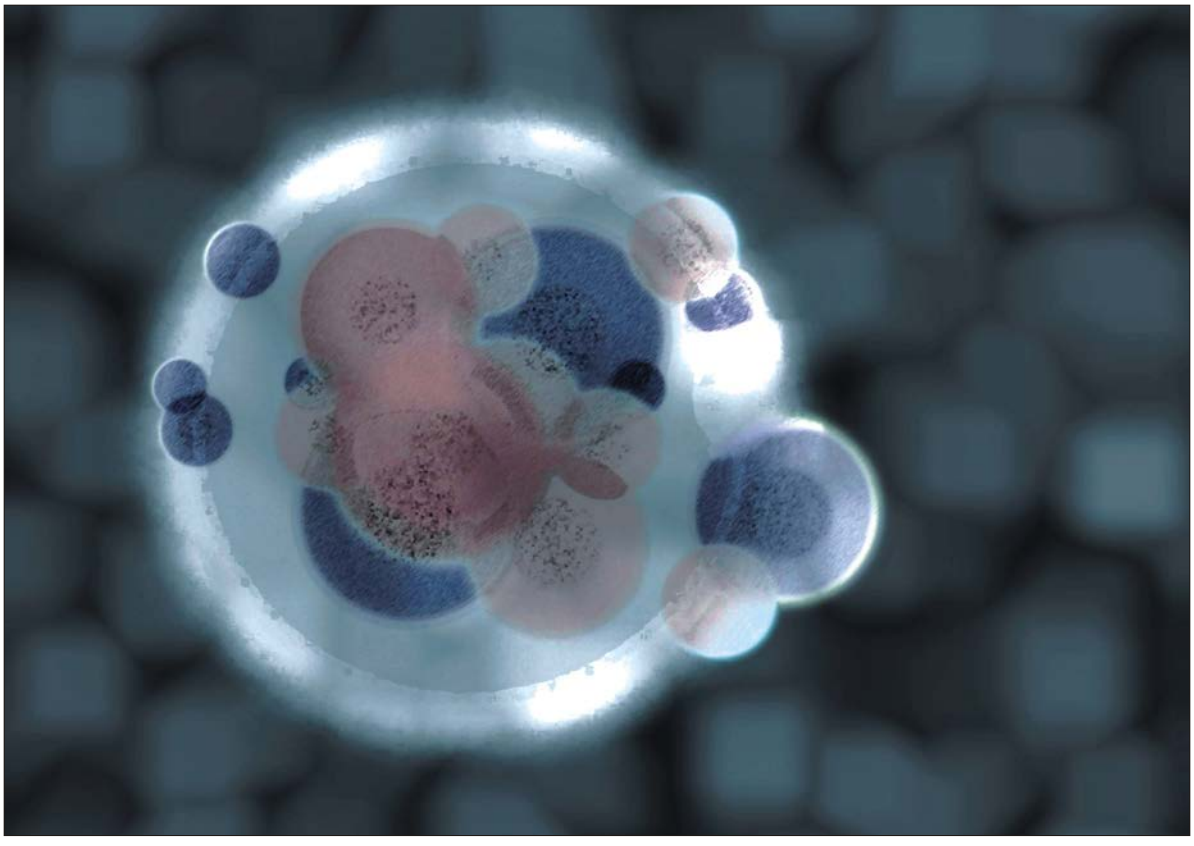

Journal of Biological Sciences

ISSN 1727-3048

science alert

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OPEN ACCESS Journal of Biological Sciences

ISSN 1727-3048
DOI: 10.3923/jbs.2017.410.415

Short Communication
Development of Internal PCR Control (IPC) for Human Mitochondrial DNA Typing Kit

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Abstract

Background and Objective: Amplification of target sequences has become a necessary technique in molecular research and has been used for various applications such as forensic investigation, genetic tracking, disease diagnosis and much more. The objective of this study was to develop an internal PCR control (IPC) for mitochondrial DNA (mtDNA) typing using allele specific PCR (asPCR) method. **Materials and Methods:** A total of 5 non-competitive IPC with 300 bp in length were synthesized by incorporating the mtDNA target sequence in the fragment. Both target sequences and IPCs shared similar forward primers. However, reverse primers were designed for each IPC in order to obtain the desired fragment length. Each developed IPC consist of 3 to 6 selected mt DNA SNPs that was used as control to determine the presence of variant in the target sequence, without the need of DNA sequencing. A total of 20 mtDNA SNPs from both coding and control regions were selected for asPCR (16 148, 3552, 16 355, 195, 1872, 1709, 16 108, 16 335, 16 274, 8440, 13 626, 16 291, 9080, 3705, 4491, 146, 1719, 16 093, 3027 and 7684). **Results:** The IPCs for all selected mtDNA SNPs in asPCR were successfully developed and amplified. The successful amplified IPCs were observed in either wild type lane or variant type lane. **Conclusion:** Development and incorporation of IPC is important and necessary as these fragments act as control to monitor the performance and amplification results of the asPCR.

Key words: Internal PCR control, mitochondrial DNA SNPs, allele specific PCR, synthetic DNA, mtDNA typing

Citation: Ishar Seri Mirianti, Abdullah NurAzeelah and Zainuddin Zafarina, 2017. Development of internal PCR control (IPC) for human mitochondrial DNA typing kit. J. Biol. Sci., 17: 410-415.

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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.

INTRODUCTION

Polymerase Chain Reaction (PCR) has become one of the most powerful molecular biological tools in the last few decades. It is used for genetic analyses, diagnosis of infectious or hereditary diseases in a huge variety of sample types[1]. In perfect condition, the target sequences will be replicated to a large copy number. However, false-positive or false-negative results may occur during PCR process. Generally, false positives results are mainly due to cross contamination from either positive samples or reaction products[2] while false-negative amplification may be a result of various factors such as inadequate amount of sample or complete inhibition of the amplification process, even in the presence of target sequence[3]. To avoid misinterpretation of the PCR results, an internal control must be included in the PCR reactions[4]. The existence of a control in PCR reactions is necessary to exclude any ambiguities[5].

The function of the incorporated controls depends on the type of the controls themselves[6]. There are several types of controls that can be distinguished according to the controlled steps (process control and amplification control) and the implementation of the control reaction (internal or external control). A process control is added during preparation steps for example before nucleic acid extraction while an amplification control is incorporated to the nucleic acid extracted from the sample and thus controlling only the performance of the PCR itself. An internal control should be analyzed in the same tube as the target, whereas an external control is included in a separate aliquot of the sample[7].

In this project, a total of 20 mtDNA SNPs (coding and control regions) were selected for allele specific PCR (asPCR) with incorporated internal control. The asPCR is used to determine the variant of the target polymorphisms, without the need of DNA sequencing. The IPC was developed specially to monitor the asPCR process and to provide assurance of the amplification results. The Malay population was use as target sample in the validation process as the Malays represent major population in Malaysia.

MATERIALS AND METHODS

Sample extraction: A total of 30 buccal swab samples were collected from Malay individuals with informed consent in March, 2016. This study has been approved by Human Research Ethics Committee of Universiti Sains Malaysia. The extracted DNA was used to validate the IPC developed for 20 mtDNA SNPs selected from both coding and control regions. DNA was extracted using commercial extraction DNA kit (Qiagen, Germany).

First round PCR reactions: In this project, PCR were carried out in two rounds and the IPC was required in the second round, PCR (asPCR). For first round PCR, the 9947A DNA that is commercially available was used as an external control. The primers were designed to amplify fragments between 100-150 bp that contain the targeted SNPs. The optimized PCR program has been listed in Table 1-2.

The primers were designed to amplify short DNA fragments since the final aim of this project is to assist in forensic investigation that normally deals with limited quality and quantity of DNA samples.

Purification of amplified products: The amplified products from first round PCR were purified using commercial DNA purification kit (Qiagen, Germany). Prior to as PCR, the amplified products were quantitated using Nanodrop. A total of 1 ng μL^{-1} of amplified product was used in the as PCR.

Second round PCR (as PCR): The second round PCR was carried out to amplify the targeted SNPs for identification of variants and haplotyping. Two types of IPC labeled as wild type (wtIPC) and variant type (vtIPC) were designed and incorporated into each corresponding reaction. The primers for IPC (Bioneer, Korea) were designed to amplify fragments between 250-300 bp in length. A total of 5 IPC fragments were synthesized of which each synthetic DNA fragment consist of 3 to 6 selected mtDNA SNPs sequences. The amplified

Table 1: PCR components and volume for the first round PCR

PCR components	Volume (µL)
10X PCR Buffer (Qiagen, Germany)	2.5
PCR Nucleotide Mix (10 mM each dATP, dCTP, dGTP, dTTP) (Qiagen, Germany)	0.5
$MgCl_2$ (Qiagen, Germany)	1
Taq DNA Polymerase (5 U μL^{-1}) (Qiagen, Germany)	0.25
ddH_2O	19.25
Forward Primer (10 uM) (Bioneer, Korea)	0.5
Reverse Primer (10 uM) (Bioneer, Korea)	0.5
DNA template	0.5
Total	25

Table 2: Optimized parameters for first round PCR

PCR cycling	Cycles	Temperature (°C)	Duration
Initial denaturation	1	95	3 min
Denaturation	25	95	30 sec
Annealing	25	45-60	30 sec
Extension	25	72	30 sec
Final extension	1	72	5
Hold	1	4	∞

Table 3: PCR components and volume used in second round PCR

PCR component	Volume (µL)
Buffer (with $MgCl_2$) (Qiagen, Germany)	5
Taq polymerase (Qiagen, Germany)	0.125
dNTPs (Qiagen, Germany)	0.5
Wild type primer (Bioneer, Korea)	0.5
Variant type primer (Bioneer, Korea)	0.5
Common reverse primer (Bioneer, Korea)	0.5
Reverse primer for IPC (Bioneer, Korea)	0.5
Template DNA	0.5
IPC (Bioneer, Korea)	0.5
ddH_2O	20.875
Total	25

Table 4: PCR conditions for second round PCR

PCR Cycling	Cycles	Temperature (°C)	Duration
Initial denaturation	1	95	3 min
Denaturation	25	95	30 sec
Annealing	25	50-60	30 sec
Extension	25	72	30 sec
Final extension	1	72	5
Hold	1	4	∞

fragments from first round PCR and the synthetic DNA for IPC shared the same forward primers that specifically designed for allele specific regions. The reverse primers were designed only specific for IPC. The PCR optimization was carried out in order to obtain the best condition for asPCR amplification. The second round PCR reaction mix and program are listed in Table 3 and 4, respectively.

Gel electrophoresis and visualization of amplified products: Electrophoresis of the amplified products was carried out on 1% agarose gel and visualized using gel documentation system.

RESULTS

Two types of IPC (wtIPC and vtIPC) were successfully developed and fully functional in the asPCR. Each IPC comprise at least 3 selected mtDNA SNPs. The target sequence was incorporated into the IPC fragment that was synthesized using available mtDNA sequence in Mitomap Each IPC (synthetic DNA) consist of the target sequences that are highlighted in Table 5. The reverse primers for each IPC were developed separately to produce amplified products around 250-300 bp. The wild type IPC (wtIPC), variant type IPC (vtIPC) and their corresponding SNPs were listed in Table 5. The sequence of SNPs in the IPC were highlighted.

According to the results as shown in Fig. 1(a-c), the wtIPC and vtIPC were observed to appear as 300 bp bands in the agarose gel electrophoresis. For this representative sample, no variant was recorded as only the IPC band appeared in the variant type lane. Both IPC and the target sequence fragments were observed in the wild type lane.

DISCUSSION

The IPC developed in this project was an application of non-competitive internal control as the sequence for IPC was totally different from the template DNA, except for the complementary sequences for forward primers[8]. The target sequences were incorporated in this IPC and it was recommended to have a larger size product than the target sequence to ensure the competitive edge of the latter and would not influence the native PCR sensitivity[9]. Hence, the length of the IPC was designed to be around 250-300 bp.

Incorporating of IPC in PCR however may cause competition between IPC and target DNA, as well as inefficient amplification of one or both fragments[10]. To ensure optimum amplification of target DNA, the IPC reaction was set according to the conditions of the target DNA reaction. Several rounds of optimization were conducted to overcome this issue, such as increasing the PCR components and optimization of annealing temperature. The optimization was mostly focused on the target DNA rather than the IPC.

One special kind of non-competitive control was due to the endogenetic control that uses the housekeeping genes, directly originate from the sample. The endogenetic control may act as process controls, which control the whole sample processing procedure[11]. Unfortunately, an extra concentration of endogenetic nucleic acids may interfere the amplification of the target sequence[5]. The method has been used in previous studies such as microbes and human plasmodium detection[12], diagnosis of herpes simplex virus[13], diagnostic control in *Cryptosporidium* PCR[14] and also in molecular study of cholerae[15]. Application of multiplex PCR with incorporation of IPC has been used in some of the studies.

After few series of optimization, the IPC was successfully co-amplified in one PCR tube. Competition between IPC and the target sequence can be seen from the brightness of the IPC band. In the PCR reaction where no amplification of the target sequence occurred, the IPC band appeared brighter as compared to the reaction with co-amplification of the target sequence.

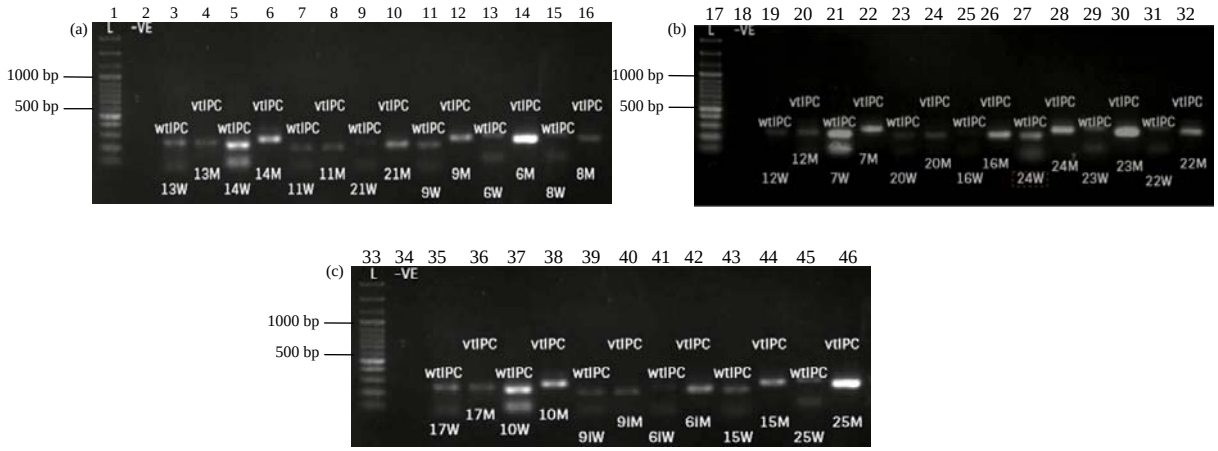

Fig. 1(a-c): Gel electrophoresis of asPCR for wild type and variant type of mtDNA SNPs

Lane	Sample	Description	Size (bp)
1	L	100 bp ladder	-
2	-VE	Negative control	-
3	wtIPC	Wild type IPC for SNP 16148	288
	13W	Wild SNP 16148	105
4	vtIPC	Variant type IPC for SNP 16148	288
	13M	Variant SNP 16148	-
5	wtIPC	Wild type for SNP 3552	257
	14W	Wild SNP 3552	110
6	vtIPC	Variant type IPC for SNP 3552	257
	14M	Variant SNP 3552	-
7	wtIPC	Wild type IPC for SNP 16355	225
	11W	Wild SNP 16355	115
8	vtIPC	Variant type IPC for SNP 16355	225
	11M	Variant SNP 16355	-
9	wtIPC	Wilt type IPC for 1872	245
	21W	Wild SNP 1872	77
10	vtIPC	Variant type IPC for SNP 1872	245
	21M	Variant SNP 1872	-
11	wtIPC	Wild type IPC for SNP 1709	203
	9W	Wild SNP 1709	89
12	vtIPC	Variant type IPC for SNP 1709	203
	9M	Variant SNP 1709	-
13	wtIPC	Wild type IPC for SNP 16093	238
	6W	Wild SNP 16093	130
14	vtIPC	Variant type IPC for SNP 16093	238
	6M	Variant SNP 16093	-
15	wtIPC	Wild type IPC for SNP 16335	243
	8W	Wild SNP 16335	82
16	vtIPC	Variant type IPC for SNP 16335	243
	8M	Variant SNP 16335	-
17	L	100 bp ladder	-
18	-VE	Negative control	-
19	wtIPC	Wild type IPC for SNP 195	278
	12W	Wild SNP 195	-
20	vtIPC	Variant type IPC for SNP 195	278
	12M	Variant SNP 195	107
21	wtIPC	Wild type IPC for SNP16274	193
	7W	Wild SNP 16274	98
22	vtIPC	Variant type IPC for SNP 16274	193
	7M	Variant SNP 16274	-
23	wtIPC	Wild type IPC for SNP 8440	265
	20W	Wild SNP 8440	108
24	vtIPC	Variant type IPC for SNP 8440	265
	20M	Variant SNP 8440	-

Lane	Sample	Description	Size (bp)
25	wtIPC	Wild type IPC for SNP 13626	241
	16W	Wild SNP 13626	62
26	vtIPC	Variant type IPC for SNP 13626	241
	16M	Variant SNP 13626	-
27	wtIPC	Wil type IPC for SNP 16291	219
	24W	Wild SNP 16291	147
28	vtIPC	Variant type IPC for SNP 16291	219
	24M	Variant SNP 16291	-
29	wtIPC	Wild type IPC for SNP 9080	198
	23W	Wild SNP 9080	83
30	vtIPC	Variant type IPC for SNP 9080	198
	23M	Variant SNP 9080	-
31	wtIPC	Wild type IPC for SNP 3705	176
	22W	Wild SNP 3705	96
32	vtIPC	Variant type IPC for SNP 3705	176
	22M	Variant SNP 3705	-
33	L	100 bp ladder	-
34	-VE	Negative control	-
35	wtIPC	Wild type IPC for SNP 4491	308
	17W	Wild SNP 4491	121
36	vtIPC	Variant type IPC for SNP 4491	308
	17M	Variant SNP 4491	-
37	wtIPC	Wild type IPC for SNP 146	285
	10W	Wild SNP 146	58
38	vtIPC	Variant type IPC for SNP 146	285
	10M	Variant SNP 146	-
39	wtIPC	Wild type IPC for SNP 1719	259
	9IW	Wild SNP 1719	55
40	vtIPC	Variant type IPC for SNP 1719	259
	9IM	Variant SNP 1719	-
41	wtIPC	Wild type IPC for SNP 16108	276
	6IW	Wild SNP 16108	145
42	vtIPC	Variant type IPC for SNP 16108	276
	6IM	Variant SNP 16108	-
43	wtIPC	Wild type IPC for SNP 3027	219
	15W	Wild SNP 3027	74
44	vtIPC	Variant type IPC for SNP 3027	219
	15M	Variant SNP 3027	-
45	wtIPC	Wild type IPC for SNP 7684	198
	25W	Wild SNP 7684	51
46	vtIPC	Variant type IPC 7684	198
	25M	Variant SNP 7684	-

Table 5: List of wild type IPC (wtIPC), variant type IPC (vtIPC) and selected mtDNA SNPs

Wild type IPC (wtIPC)	Variant type IPC (vtIPC)	SNP
5'- AGT AG**C GGT ACC ATA AAT ACT TGA AC**A GTG CTT AGT **GAC CTT AGC TCT CAC CAT CGT T**TG AAC AGG GCC ATA GCA CAT TAC AGT CAA ATC ACC CTG AAG CGC GTA CAC ACC GCC CGT CAC CCT CCT CC**T TAC ACT ATT CCT CAT CAC CCT A**AA GTA TAC TTC AAA GGA CAT CTC GAA TAA TTC TTC TCA ACT TAA CTA AAA CCC CTA CGC ATT TAT ATA GAG GAA AGT GCA ACA AAC CTA CCC ACC CTT ATC CTT GGA CGA ACA ACC TTA GCC AAA CCA TTT CCT AGC AAT ATC AAC CAT TTA ACC CAA ATA A **-3'**	**5'**- AGT AG**C GGT ACC ATA AAT ACT TGA AT**A GTG CTT AGT **GAC CTT AGC TCT CAC CAT CGT A**TG AAC AGG GCC ATA GCA CAT TAC AGT CAA ATC ATC CTG AAG CGC GTA CAC ACC GCC CGT CAC CCT CCT **CCT TAC ACT ATT CCT CAT CAC CCT G**AA GTA TAC TTC AAA GGA CAT CTC GAA TAA TTC TTC TCA ATT TAA CTA AAA CCC CTA CGC ATT TAT ATA GAG GAA AGT GCA ACA AAC CTA CCC ACC CTT ATT CTT GGA CGA ACA ACC TTA GCC AAA CCA TTT CCT AGC AAT ATC AAC CAT TTG ACC CAA ATA A -3'	• 16148 • 3552 • 16355
5'- CTA GAC **TCA ATA TTA CAG GCG AAC ATA AT**C CAA AGC TGG **CTG CAT AAT GAA TTA ACT AGA ACT** TTT CAA GCC AAC AAA AGG **AAA CCC ACT CCA CCT TAC** TAC CAG TTG TCA AAG TTA AAT GAT TGT TTG GGC TAC TGC TCT CTA ATG GCA CAT GCA GCG CAC AAA GAT GGT AGA GTA GAT GCC CCC CTA TCA TAG AAG AGC TTA TCA CCT TTA TCT GTC TTT GAT TCC TGC ATA TTT TCC TTA TCT GCT TCC TAG TCC TGT ATG CAC CTT ACT ACC AGA CAA CCT TCG AAC TAA CTA A **-3**	**5'**- CTA GAC **TCA ATA TTA CAG GCG AAC ATA AC**C CAA AGC TGG **CTG CAT AAT GAA TTA ACT AGA ACC** TTT CAA GCC AAC AAA AGG **AAA CCC ACT CCA CCT TAC TAC CAA** TTG TCA AAG TTA AAT GAT TGT TTG GGC TAC TGC TCT TTA ATG GCA CAT GCA GCG CAC AAA GAT GGT AGA GTA GAT GCT CCC CTA TCA TAG AAG AGC TTA TCA CCT TTA TCT GTC TTT GAT TCC TGC ACA TTT TCC TTA TCT GCT TCC TAG TCC TGT ATG CAC CTT ACT ACC AGA CAA CCT TCA AAC TAA CTA A **-3'**	• 195 • 1872 • 1709
5'- TGT ATG T**CA ATA TTC ATG GTG GCG G**GT CTT ACT CTT TTA G**GG GAT TTG ACT GTA ATG TGA T**CC AAT TAA CTA GTT TTG ACA ACA TTC AAA **TGG GTA GGT TTG TTG GTA GC**T TAA TTT TAA TAA TCA ACA CCC TCC TAG CCT TAC TAC TAA CTG GCA GTA ATG TAC GGA TAC CAC AAC TCA ACG GCT ACA TGT TGA ACA AAC GAA CCT TGA AGT GCG GCT TCG ACC CTA TAT CCC CCG CCC GCG TCC CTT TCT CCA TAA AAT TCT TCT TAG ATA CAG GAC TAG GAA GCA GAG ATG ATC TAG **-3'**	**5'-** TGT ATG T**CA ATA TTC ATG GTG GCG A**GT CTT ACT CTT TTA G**GG GAT TTG ACT GTA ATG TGA C**CC AAT TAA CTA GTT TTG ACA ACA TTC AAA **TGG GTA GGT TTG TTG GTA GT**T TAA TTT TAA TAA TCA ACA CCC TCC TAG CCT TAC TAC TAA CTG GCA GTA ATG TAC GGT TAC CAC AAC TCA ACG GCT ACA TGT TGA ACA AAC GAA CCT TGG AGT GCG GCT TCG ACC CTA TAT CCC CCG CCC GCG TCC CTT TCT CCA TAA AAT TCT TCT TAG ATA CAG GAC TAG GAA GCA GAG GTG ATC TAG **-3'**	• 16108 • 16335 • 16274
5'- GGT AG**T TAC ACT ATT CCT CAT CAC CCT A**G**C TCG AAT AAT TCT TCT CAC C**T**A CAA ACC TAC CCA CCC TTA TC**A **CCT AGC AAT ATC AAC CAT TTA** T**GA TTG TTT GGG CTA CTG CTC TC**C TTA GTC CAA AGA GGA ACA GCT CTT TGG ACA CTA GGA AAA AAC CTT GTA GAG AGA GTA AAA AAT TTA ACA CCC ATA GTA GGC CTA AAA GCA GCC ACC AAT TAA GAA AGC GTT CAA GCT CAA CAC CCA CTA CCT AAA AAA TCC CAA ACA TAT AAC TGA ACT CCT CAC ACC CAA TTG GAC CAA TCT ATC **-3'**	**5'-** GGT AG**T TAC ACT ATT CCT CAT CAC CCT G**G**C TCG AAT AAT TCT TCT CAC T**T**A CAA ACC TAC CCA CCC TTA TTA CCT AGC AAT ATC AAC CAT TTG** T**GA TTG TTT GGG CTA CTG CTC TTC TTA GTC CAA AGA GGA ACA** GCT CTT TGG ACA CTA GGA AAA AAC CTT GTA GAG AGA GTA AAA AAT TTA ACA CCC ATA GTA GGC CTA AAA GCA GCC ACC AAT TAA GAA AGC GTT CAA GCT CAA CAC CCA CTA CCT AAA AAA TCC CAA ACA TAT AAC TGA ACT CCT CAC ACC CAA TTG GAC CAA TCT ATC **-3'**	• 8440 • 13626 • 16291 • 9080 • 3705
5'- TAG CA**C AAA GAT GGT AGA GTA GAT GCC C**T**A TCT GTC TTT GAT TCC TGC AT**T **ACC TTA CTA CCA GAC AAC CTT CG**C **CTG GCA GTA ATG TAC GGA** G**GT TGA ACA AAC GAA CCT TGA** T**AT ACA GGA CTA GGA AGC AGA GA**C GCA CCC ACC CTT AAT TCC ATC CAC CCT CCT CTC CCT AGG AGG CCT GCC CCC GCT AAC CGG CTT TTT GCC CAA ATG GGC CAT TAT CGA AGA ATT CAC AAA AAA CAA TAG CCT CAT CAT CCC CAC CAT CAT AGC CAC CAT CAC CCT CCT TAA CCT CTA CTT CTA CCT ACG CCT AAT CTA CTC CAC CTC AAT CAC ACT ACT CCC CAT A **-3'**	**5'-** TAG CAC **AAA GAT GGT AGA GTA GAT GCT C**T**A TCT GTC TTT GAT TCC TGC AC**T **ACC TTA CTA CCA GAC AAC CTT CAC CTG GCA GTA ATG TAC GGT** G**GT TGA ACA AAC GAA CCT TGG TAT ACA GGA CTA GGA AGC AGA GG**C GCA CCC ACC CTT AAT TCC ATC CAC CCT CCT CTC CCT AGG AGG CCT GCC CCC GCT AAC CGG CTT TTT GCC CAA ATG GGC CAT TAT CGA AGA ATT CAC AAA AAA CAA TAG CCT CAT CAT CCC CAC CAT CAT AGC CAC CAT CAC CCT CCT TAA CCT CTA CTT CTA CCT ACG CCT AAT CTA CTC CAC CTC AAT CAC ACT ACT CCC CAT A **-3'**	• 4491 • 146 • 1719 • 16093 • 3027 • 7684

*Bold letter represent sequence for each selected mtDNA SNPs

CONCLUSION

Incorporation of IPC in PCR has been important and compulsory under certain conditions as it may exclude especially the false-negative results. The optimization of IPC concentration was the most critical part, as too much IPC will compete for the genomic DNA template and thus giving wrong results. The concentration of IPC-primers must also be minimal in order to limit the competition for oligonucleotides and DNA polymerase. In general, any IPC shall not lower the sensitivity and efficiency of the target DNA amplification. Successful application of the mtDNA typing kit developed in this study may help human identification in mass disaster, which is lower in cost and does not require the use of high-end technology.

SIGNIFICANCE STATEMENT

This study discovers the possibility of designation of synthetic DNA as internal PCR control and incorporation of internal PCR control in allele specific PCR that can be beneficial for validation of PCR results and to avoid such as negative results in PCR reactions. This study will help the researcher to uncover the critical areas of development of internal PCR control that many researchers were not able to explore. Thus a new theory on possibility of internal PCR control designation through modification of target sequence maybe arrived at.

ACKNOWLEDGMENT

This study was supported by USM Short Term Grant No: 304/PPSK/61313150.

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