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Multiplex PCR Construction Assay for Short Tandem Repeats (STRs) Loci Separated with Agarose and Polyacrylamide Gels

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ABSTRACT

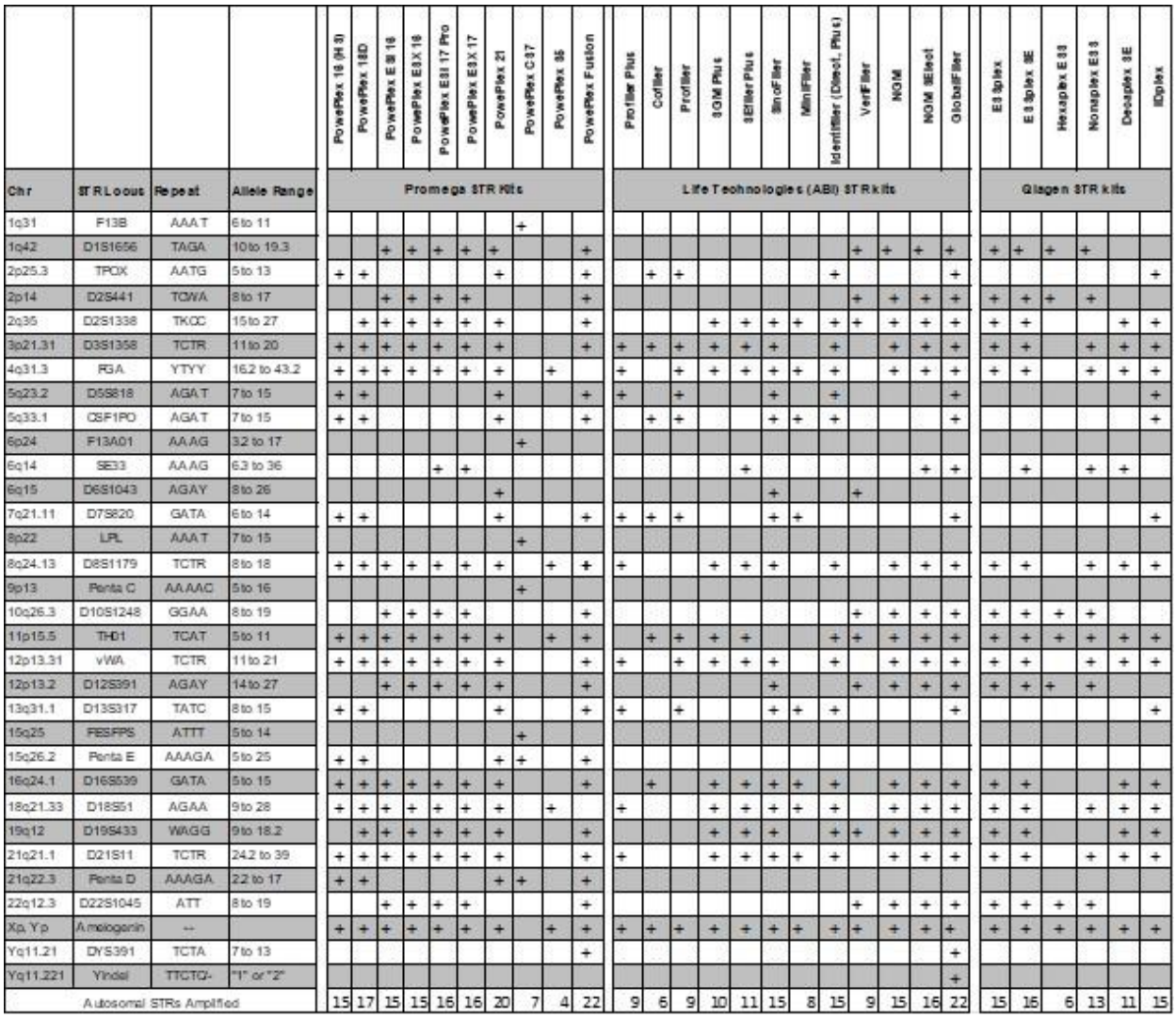
Multiplex polymerase chain reaction (PCR) is an important tool of modern molecular biology because it offers several important advantages over amplifications involving one DNA marker at a time (singleplexes). The use of multiplex PCR requires careful design of the primers for the loci of interest and rigorous optimization of cycling conditions to obtain results. The aim of this study was to design, construct and optimize multiplex PCR assays for the 10 STR loci using simple tools. DNA was extracted from 5 human blood samples obtained from Nigerian individuals. Primer sequences were obtained from literature. Ten (10) loci were combined into multiplex assays and tested by PCR followed by polyacrylamide gel electrophoresis and silver staining. The ten loci were successfully combined into four multiplexes M01 (Amelogenin and D10S1248), M02 (D6S1017 and D18S51), M03 (D22S1045, D21S11, and D2S1338), and M04 (D9S2157, FGA and D8S1179). A 20bp DNA ladder was used as DNA size marker while the gels were scored using the Gel Analyzer software. Generally, loci amplification improved with DNA concentration above 2ng, $MgCl_2$ concentration between 1.5- 4.5mM, and 30-35 PCR cycles with final extension times between 10-30 minutes.

Keywords: *Multiplex, Amplification, Combinations, Loci, Primers*

INTRODUCTION

The simultaneous amplification of two or more regions of DNA in what is commonly referred to as multiplex PCR has become an important facet of modern molecular biology because it offers several important advantages over amplifications involving one DNA marker at a time or "singleplexes (Amazan *et al.,* 2023). The markers commonly used in human forensic and identification purposes are usually genotyped using multiplex assays, and many commercial multiplex assay kits exist for different combinations of short tandem repeats (STRs). Several commercially available multiplex forensic STR kits have been developed which allow the synthesis of the desired PCR products with high specificity e.g. (a) AmFlSTR® ProfilerPlus® (b)AmFlSTR® Cofiler® (c) AmFlSTR® Identifier® (all three from Applied Biosystems [Foster City, CA] and (d) PowerPlex® 16 Systems (manufactured by Promega [Madison, WI]) (Butler, 2015). The kits are however limited to only STR loci that are included in them and research into loci not included in them can present PCR genotyping difficulties not encountered in the commercial set (*Figure 1*). Additionally, typing forensic DNA multiplexes often require the use of capillary electrophoresis instrument which is rarely available in many laboratories and localities (Butler, 2015).

When primers are designed with 24- base and 50% GC content, all reactions will have similar melting temperature (T_m) and thus all reactions can be performed under identical amplification conditions (Butler, 2015; Xie et al., 2022). These primers need to be compatible with one another in order for the multiplex to work well (Basu, 2022). Multiplex PCR has become an important facet of modern molecular biology because it offers several important advantages over amplifications involving one DNA marker at a time or "singleplexes". First, the cost of analysis and labor to obtain a set of results from multiple markers can be reduced. Additionally, the amount of information obtained per unit time improves and the amount of template DNA required to obtain results is reduced (Cupples Connon, 2023). These have led to various attempts to improve STR amplification speed, sensitivity and throughput at low cost (Giese *et al.,* 2009; (Butler, 2004; Cornelis et al., 2018; Shrivastava et al., 2021).



				Promega STR Kits										Life Technologies (ABI) STR kits											Qiagen STR kits					
				PowerPlex 16 (HS)	PowerPlex 16D	PowerPlex ESI 16	PowerPlex ESX 16	PowerPlex ESI 17 Pro	PowerPlex ESX 17	PowerPlex 21	PowerPlex C37	PowerPlex 35	PowerPlex Fusion	Profiler Plus	Cofiler	Profiler	SGM Plus	SEfiler Plus	SinoFiler	MiniFiler	Identifiler (Direct. Plus)	VenFiler	NGM	NGM SElect	GlobalFiler	ESIplex	ESSplex SE	Hexaplex ESS	Nonaplex ESS	Decaplex SE	IDplex
Chr	STR Locus	Repeat	Allele Range																											
1q31	F13B	AAAT	6 to 11								+																			
1q42	D1S1656	TAGA	10 to 19.3			+	+	+	+	+			+									+	+	+	+	+	+	+		
2p25.3	TPOX	AATG	5 to 13	+	+					+			+		+	+					+				+					+
2p14	D2S441	TCWA	8 to 17			+	+	+	+				+									+	+	+	+	+	+	+		
2q35	D2S1338	TKCC	15 to 27		+	+	+	+	+	+			+				+	+	+	+	+	+	+	+	+	+	+			+	+
3p21.31	D3S1358	TCTR	11 to 20	+	+	+	+	+	+	+			+	+	+	+	+	+	+		+		+	+	+	+	+		+	+	+
4q31.3	FGA	YTYY	16.2 to 43.2	+	+	+	+	+	+	+		+		+		+	+	+	+	+	+		+	+	+	+	+		+	+	+
5q23.2	D5S818	AGAT	7 to 15	+	+					+			+	+		+			+		+					+					+
5q33.1	CSF1PO	AGAT	7 to 15	+	+					+			+		+	+			+	+	+					+					+
6p24	F13A01	AAAG	3.2 to 17								+																				
6q14	SE33	AAAG	6.3 to 36					+	+									+						+	+		+	+		
6q15	D6S1043	AGAY	8 to 26							+									+			+									
7q21.11	D7S820	GATA	6 to 14	+	+					+			+	+	+	+			+	+						+					+
8p22	LPL	AAAT	7 to 15								+																				
8q24.13	D8S1179	TCTR	8 to 18	+	+	+	+	+	+	+		+	+	+			+	+	+		+		+	+	+	+	+		+	+	+
9p13	Penta C	AAAAC	5 to 16								+																				
10q26.3	D10S1248	GGAA	8 to 19			+	+	+	+				+									+	+	+	+	+	+	+		
11p15.5	TH01	TCAT	5 to 11	+	+	+	+	+	+	+		+	+		+	+	+	+			+	+	+	+	+	+	+	+	+	+
12p13.31	vWA	TCTR	11 to 21	+	+	+	+	+	+	+			+	+		+	+	+	+		+		+	+	+	+	+		+	+	+
12p13.2	D12S391	AGAY	14 to 27			+	+	+	+	+			+						+			+	+	+	+	+	+	+		
13q31.1	D13S317	TATC	8 to 15	+	+					+			+	+		+			+	+	+					+					+
15q25	FESFPS	ATTT	5 to 14								+																				
15q26.2	Penta E	AAAGA	5 to 25	+	+					+	+		+																		
16q24.1	D16S539	GATA	5 to 15	+	+	+	+	+	+	+			+		+		+	+	+	+	+		+	+	+	+	+			+	+
18q21.33	D18S51	AGAA	9 to 28	+	+	+	+	+	+	+		+		+			+	+	+	+	+		+	+	+	+	+		+	+	+
19q12	D19S433	WAGG	9 to 18.2		+	+	+	+	+	+			+				+	+	+		+	+	+	+	+	+	+			+	+
21q21.1	D21S11	TCTR	24.2 to 39	+	+	+	+	+	+	+			+	+			+	+	+	+	+		+	+	+	+	+		+	+	+
21q22.3	Penta D	AAAGA	2.2 to 17	+	+					+	+		+																		
22q12.3	D22S1045	ATT	8 to 19			+	+	+	+				+									+	+	+	+	+	+	+		
Xp, Yp	Amelogenin	--		+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Yq11.21	DYS391	TCTA	7 to 13										+												+						
Yq11.221	Yindel	TTCTC/-	"1" or "2"																						+						
Autosomal STRs Amplified				15	17	15	15	16	16	20	7	4	22	9	6	9	10	11	15	8	15	9	15	16	22	15	16	6	13	11	15

Figure 1: Thirty-one (31) commercially available autosomal STR kits. Plus sign (+) indicates the loci is included the kit (Adapted from Butler, 2015).

Multiplexed reactions are carefully balanced by optimizing the reaction conditions and primer sequences so that one locus with its respective primer set does not preferentially amplify over the others (Amazan, 2023; Basu, 2022; Xie et al., 2022; Yuan, 2021). Multiplex PCR reactions also require rigorous optimization of all reaction mix components and cycling parameters (Saiki, 1989; Krüger & Schleinitz, 2017; Tariq & Tahir, 2023; Zhang et al., 2013). In multiplexes, apart from PCR conditions, amplification product size determines the groups of loci and their alleles that can be co-amplified or resolved by sequencing gel separation. (Akpan et al., 2018) demonstrated the ability of 4% agarose gels to successfully discriminate between small-sized products of amplified mini -STR.

The forensic DNA loci D21S11, FGA, D18S51, D2S1338, D10S1248, D22S1045, D9S2157, D6S1017, D8S1179, D9S1122, D14S1434, D1GATA113, D5S2500, D20S482 have been

shown to have high heterozygosity in some African populations and in the US African American population and are therefore potential useful markers for studying African populations (Hohoff et al., 2009; Butler and Hill, 2012; Agbo et al., 2017; Okolie et al; 2017; Akpan et al; 2018). However, several of these loci are not included in any commercial STR kits, while others are included across many kits. The loci listed above can only be studied by combining several STR kits or developing multiplex assays that include these markers. The need for developing multiplex assay becomes further heightened in studies involving sex-linked STR markers and miniSTRs many of which are not included in commercial kit sets though they have been shown to be highly discriminating in several populations. There is also the need to employ alternative equipment and analytical tools for typing forensic DNA loci that does not involve the demand for capillary electrophoresis instrument and proprietary software which are often difficult to access in resource limited settings.

The aim of this study was to design, construct and optimize multiplex simple PCR assays for the 15 forensic DNA loci D21S11, FGA, D18S51, D2S1338, D10S1248, D22S1045, D9S2157, D6S1017, D8S1179, D9S1122, D14S1434, D1GATA113, D5S2500, D20S482 and Amelogenin. The simple assays can be performed with the use of regular molecular biology reagents and tools and the result can be evaluated on open-sourced software.

MATERIALS AND METHODS

DNA was extracted from 5 human blood samples obtained from Nigerian individuals. Genomic DNA was extracted from white blood cells present in the blood samples, using Omega^{TM} DNA extraction kit. The quality of the DNA was assessed on 1% agarose gel run 70mV for 45 minutes. The DNA was quantified using a nanodrop and a spectrophotometer. The autosomal STR markers used in the study were selected from the lists of STRs with a high number of genotypes observed, high heterozygozity, high polymorphism information content value and good probability of identity values in African American, African and other black populations. A total of 15 STR loci (including miniSTR) and the sex-typing marker amelogenin were chosen. The loci are D21S11, FGA, D18S51, D2S1338, D10S1248, D22S1045, D9S2157, D6S1017, D8S1179, D9S1122, D14S1434, D1GATA113, D5S2500, D20S482 and Amelogenin. *(See Table 1 for List of Primers)*

Table 1: List of Primers

S/NO	LOCUS NAME	REPEAT MOTIF CATEGORY	GENBANK ACCESSION (ALLELE REPEAT #)	ALLELE RANGE	PRIMER SEQUENCE	PCR PRODUCT SIZES (bp)	REFERENCES
1.	D2S1338	TGCC/TTCC Compound	AC010136 (23)	10-13	ACTGCAGTCCAATCTGGGT ATGAAATCAACAGAGGCTTGC	277-345	Krenke *et al*., 2002
2.	FGA alpha fibrinogen 3rd intron	CTTT/TTCC Compound	M64982 (21)	12.2-51.2	GGCTGCAGGGCATAACATTA ATTCTATGACTTTGCGCTTCAGGA	308-464	Krenke *et al*., 2002
3.	D6S1017	ATCC Simple	AL035588 (10)	7-14	CCACCCGTCCATTTAGGC GTGAAAAAGTAGATATAATGGTTGGTG	81–109	Hill *et al*., (2008)
4.	D8S1179	TCTA/TCTG Compound	AF216671 (13)	6-20	ATTGCAACTTATATGTATTTTTGTATTTCATG ACCAAATTGTGTTCATGAGTATAGTTTC	203-255	Krenke *et al*., 2002
5.	D9S2157	ATA Simple	AL162417 (10)	7-19	CAAAGCGAGACTCTGTCTCAA GAAAATGCTATCCTCTTTGGTATAAAT	71-107	Hill *et al*., (2008)
6.	D10S1248	GGAA Simple	AL391869 (13)	7-19	TTAATGAATTGAACAAATGAGTGAG GCAACTCTGGTTGTATTGTCTTCAT	83–123	Hill *et al*., (2008)
7.	D18S51	AGAA Simple	AP001534	5.3-40	TTCTTGAGCCCAGAAGGTTA ATTCTACCAGCAACAACACAAATAAAC	286-373	Krenke *et al*., 2002
8.	D21S11	TCTA/TCTG Compound	AP001752 (13)	12-43.2	ATATGTGAGTCAATTCCCCAAG TGTATTAGTCAATGTTCTCCAGAGAC	155-273	Krenke *et al*., 2002
9.	D22S1045	ATT Simple	AL033314 (17)	7-20	ATTTTCCCCGATGATAGTAGTCT GCGAATGTATGATTGGCAATATTTTT	76–109	Hill *et al*., (2008)
10.	Amelogenin		M55418 M55419		ACCTCATCCTGGGCACCCTGG AGGCTTGAGGCCAACCATCAG	212 218	Sullivan *et al.,* 1993

Based on these sequences, PCR primer oligonucleotides were purchased from Inqaba Biotec, South Africa. Five samples were run with all primers in single PCR reaction in volumes of ~25ul to test sensitivity using PCR and electrophoresis. For the different loci, different concentrations of the reagents were optimized but the concentration of dNTP's was kept constant (Aslam *et al.*, 2002; Tariq & Tahir, 2023). Ten (10) of the 14 loci (including amelogenin) had consistent amplification in the singleplex testing and had product sizes ranges that allowed for combination. These were then selected for multiplex testing. They were then sorted into initial four miniplexes of two 2-primer pair and two 3-primer pair. A visual schematic of the multiplex loci was prepared to provide for visual representation of the loci and highlight areas of possible size overlap *(Figure 2).*

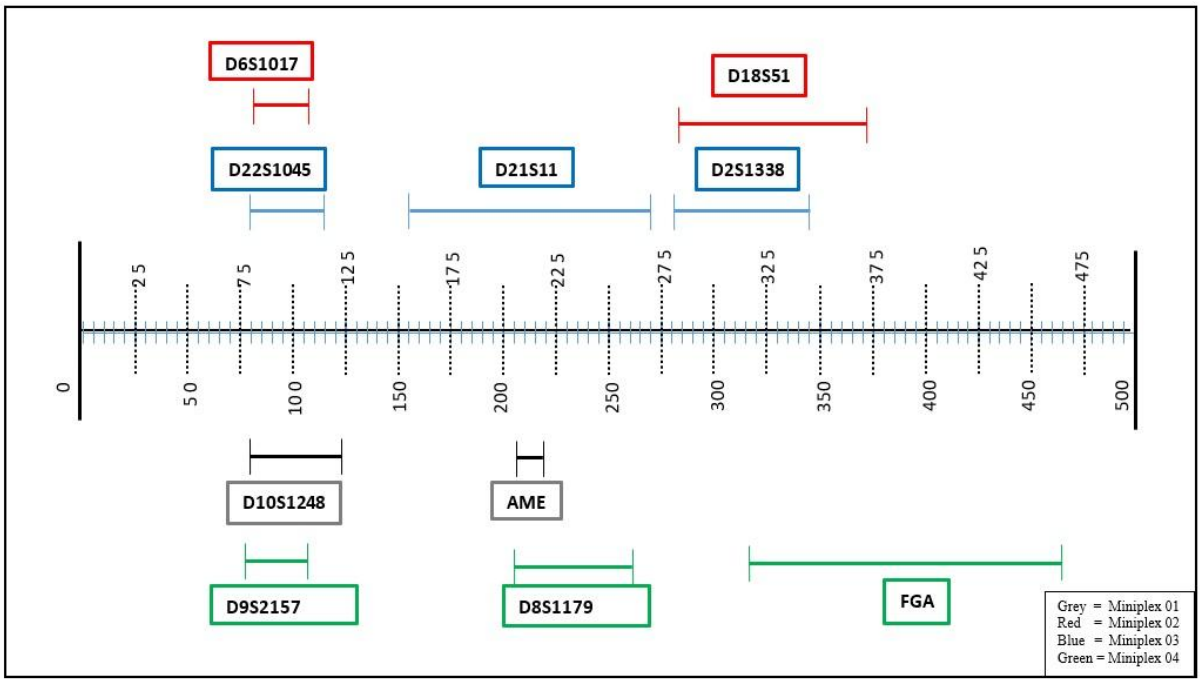


Figure 2: [Title] Schematic representation of loci in each multiplex based on PCR product size (bp).

[Figure 2 Legend] The loci are combined into groups and each group is identified here by boxes of the same colour.

Primer-primer comparison was performed to avoid excessive regions of complementary between primers using the software AutoDimer (Vallone and Butler, 2004). The miniplexes were then named Miniplex 01(Amelogenin and D10S1248), Miniplex 02 (D6S1017 and D18S51), Miniplex 03 (D22S1045, D21S11, and D2S1338), and Miniplex 04 (D9S2157, FGA and D8S1179). During the PCR, the oligonucleotides of all of the primer pairs for each

of the miniplex were combined to yield a selected final concentration. (*See Table 2 for PCR parameters*). Samples was then run with all primers in each miniplex in a single PCR reaction in volumes of ~25ul containing between 5X to 10X PCR buffer (Refaat *et al*, 2008; Chatumal *et al.*, 2010), 200 – 250 um of dNTPs (dATP, dCTP, dGT, and DTTP), STR primers (10p mole each of reverse and forward), 0.5-1 unit of Taq polymerase and a negative control. The concentration and volume of genomic DNA, $MgCl_2$ concentration, the thermocycling conditions (i.e., number of cycles and temperatures) were all varied and evaluated **(**Hammond *et al.*, 1994; Aslam *et al.*, 2002; Liu et *al.*, 2008; Chatumal *et al.*, 2010) *(See Figure 3)*

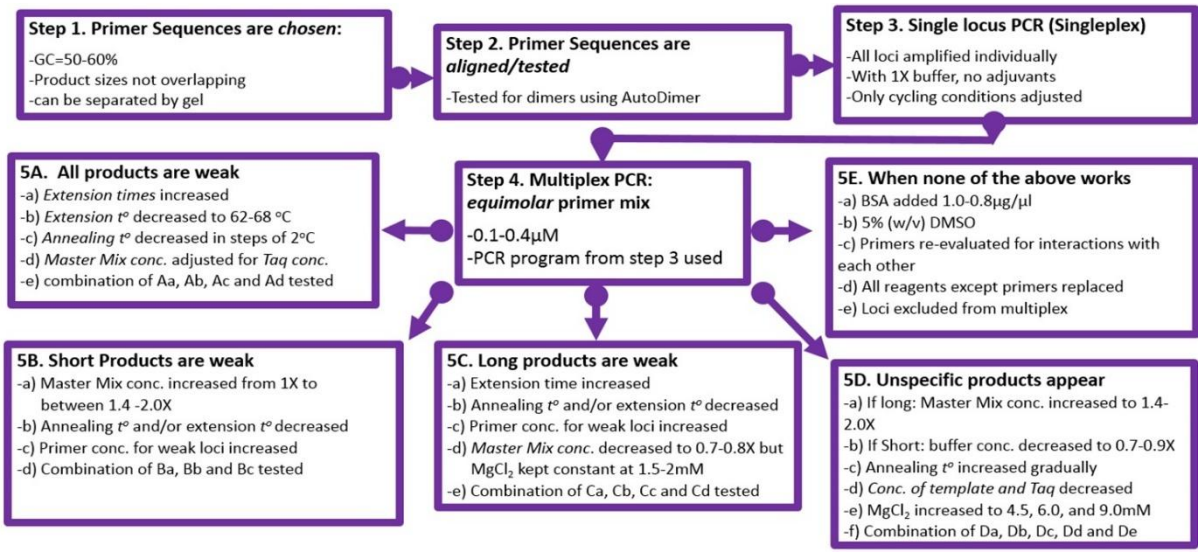


Figure 3: [Title] Steps in Evaluating PCR success for the multiplex testing

Electrophoresis of the amplified products was carried out on 4% Agarose and Polyacrylamide gel electrophoresis according to Refaat *et al.*, (2008). A twenty base-pair (20bp) DNA ladder (O'rangeRuler) was loaded as DNA size marker in the first well followed by the negative control in the 2nd well. The gels were then viewed with a UV transilluminator and images capture with a camera for gel documentation. The gels were scored using the Gel Analyzer software (2010).

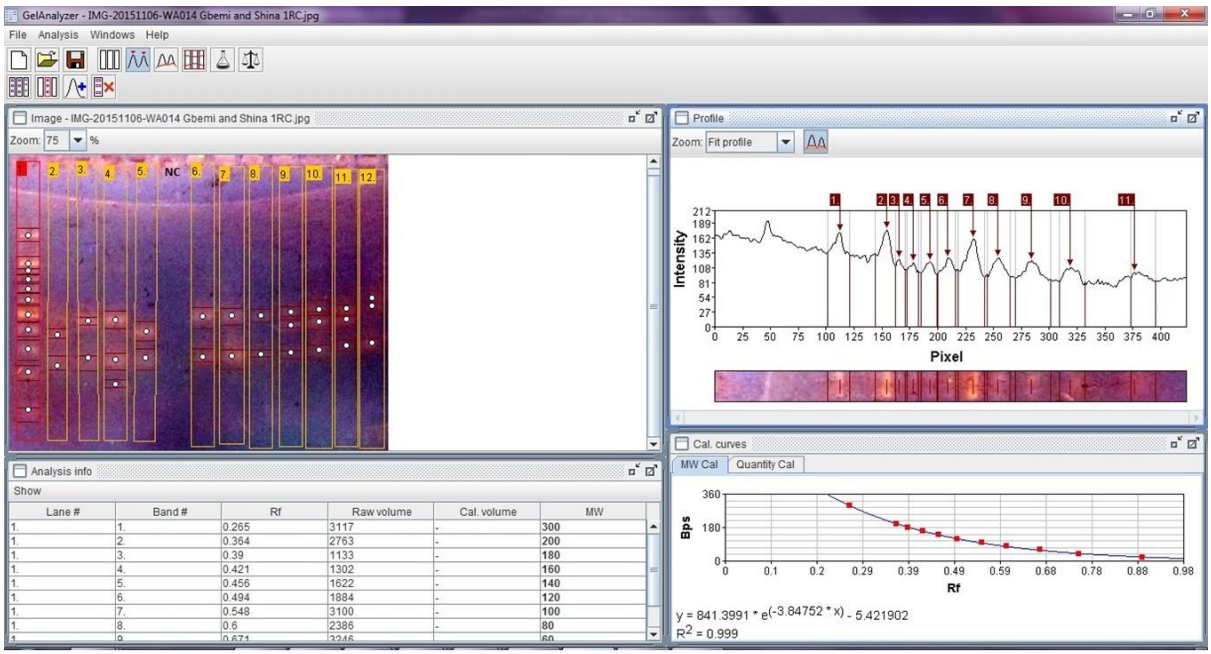


Figure 4: [Title] Screenshot of the GelAnalyzer software showing template of analysis of the gels in this study.

RESULTS AND DISCUSSION

DNA concentration and overall volume is known to affect the amplification efficiency of the various loci in a multiplex. Too much DNA may cause extra peaks in the electrophoregram creating difficulties during interpretation (Henegariu et al., 1997). In In this study four DNA concentrations were used (1ng, 2ng, 5ng and 10ng) in two volumes of 1ul or 5ul respectively. [Amelogenin and D10S1248], and [D6S1017 and D18S51] had their best amplification measured by band intensity (height of peaks) and number of peaks at 5ng DNA concentration (Table 2). The best amplification for [D22S1045, D21S11, and D2S1338] was obtained when 2ng of template DNA was used. 10ng was the concentration at which optimal amplification was observed for the [D9S2157, FGA and D8S1179] though a good amplification was also observed at 2ng (as 2ng_1ul) concentration. Amplification of [D22S1045, D21S11, and D2S1338] was generally good with DNA concentration above 2ng. Generally, loci amplification improved with DNA concentration above 2ng for all the Miniplexes. The 1ng template DNA concentration consistently had the lowest detectable DNA amplification (Table 2).

The different concentrations of DNA were however tested with different annealing temperatures based on estimates from the average melting temperatures for the all the oligonucleotides in the multiplex (Table 3). [Amelogenin and D10S1248] required the highest annealing temperature to obtain amplification without non-specific bands. These were done with a 1.5mM concentration $MgCl_2$ concentration which was adjusted in increments of 1.5mM to 3.0mM and 4.5mM concentrations with repeated attempts to obtain better, more specific amplification (Table 2). The amplification for the [Amelogenin and D10S1248] improved when the concentration of $MgCl_2$ was increased to 4.5mM. Allele dropout was observed at 1.0mM $MgCl_2$. Concentrations of 1.5-3.0mM were optimal.

Increasing the number of amplification cycles of any PCR based assay is one way to improve sensitivity of the assay. The number of cycles used were 28, 30, 33, 35 and 40 cycles. At 28 cycles, amplification products were observed but signal intensity was poor for all the four miniplexes in the study (Table not included). At 30 cycles, D21S11, FGA, D18S11, D10S1248 and Amelogenin respectively amplified with 1ng of DNA in each of their multiplexes. At 40 cycles however, all multiplex loci were over-amplified and problems with non-specific binding problems were detected.

The optimal amplification depends on several factors including temperature profile, and the concentration of reagents in the buffer. Specificity is affected by factors that impact mis-priming such as annealing temperatures, concentration of primers, concentration of DNA polymerase and concentration of magnesium chloride (Giese *et al.,* 2009). Aslam *et al.* (2002) suggest that the most straight forward way of optimizing a PCR with a given primer pair is to change the concentration of $MgCl_2$ or the annealing temperature. The normal range of annealing temperature is 36 – 75 ^{o}C. Moreover, increased concentration of Tris in the buffer reduced specificity (Blanchard *et al.,* 1993) and modest concentration of salts ($MgCl_2$, etc.) is known to stimulate the synthesis rate of Taq polymerase (Gelfand, 1989). Variation in the $MgCl_2$ concentration has strong and complex effects on nucleic acid interactions because the Mg^{2+} binds tightly to the sugar phosphate backbone for nucleotides. Variations of Mg^{2+} concentration below 4mM improved PCR by affecting specificity (lower concentration raise specificity, high concentrations lower specificity) (Blanchard et al., 1993).

Table 2: DNA concentration and volume with measures of PCR success (number of peaks and peak intensity)

Miniplex		1ul_1ng/ul		1ul_2ng/ul		5ul_1ng/ul		5ul_2ng/ul	
		Total no. of peaks	**Intensity Mean±SD**	**Total no. of peaks**	**Intensity Mean±SD**	**Total no. of peaks**	**Intensity Mean±SD**	**Total no. of peaks**	**Intensity Mean±SD**
M 01	Amelogenin_1	3	91±76	5	111±31	5	155±11	4	103±65
	Amelogenin_2	3	92±75	5	129±28	5	113±61	3	42±62
	D10S1248_1	4	103±59	4	92±52	4	124±62	3	78±65
	D10S1248_2	2	43±60	3	68±62	5	135±32	2	49±63
M 02	D6S1017_1	5	50±10	0	0	5	138±20	5	135±14
	D6S1017_2	0	0	5	23±5	5	146±5	5	142±9
	D18S51_1	5	115±4	5	102±16	5	137±27	5	134±14
	D18S51_2	0	0	0	0	0	0	0	0
M 03	D22S1045_1	5	46±9	5	145±4	5	154±4	4	125±63
	D22S1045_2	5	47±5	5	147±2	5	156±4	4	119±60
	D21S11_1	5	120±4	5	156±3	5	136±3	5	130±34
	D21S11_2	5	116±7	5	156±2	5	134±3	5	164±29
	D2S1338_1	5	153±6	5	150±8	5	150±14	5	158±4
	D2S1338_2	5	153±6	5	143±4	5	152±6	5	159±4
M 04	D9S2157_1	0	0	5	12±4	5	12±4	5	17±4
	D9S2157_2	0	0	5	12±4	5	12±2	5	13±2
	FGA_1	5	86±8	5	112±11	4	60±33	5	94±44
	FGA_2	5	83±9	5	125±16	3	88±72	5	123±39
	D8S1179_1	5	11±2	5	80±7	4	113±58	5	133±8
	D8S1179_2	5	10±1	5	83±10	4	101±61	5	137±12

Table 3: Optimal PCR conditions for the four multiplexes

		Annealing temp. (^oC)	$MgCl_2$ Concentration (mM)	Number of Cycles	DNA volume and concentration
Miniplex 01	Amelogenin; D10S1248	62.0	4.5 mM	30	5ul_1ng/ul
Miniplex 02	D6S1017; D18S51	59.0	1.5 mM	35	5ul_1ng/ul
Miniplex 03	D22S1045; D21S11; D2S1338	57.4	1.5 mM	35	1ul_2ng/ul
Miniplex 04	D9S2157; FGA; D8S1179	59.5	3.0 mM	35	5ul_2ng/ul

DECLARATIONS

Ethics approval and consent to participate

Approval of the research design, with the consent forms and biosample collection methods was obtained from the Ethics Review Board of the Lagos University Teaching hospital with reference ADM/DCST/HREC/1921

Consent for publication

All the authors have given consent for publication

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

AUU, AKO and OJ designed the experiments. AKO and OJ applied for and obtained funding from the University of Lagos. AAU, GBO, SOU, AAD, JHO and AMA collected samples and went on sampling trips. AUU performed the experiments and analysed the results. AKO and OJ reviewed the laboratory results. AUU obtained funding from the Association of African Universities for thesis writing. AUU, GBO, SOU and JHO wrote the manuscript. All authors read and approved the final manuscript.

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