycles. After completion, the products were separated through gel electrophoresis using 1.2% agarose gel and visualized under UV light.

The results of gel observation under UV light were summarized in Table 1. The pattern of annealing temperature of mtDNA primers lies between 49.5-51.3°C. The designed primers successfully amplified the target region within a narrow annealing temperature range. The current focus to

Table 1: Summary of optimum annealing temperature of mDNA primers

	Primers			Ta trial (°C)																						
Target region	Primer sequence (5' to 3')	Tm	GC (%)	49	49.4	50	49.6	49.7	49.8	49.9	50.0	50.1	50.2	50.3	50.4	50.5	50.6	50.7	50.8	50.9	51.0	51.1	51.2	51.3	Optimum Ta (°C)	Product size (bp)
16062-16355	F-ATTGACTCACCCATCAACAA	53.9	40										✓	✓✓											50.3	294
	R-GGGATTTGACTGTAATGTGC	53.7	45																							
131-465	F-TCTTTGATTCCTGCCTCATC	54.2	45																✓	✓✓					50.9	335
	R-GTGGGAGGGGAAAATAATGT	54.0	45																							
11488-11790	F-AATACGCCTCACACTCATTC	54.6	45																		✓	X	X		51.0	303
	R-AGGATTATGATGCGACTGTG	54.4	45																							
7942-8363	F-CTCCTACATACTTCCCCCAT	54.4	50																		X	X	✓✓		51.2	422
	R-CACTGTAAAGAGGTGTTGGT	54.3	45																							
4014-4452	F-CCTCACCACTACAATCTTCC	54.2	50																	X	✓	✓✓			51.1	439
	R-AACCAACATTTTCGGGGTAT	54.2	40																							
9848-10153	F-CCTCACTATCTGCTTCATCC	54.1	50																	✓✓					50.9	306
	R-TCTATGTAGCCGTTGAGTTG	54.0	45																							
3932-4197	F-CAGGCTTCAACATCGAATAC	53.6	45												✓✓	✓									50.4	266
	R-GGTGAGTGGTAGGAAGTTTT	53.9	45																							
14948-15206	F-CACATCACTCGAGACGTAAA	54.3	45																✓✓	✓					50.8	259
	R-GGATGGCGGATAGTAAGTTT	54.0	45																							

Table 1: Continue

Target region	Primers: Primer sequence (5' to 3')	Tm	GC (%)	Ta trial (°C): 49	49.4	50	49.6	49.7	49.8	49.9	50.0	50.1	50.2	50.3	50.4	50.5	50.6	50.7	50.8	50.9	51.0	51.1	51.2	51.3	Optimum Ta (°C)	Product size (bp)
9033-9382	F-ACTCATGCACCTAATTGGAA	53.6	40									✓✓		X		✓									50.1	350
	R-CGCCATCATTGGTATATGGT	54.4	45																							
903-1227	F-TCACACGATTAACCCAAGTC	54.5	45						✓	✓✓	✓	✓	✓		X	X	X								49.9	325
	R-CGGGGTTTATCGATTACAGA	53.7	45																							
4434-4732	F-TACCCCGAAAATGTTGGTTA	53.6	40	X		✓✓		X	✓																49.5	299
	R-TTGGTTATGGTTCATTGTCC	52.5	40																							
7707-8011	F-CCCTTTTCCTAACACTCACA	53.9	45									X	✓	✓✓	✓	✓									50.3	305
	R-TACTCGATTGTCAACGTCAA	53.9	40																							
8507-8947	F-AACAAACCCTGAGAACCAAA	54.3	40																		X	X	✓✓	✓	51.2	441
	R-GTATGGGGATAAGGGGTGTA	54.4	50																							
1414-1713	F-CGAAGGTGGATTTAGCAGTA	53.9	45											✓✓											50.3	300
	R-TTGTCTGGTAGTAAGGTGGA	54.1	45																							
2584-2874	F-CGTGCAAAGGTAGCATAATC	53.9	45											✓✓											50.3	291
	R-TTGACTGGTGAAGTCTTAGC	54.1	45																							
3457-3758	F-GACGCCATAAAACTCTTCAC	53.8	45											X	✓✓	✓*	X								50.4	302
	R-AGTAGAATGATGGCTAGGGT	54.0	45																							

All reactions were optimized using the gradient PCR method. The 16 target regions listed above contain SNPs that are specific to the Malay population. This first round of PCR reactions was carried out according to the manufacturer's protocol. X: No DNA band was observed during the trial, ✓: DNA band observed at low intensity, ✓✓: DNA band was observed at high intensity, ✓*: DNA band appeared on gel with the additional non-specific band and Columns left blank under trials showed no trial has been carried out at a particular temperature

determine the optimum binding of primers is according to where the mtDNA primers bind to the template without the formation of secondary structures and high PCR product yield observed from the bright intensity of the DNA band on the gel. Initially, the annealing temperature of primers has been tried 1-2°C below the melting temperature of primers as suggested by the Vazyme manufacturer. Nevertheless, the primers failed to amplify in the suggested temperature range. After a series of amplification, the annealing temperature range of primers was determined from 3-5°C below the melting temperature of mtDNA primers. Besides that, significant changes in presence of DNA bands or their intensity were observed at ±0.1°C differences in annealing temperature during PCR optimization as indicated in Table 1.

In a previous study on the optimization of first round PCR, the amplified fragments were seen in lower quantity This might be due to optimizing primer annealing temperature in integers forms only. For instance, there was the presence of a DNA band (target region 903-1227) at Ta of 50°C but not when it is adjusted to 49 or 51°C. Even though, the amplification occurred at 50°C but the low intensity of the band shows low product yield. Thus, in this study, the annealing temperature was tested between the intervals of 49.8-50.6°C. It was proven that 49.9°C is the best optimal temperature for amplification of mtDNA target region from 903-1227 since non-specific binding did not occur and the DNA band on gel appeared the brightest compared to other temperature intervals.

CONCLUSION

It is vital to take note of the slight annealing temperature differences during PCR amplification since they might greatly influence the final PCR product. The low yield of the product during first round PCR optimization may further reduce the final yield during second round PCR (allele-specific PCR) causing the DNA band undetected in gel electrophoresis. Therefore, the data presented here provide better insights to researchers in determining optimal annealing temperatures for the development of allele-specific PCR in forensic human identification.

SIGNIFICANCE STATEMENT

This study discovered the slight annealing temperature differences possible influence the result of the final PCR products which could be beneficial in a crucial investigation. This study will help the researchers to uncover the critical areas of mtDNA analysis that many researchers were not able to explore. Thus, a new theory on mtDNA annealing temperature may be arrived at.

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