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Optimisation of a reduced volume PCR amplification for PowerPlex® Fusion kit using FTA™ cards and generation of population genetic data for Brunei population

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Abstract

The commercial PowerPlex® Fusion kit is an autosomal STR multiplex kit that has high discrimination power and is more informative in forensic, paternity and relationship-testing cases. Key features of this multiplex system are the possibility to direct amplify FTA™ card punches as well as non-FTA cards and commonly used swabs; optimised inhibitor tolerance and high sensitivity generating full profiles from as little as 100 pg of human DNA.

This study focused on the optimization of performance variables such as FTA™ punch sizes, reduced reaction volumes, and FTA™ purification reagent aiming to increase the analytical sensitivity, decrease the sample consumption and cost effectiveness. LOD and LOQ values demonstrated high

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sensitivity of the PowerPlex® Fusion system. In addition, population databases of Brunei Malay and Chinese from the Brunei Darussalam were established, and parameters of forensic importance were calculated. Overall, the forensic parameters indicated an enhanced utility of the PowerPlex® Fusion kit for forensic evidence analysis and paternity testing in Brunei Malay and Chinese populations.

Keywords: Brunei population, Forensic genetics, FTA™ cards, PCR optimization, PowerPlex® Fusion, STRs

Additional supporting information may be found in the online version of this article at the publisher's web-site.

1 Introduction

Forensic DNA typing is constantly evolving and new commercial STR kits have been released with increased number of loci, improving the discrimination capacity of the kits. PowerPlex® Fusion kit allows simultaneous amplification of 22 autosomal STR loci using extracted DNA or FTA™ punches, generating profiles suitable for comparison with databases like the expanded CODIS or European Standard Set (ESS) requirements. The system has some key features like the inclusion of DYS391, which serves as an additional gender confirmation marker catering for individuals exhibiting Amelogenin deletion and an expanded STR loci panel improving genotyping accuracy and efficiency [1, 2]. Furthermore, nine loci yielding PCR products under 220 bp are integrated into the PowerPlex® Fusion kit ensuring a higher success rate with degraded casework samples. Sensitivity of the kit is able to meet the challenges of low template DNA samples as it can reliably generate full profiles from as little as 100 pg of human DNA (<https://ita.promega.com/resources/webinars/worldwide/archive/powerplex-fusion-system-overview-and-developmental-validation-preliminary-summary/>).

FTA™ cards have become a standard substrate for collection of DNA samples. DNA profiles generated from FTA™ card punches usually produce higher peak heights, and proved better than extracted DNA in an EDTA titration study displaying a higher allele call rate even in the presence of inhibitors [1]. The gold standard to avoid PCR inhibition is to purify DNA from the sample, but for FTA™ card punches this is unavoidable to some extent as they are directly amplified or are washed and amplified. In body fluids like blood, polypeptides, haemoglobin and lactoferrin have been identified as PCR inhibitors which interact with DNA polymerase blocking its activity [1]. Proven methods to overcome inhibition are increasing the amount of DNA polymerase, adding amplification facilitators such as Bovine Serum Albumin (BSA) or filtering or diluting the DNA extract [2]. Due to the nature of FTA™ card, reducing the punch size would result in DNA template dilution in the PCR reaction and the advantage would be conservation of the sample. However, a reduction of the PCR reaction volume poses some challenges as the kinetics of the reaction lead to stochastic effects due to enhanced sensitivity [3, 4]. Conversely, the increased sensitivity of the reduced volume reaction can enhance the interpretation of DNA mixtures favouring the detection of peaks from the minor contributor [3, 5]. Reduced volume PCR for the STR multiplex kits used for forensic purposes has been employed with normal and fast PCR protocols/different enzymes with positive results [6, 7]. The main advantage being the ability to amplify low template samples shown through sensitivity studies. However, the PCR optimisation needs to be carefully carried out so that PCR artefacts do not compromise the results.

The aim of this study was to optimise a reduced volume PCR reaction for the PowerPlex® Fusion kit in order to increase the analytical sensitivity while decreasing sample consumption. Performance variables crucial in determining the reliability and reproducibility of an optimised assay such as FTA™ punch sizes, reduced reaction volumes, and FTA™ purification reagent, were tested, and statistically analysed.

The population samples from Brunei Malay and Chinese were then analysed using optimised conditions and evaluated, to establish population databases. All work was conducted at the Forensic

Biology/DNA Laboratory of the Department of Scientific Services, Brunei Darussalam and School of Forensic and Applied Sciences, University of Central Lancashire, Preston, UK.

2 Material and Methods

2.1 PCR optimisation study

The influence of FTA[™] punch sizes, reduced reaction volumes, and FTA[™] purification reagent (Whatman, Maidstone, UK) on PCR was assessed using FTA[™] punches of 0.5 or 1.2 mm taken blood stained FTA[™] Micro Card (GE Healthcare, Buckinghamshire, UK), from 8 donors (2 males and 6 females). DNA samples from these donors were amplified in triplicate for establishing the genotypes. All replicate punches were made within a few mm area preventing intra-sample variation. The punch was cleaned by punching a fresh FTA[™] card twice in between punching different samples. Ethical approval for conducting the study was granted by the Department of Scientific Services, Ministry of Health in Brunei Darussalam and University of Central Lancashire.

2.1.1 FTA[™] punch size study

According to manufacturer's protocol a 1.2 mm FTA[™] punch contains about 5-20 ng of DNA, whereas a 0.5 mm punch would yield approximately 2-8 ng [4]. These were used to evaluate the impact of the reduced amount of input DNA in terms of sensitivity, fluorescence intensity, and STR peak morphology [8]. Allelic ladders and positive/negative controls were verified against manufacturer's data to determine PCR efficiency, null alleles and artefacts such as stutters, split peaks, microvariants, tri-allelic patterns, spikes, and mutations [8].

2.1.2 Reaction volume study

An equivalent amount of input DNA was amplified in a final volume of 12.5 and 6.25 μ L to determine PCR sensitivity, profile accuracy/quality and stochastic effects of reduced volume reactions. Triplicate PCRs were prepared for this study including the positive and negative controls.

2.1.3 Purification study

A batch of the 10 FTA™ punches (0.5 mm) were washed thrice with 200 µl of FTA™ purification reagent (Whatman, Maidstone, UK) and twice in 200 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). They were then dried on a hot block at 72°C for 3 minutes.

2.2 Population samples collection

Blood stains spotted on FTA™ Micro Card (GE Healthcare, Buckinghamshire, UK) were collected using finger prick method from 505 healthy unrelated individuals (age 12-60 yr) after obtaining written informed consent. Participants were citizens or permanent residents of Brunei Darussalam and were asked to provide detailed ethnic information through a questionnaire; also four-generation pedigree charts were recorded. Only participants having at least three-generation of consistent Brunei Darussalam Malay or Chinese heritage were included. Subjects of mixed ethnic background were not included in this study.

2.3 PCR amplification

PCR amplification was performed using the PowerPlex® Fusion kit (Promega, Madison, WI) according to the manufacturer's instructions, except for the reduced reaction volumes (6.25/12.5 µL) and 26 PCR cycles. For the FTA™ punch size study as well as for the purification study, all PCRs were carried out using 0.5/1.2 mm punches in a final reaction volume of 6.25 and 12.5 µL. For comparison, 10 PCR amplifications were carried out using 0.5/1.2 mm punches in 25 µL volume as well. Furthermore, 50 negative controls were amplified in triplicate in PCR volumes of 6.25 and 12.5 µL.

FTA™ card samples collected from Brunei Darussalam Malay and Chinese populations were punched using the Harris Micro-Punch® (Whatman, Maidstone, UK) with 1.2 mm punches which were purified using FTA™ purification reagent and amplified in a final reaction volume of 12.5 µL. The puncher was cleaned by punching a fresh FTA™ card twice in between punching successive samples.

2.4 Capillary electrophoresis

1 μ l PCR products were prepared in 8.7 μ l formamide and 0.8 μ l CC5 ILS and injected into a 3130xl Genetic Analyser (ThermoFisher Scientific, Waltham, MA, USA) using an injection time of 10 s at 3 kV using. Reference allelic ladder provided with the PowerPlex® Fusion kit (Promega, Madison, WI) were prepared like the amplifications and were used in each injection. Raw data were analysed using the GeneMapper® ID-X v1.2 software using 50 RFU allele calling threshold and all other parameters were kept at default values (ThermoFisher Scientific, Waltham, MA, USA). A GeneMapper® ID-X minus 4 stutter filter was set at 15%.

2.5 Statistical analysis

2.5.1 Optimisation study

The STR profile quality was evaluated following the routine protocol employed at the Department of Scientific Services, Ministry of Health of Brunei Darussalam [9]. Furthermore, the average peak heights for each locus, the mean heterozygote peak height ratios, and the percentage of the known DNA profile detected were measured for all the variables considered in the optimisation study [10].

2.5.2 Limit of detection (LOD) and limit of quantification (LOQ)

Data from 50 negative amplification controls were pooled to assess baseline noise in order to calculate the LOD and LOQ. The peak amplitude threshold of the GeneMapper® ID-X software analysis method was adjusted to 1 relative fluorescent unit (RFU) to capture all data points, and the analysis range was modified to correspond to the expected range of fragment sizes (75-475bp). Peak heights attributed to spikes were removed from the data set, and the remaining data exported to a Microsoft® Excel spreadsheet to calculate the average RFU values as well as the standard deviation values of peak heights for each dye. LOD threshold was set to the average noise of the negative controls plus 3 standard deviations, and LOQ was set at 10 standard deviations.

2.5.3 Population study

AmpF Φ STR[®] Identifiler kit (unpublished data) results from 203 Malay and 198 Chinese samples which were previously genotyped using the kit, were used to perform a concordance check of genotypes. For any observed discrepancy a re-amplification was performed to confirm it.

PowerStats 1.2 Microsoft[®] Excel spreadsheet (Promega, Madison, WI) [11] was used to calculate allele frequencies and bio-statistic forensic parameters useful to assess the utility of the loci for forensic and paternity purposes, namely Observed (H_o) and Expected Heterozygosity (H_e), Power of Discrimination (PD), Power of Exclusion (PE), Match Probability (MP), Polymorphic Information Content (PIC), Typical Paternity Index (TPI) and exact test (p) were estimated.

Analysis of Molecular Variance (AMOVA), departures from Hardy–Weinberg Equilibrium (HWE) expectations and Linkage Disequilibrium (LD) between each pair of loci and pairwise F_{ST} values and non-differentiation exact tests were performed using the software Arlequin version 3.5 [12]. Allele frequencies of the Brunei Darussalam Malay were compared with previously published data from Singapore and Malaysia Malay, East Timor population and Filipinos from the Philippines [13, 14, 15]. Brunei Chinese were compared with previously published allele frequencies from Singapore and Malaysia Chinese, Hong Kong Chinese, Taiwanese, Koreans and Japanese [13, 16, 17, 18, 19].

3 Results

3.1 Profile quality assessment

3.1.1 FTA[™] punch size study

The 0.5 and 1.2 mm FTA[™] punches from four samples were amplified in a final volume of 6.25 μ L at 26 PCR cycles (Supplemental Fig. 1). All loci showed balanced heterozygote peaks in the replicates though differences in peak heights across the four samples were observed (Supplemental Table 1).

One of the two 0.5 mm punches showed dropouts at TPOX and D22S1045 loci, as well as low peak heights at the D19S433 and FGA loci (Supplemental Fig. 2).

3.1.2 Reaction volume study

Reducing reaction volumes to half (12.5 μ L) or to a quarter (6.25 μ L) produced EPGs with higher peak intensities (Supplemental Fig. 3), and peak height balance was maintained in all the samples (Supplemental Table 2).

3.1.3 Purification study

Purified samples showed a better balance of peak heights compared to all the other tested conditions (Supplemental Table 3 and 4), and samples exhibited higher peak intensities (Supplemental Fig. 4).

3.2 Average peak heights

Peak heights for each locus were averaged between samples for two punch sizes (0.5 and 1.2 mm) and both 6.25 and 12.5 μ L PCR amplifications (Supplemental Table 4).

3.3 Peak height ratio

Profiles generated from 0.5 and 1.2 mm punches, in different PCR reaction volumes for un purified punches and purified with the FTA™ purification reagent, were used to calculate the peak height ratio (PHR) between sister alleles. Mean PHRs with standard error are reported in Supplemental Table 5.

3.4 Percentage of the known DNA profile detected

Full, concordant profiles were obtained from most of the samples assigning 100% of the expected alleles for all the variables tested (see concordance section for details).

3.5 Additional DNA samples

Balanced peaks were observed across loci for all the eight samples analysed in triplicate, except for one sample which failed to amplify once. Loci affected by low peak heights were D10S1248,

D13S317, D2S1338, CSF1PO, TPOX, D22S1045, D19S433, FGA plus the Y-chromosome marker DYS391; stutters, spikes, and microvariant were the observed artefacts. Except locus D2S1358 where the minus 4 stutter was noted at approx 10 RFU of the corresponding allelic peak; all other loci had minus 4 stutter below 10% of the allelic peak (data not shown). In 3 amplifications reactions dropouts occurred at TPOX, D22S1045, and D10S1248 loci. We attributed this to less number of cells deposited on the FTA paper rather than the technique.

Average peak height spanned from 213 RFU at DYS391 to 4410 RFU at the amelogenin locus, while the highest value of mean peak height ratio was 98.7% for the D5S818 marker reducing to 27.2% for TH01. Finally, all the profiles showed a 100% allele-calling consistency across the three replicates.

3.6 LOD and LOQ baseline calculations

Values for LOD and LOQ calculated from amplification of 50 negative samples (Supplemental Table 6).

3.7 Population study

3.7.1 Concordance

The concordance rate for the Brunei Darussalam Malay and Chinese datasets for Identifiler and Fusion kits were 99.94% and 99.98% respectively (Supplemental Table 7). Five discordant calls occurred at loci D16S539, D8S1179 and D21S11 within four Malay samples and one Chinese sample (Supplemental Table 8). Discrepancies observed at D16S539 and D8S1179 loci were probably due to PCR primer position differences causing the large-allele drop out. PowerPlex® Fusion kit (Promega, Madison, WI) recovered the microvariant allele 30.3 at D21S11 locus which the AmpFℓSTR® Identifiler kit failed to assign. The discordant samples were excluded from the calculation of allele frequencies. All samples showing discordant alleles were amplified and injected in the genetic DNA analyser twice for confirmation.

3.7.2 Allele frequencies

Allele frequencies and forensic parameters for all the loci included in the PowerPlex® Fusion multiplex kit were calculated for the Brunei Darussalam Malay (Table 1) and Chinese (Table 2) separately, and as one population (Table 3).

The Malay population showed significant departure from Hardy-Weinberg equilibrium at Penta D ($p = 0.0060$) and D10S1248 ($p = 0.0351$) loci. Conversely, no departures were observed in the Chinese population. The combined data showed Hardy-Weinberg equilibrium for all loci except D10S1248 ($p = 0.0386$). However, Bonferroni's correction ($p = 0.0023$) yielded no significant departures for this locus.

[illegible]

The observed gene diversity for the Y-chromosome marker DYS391 was 0.3680 in Malay, 0.3185 in Chinese, and 0.3502 when combine; while match probability 0.6294, 0.6841 and 0.6504 respectively, and PD values were 0.3706, 0.3159 and 0.3496.

The AMOVA analysis ($p = 0.051639$) showed no significant differences between Brunei Malay and Chinese. Furthermore, linkage disequilibrium was tested on the combined population, as well as on both populations separately, and it was observed only in the Brunei Malay population for the D7S820 locus with Penta D ($p = 0.00391$) and D10S1248 ($p = 0.02151$). However, the statistical significances were corrected after applying Bonferroni's correction ($p = 0.0002$).

4 Discussion

Reducing total PCR volume of commercial kits can help increase detection limits, sensitivity, and reduce sample consumption which is crucial for Forensic DNA laboratories. However, altering manufacturer's recommended protocols requires optimisation in order to generate robust and

acceptable results in terms of signal intensity and heterozygote balance. In DNA profiling, different parameters can be altered to find out the best conditions resulting in an optimal performance. In this study we choose to use the manufacturer's recommended PCR conditions and tested reduced volume PCR. Furthermore, the influence of FTA™ punch sizes and use of FTA™ purification reagent were evaluated for amplification of FTA™ punches using PowerPlex® Fusion kit.

Reduction of FTA™ punch sizes from 1.2 to 0.5 mm was challenging due to static forces causing punches to jump out into another tube or well, requiring re-punching. Moreover, pipetting represented a critical factor as small punches could be sucked into the tip when performing washes with FTA™ purification reagent. Generally 0.5 mm punches gave better intensity peaks than 1.2 mm punches probably due to less amount of inhibitors competing with PCR products.

The peak intensities when using 0.5 mm punches were around 3000 RFU in height indicating efficient PCR amplification. Low peaks observed at the DYS391 locus were expected and previously described [2]. Some other markers showed low signal intensity (Supplemental Table 2). Most of these markers were located in the mid molecular weight region of the PowerPlex® Fusion panel: D10S1248 (250-300bp), D13S317 (300-350bp), D2S1338 (225-300bp), CFS1PO (320-350bp), D19S433 (200-250bp) and FGA (270-410bp) [1]. The minimum and maximum average peak heights were higher for 1.2 mm punch samples than the 0.5 mm, conversely, the minimum PHR of the 1.2 mm punches was lower than that of 0.5 mm punches spanning from 64.8% to 98.1%. The profiles generated from both punch sizes were correctly called for all the samples with two dropouts occurring at TPOX and D22S1045 loci in two samples.

The reduced volume of 6.25 µL PCR reaction yielded the highest minimum and maximum average peak heights. Also PHR was not affected by PCR volume as comparable average values for 6.25 µL and 12.5 µL reactions were observed. Furthermore, testing of additional samples in triplicates allowed to test the reliability of the 6.25 µL reaction and all but three amplifications showed full profiles. Amplification of 0.5 mm size punches allowed also to calculate the LOD and LOQ values demonstrating the high sensitivity of the PowerPlex® Fusion system.

Washing the 0.5 mm punches with the FTA™ purification reagent prior to the 6.25/12.5 µL PCR resulted in higher signal intensity and a better PHR.

Concordance evaluations for the PowerPlex® Fusion kit had highlighted a severely imbalanced allele 9 at D16S539 before and this was corrected in the developmental validation study of the PowerPlex® Fusion system [1], however our results showed that it still existed. A severe imbalance was also observed at D8S1179 locus which might be due to sequence differences of the different primer set used in the two kits. The microvariant allele 30.3 at D21S11 locus was correctly assigned using the PowerPlex® Fusion system demonstrating an increased genotyping accuracy. However, sequencing of the discordant samples might help to determine the nature of the discordances.

In this study population databases for Brunei Malay and Chinese, as well as the combined allele frequency database were established for the Brunei population. Among the 22 STR loci, the Penta E locus appeared to be the most informative marker showing similar Power of discrimination (PD) values 0.9804 in Malay, 0.9829 in Chinese, and 0.9842 in the Brunei pooled population. TPOX locus had lower PD values of 0.7716, 0.7506 and 0.7625 for the Malay, Chinese and Brunei populations respectively. This was consistent with the Polymorphic Information Content (PIC) values observed. Overall, the forensic parameters indicated quite an enhanced utility of the PowerPlex® Fusion kit for forensic evidence analysis and paternity testing in Brunei Malay and Chinese. Since AMOVA results indicated no significant genetic variation between Brunei Malay and Chinese, the combined allele frequencies of these two ethnic groups can be used to calculate match probability.

Based on pairwise F_{ST} comparison between Brunei Malay and neighbouring populations the most distant populations were East Timorese, Singapore and Malaysia Malay followed by Indonesian and Filipinos. The Malays from the peninsula of Malaysia and Singapore consist of various sub-ethnic groups which might have different ancestral origins based on their migrations centuries ago. The exact origins of the Malaysia and Singapore Malays are still unknown due to migrating populations from surrounding areas which brought varying degrees of genetics admixtures [20], but Singapore Malays might have underwent more admixture due to the geographical position of Singapore and to

international migrations contributing to shape the Singapore population as it is today. Perhaps Brunei Malay genetic makeup derives from the Borneo indigenous groups. (<http://www.dnatribes.com/dnatribes-digest-2013-06-01.pdf>).

We show that Brunei Chinese were equally distant from Malaysia Singapore, Taiwan and Hong Kong Chinese, this could be explained by migration and ethnical crossbreeding or admixture of the Brunei Chinese population [21].

The Brunei Chinese were initially brought in by the British during the British protectorate period to develop Brunei back in 1905 (<http://www.dnatribes.com/dnatribes-digest-2013-06-01.pdf>). They came from Kinmen or Quemoy, a Taiwanese county. After the discovery of oil in 1929, there was an influx of Chinese from Sarawak, Singapore and Hong Kong. This explains the high similarity of the Brunei Chinese with the Chinese from Malaysia, Singapore, Hong Kong, and Taiwan. The Koreans and Japanese the two groups most distant from Brunei population among the populations studied here. Geographically, Japan and Korea are very distant from Brunei and the genetic dissimilarity is well accepted.

The authors have declared no conflict of interest.

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Table captions:

Table 1: Allele frequencies and forensic statistical parameters for the 22 STR loci in Brunei Malay population

All ele	D3S 1358	D1S 1656	D2S 441	D10S 1248	D13 S317	Pen ta E	D16 S539	D18 S51	D2S 1338	CSF 1PO	Pen ta D	TH 01	vW A	D21 S11	D7S 820	D5S 818	TP OX	DYS 391	D8S 1179	D12 S391	D19 S433	FG A	D22S 1045
5						0.0 31																	
6											0.0 04	0.0 98											
7					0.00 2					0.00 2	0.0 25	0.3 2			0.00 2	0.01							
8					0.24 7	0.0 02	0.01 2			0.00 4	0.0 18	0.1 43			0.17 8	0.00 4	0.5 27						
9					0.10 8	0.0 12	0.19 6	0.00 2		0.03 1	0.4 45	0.2 86			0.05 3	0.04 5	0.1 02	0.06 1	0.00 4				
9.1			0.01																				
9.3												0.0 51											

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10		0.00 2	0.18 6		0.11 4	0.0 39	0.16 5	0.00 2		0.25 1	0.1 2	0.1 02			0.2	0.28	0.0 12	0.77 3	0.07 8				
11		0.12 4	0.29 4		0.30 6	0.1 84	0.31	0.01		0.31	0.0 55				0.37 6	0.26 9	0.3 39	0.16 6	0.13 3		0.00 2		0.28
11. 3			0.14 9																				
12		0.02 5	0.17 1	0.078	0.19 6	0.1 08	0.17 8	0.06 7		0.35 7	0.2 39				0.16 1	0.25 9	0.0 2		0.07 5		0.02 4		
12. 3			0.00 2																				
13	0.00 2	0.10 4	0.02 5	0.267	0.02 2	0.0 73	0.11 4	0.06 7		0.04 3	0.0 78				0.02 4	0.12 2			0.24 1		0.23 7		0.008
13. 2																					0.04 5		
13. 3								0.00 2															
14	0.01 2	0.11 6	0.14 9	0.28	0.00 6	0.1 08	0.02 5	0.21		0.00 2	0.0 06		0.1 57		0.00 6	0.01			0.17 8		0.18		0.027
14. 2																					0.11 6		
15	0.29 6	0.23 1	0.01 2	0.194		0.1 12		0.28 8			0.0 1		0.1 02			0.00 2			0.15 5	0.00 6	0.09 4	0.0 02	0.371

15. 2								0.00 2													0.26 3		
15. 3		0.00 8																					
16	0.29 6	0.19 8	0.00 2	0.143		0.0 69		0.12 5	0.02				0.1 37						0.11 6	0.02 2	0.00 4		0.124
16. 2																					0.02 4		
16. 3		0.00 8																					
17	0.31 8	0.05 9		0.035		0.0 73		0.08 2	0.11 2				0.2 2						0.01 4	0.09 2	0.00 6		0.175
17. 2																					0.00 6		
17. 3		0.07 8																					
18	0.07 1	0.01 4		0.002		0.0 53		0.04 9	0.05 1				0.2 75						0.00 6	0.15 7		0.0 12	0.004
18. 3		0.03 1																		0.00 2			
19	0.00 6	0.00 2				0.0 41		0.04 7	0.16 3				0.0 96							0.18 4		0.0 86	0.012

19. 2																						0.0 02	
20						0.0 49		0.01 6	0.09 8				0.0 14							0.21 2		0.0 27	
20. 2																							
21						0.0 2		0.01 2	0.02 4											0.09 4		0.2 18	
21. 2																						0.0 02	
22						0.0 2		0.00 2	0.10 2											0.12 5		0.2 12	
22. 2																						0.0 12	
23						0.0 02		0.00 8	0.16 5											0.04 9		0.1 73	
23. 2																						0.0 04	
24						0.0 02		0.00 8	0.16 7											0.02 4		0.1 12	
24. 2																						0.0 02	

25						0.0 04		0.00 2	0.07 1					0.00 6						0.02 4		0.0 57	
25. 2																						0.0 04	
26									0.02 4											0.00 8		0.0 45	
26. 2																						0.0 02	
27									0.00 6					0.00 4						0.00 2		0.0 2	
28														0.03 5								0.0 08	
28. 2														0.00 2									
29														0.21 4								0.0 02	
30														0.24 5									
30. 2														0.02 2									
31														0.16 5									

31. 2														0.07 1									
32														0.04 9									
32. 2														0.10 2									
33														0.00 2									
33. 2														0.07 1									
34														0.00 2									
34. 2														0.00 8									
35. 2														0.00 4									
Ho	0.73 26	0.84 88	0.79 46	0.728 7	0.75 97	0.8 876	0.77 91	0.84 88	0.89 54	0.74 03	0.6 783	0.7 636	0.8 14	0.83 33	0.72 48	0.79 07	0.5 543	-	0.83 72	0.82 95	0.82 17	0.8 333	0.724 8
He	0.71 95	0.85 82	0.80 67	0.785 3	0.78 45	0.9 07	0.79 46	0.83 82	0.87 9	0.71 26	0.7 213	0.7 737	0.8 151	0.84 45	0.75 86	0.76 7	0.5 981	-	0.84 35	0.86 2	0.81 79	0.8 542	0.739
p	0.82 29	0.52 17	0.47 53	0.035 1	0.11 73	0.7 497	0.62 69	0.99 17	0.32 52	0.45 67	0.0 06	0.9 175	0.1 829	0.80 79	0.46 34	0.05 27	0.6 726	-	0.45 02	0.59 98	0.76 41	0.7 676	0.479 5

SD	0.00 03	0.00 04	0.00 03	0.000 2	0.00 03	0.0 003	0.00 05	0.00 01	0.00 05	0.00 06	0.0 001	0.0 003	0.0 003	0.00 02	0.00 05	0.00 02	0.0 004	-	0.00 04	0.00 04	0.00 03	0.0 003	0.000 5
PD	0.86 15	0.96 15	0.93 26	0.920 8	0.91 82	0.9 804	0.92 57	0.95 61	0.96 8	0.85 34	0.8 802	0.9 136	0.9 353	0.95 54	0.90 72	0.89 9	0.7 716	0.37 06	0.95 43	0.96 4	0.93 82	0.9 596	0.885 3
PIC	0.66 5	0.84 06	0.77 66	0.751 5	0.74 86	0.8 973	0.76 26	0.81 86	0.86 45	0.65 66	0.6 845	0.7 39	0.7 874	0.82 43	0.72 16	0.72 62	0.5 232	0.33 31	0.82 38	0.84 38	0.79 23	0.8 345	0.694 6
M P	0.13 85	0.03 85	0.06 74	0.079 2	0.08 18	0.0 196	0.07 43	0.04 39	0.03 2	0.14 66	0.1 198	0.0 864	0.0 647	0.04 46	0.09 28	0.10 1	0.2 284	0.62 94	0.04 57	0.03 6	0.06 18	0.0 404	0.114 7
PE	0.48 82	0.68 91	0.59 18	0.475 2	0.52 16	0.7 754	0.57 74	0.68 91	0.78 34	0.49 48	0.3 957	0.5 353	0.6 211	0.65 85	0.46 88	0.57 74	0.2 341	0	0.66 61	0.65 09	0.63 59	0.6 585	0.468 8
TPI	1.9	3.27	2.45	1.85	2.06	4.5 5	2.36	3.27	4.72	1.93	1.5 5	2.1 3	2.6 6	2.97	1.82	2.36	1.1 1	0.5	3.04	2.9	2.77	2.9 7	1.82

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Table 2: Allele frequencies and forensic statistic parameters for the 22 STR loci in Brunei Chinese population

All ele	D3S 1358	D1S 1656	D2S 441	D10S 1248	D13 S317	Pen ta E	D16 S539	D18 S51	D2S 1338	CSF 1PO	Pen ta D	TH 01	vW A	D21 S11	D7S 820	D5S 818	TP OX	DYS 391	D8S 1179	D12 S391	D19 S433	FG A	D22S 1045
5						0.0 58																	
6							0.00 2					0.1 28											
7										0.00 6	0.0 06	0.2 7			0.00 2	0.02 6							
8			0.00 2		0.29 2	0.0 04	0.00 2			0.00 4	0.0 66	0.0 52			0.15 2	0.01 6	0.5 8						
9					0.11 4	0.0 2	0.24 2			0.03	0.3 56	0.4 56			0.06 4	0.06 6	0.0 92	0.01 8					
9.1			0.02 4																				
9.3												0.0 28											
10		0.00	0.24		0.15	0.0	0.12	0.00		0.25	0.1	0.0			0.13	0.19	0.0	0.81	0.14				

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		2	8		4	48	8	4		4	3	66			4	8	26	3					
10. 1			0.00 2																				
11		0.06 2	0.33		0.26 6	0.1 56	0.29 6	0.00 2		0.25 8	0.1 04				0.38 4	0.33 4	0.2 86	0.16 1	0.08 4		0.00 2		0.192
11. 3			0.07 4																				
12	0.00 2	0.03 8	0.16	0.092	0.12 2	0.1 1	0.21 6	0.05 2		0.37 8	0.1 66				0.23 8	0.19 4	0.0 14	0.00 9	0.14 6		0.04 2		0.004
12. 2																					0.00 2		
13		0.09 6	0.01 8	0.254	0.04	0.0 54	0.09 6	0.16 2		0.04 8	0.1 2				0.02 2	0.16	0.0 02		0.21 6		0.29 8		0.004
13. 2																					0.03		
14	0.03 8	0.10 8	0.13 4	0.272	0.01 2	0.0 62	0.01 6	0.18 6		0.02 2	0.0 44		0.2 74		0.00 4	0.00 6			0.17 4		0.25		0.038
14. 2																					0.15		
15	0.34 6	0.31 2	0.00 8	0.246		0.0 82	0.00 2	0.21 6			0.0 06		0.0 32						0.16 8	0.01 2	0.06		0.34
15.																					0.12		

2																				2		
15. 3																						
16	0.30 2	0.20 2		0.112		0.0 92		0.13 8	0.01 8		0.0 02		0.1 42					0.05 6	0.00 2	0.01 4	0.0 04	0.216
16. 2																				0.02 6		
16. 3		0.00 6																				
17	0.25 6	0.06 2		0.022		0.0 98		0.08 8	0.06 2				0.2 4					0.01 6	0.07 2	0.00 2	0.0 02	0.178
17. 2																				0.00 2		
17. 3		0.07																				
17. 4						0.0 02																
18	0.05 4	0.02		0.002		0.0 7		0.04	0.09 6				0.2 06						0.23 2		0.0 44	0.026
18. 3		0.01 6																				
19	0.00					0.0		0.04	0.21				0.0						0.18		0.0	0.002

	2					3							88							8		5	
19. 3		0.00 2																					
20						0.0 44		0.02 2	0.1				0.0 16							0.16 4		0.0 62	
20. 3		0.00 4																					
21						0.0 38		0.01 8	0.04 6				0.0 02							0.13		0.1 42	
21. 2																						0.0 04	
22						0.0 14		0.02 6	0.03 4											0.09		0.1 76	
22. 2																						0.0 02	
23						0.0 06		0.00 4	0.19 2											0.07 6		0.1 78	
23. 2																						0.0 04	
24						0.0 06		0.00 2	0.14 8											0.02 2		0.1 62	
24.																						0.0	

2																						1	
25						0.0 06			0.07 8										0.01 2			0.0 94	
25. 2																						0.0 02	
26									0.01 6													0.0 46	
26. 2																						0.0 12	
27																						0.0 06	
28													0.05 8										
28. 2													0.00 2										
29													0.24 4										
30													0.26										
30. 2													0.00 6										
30. 3													0.00 2										

31														0.09 2									
31. 2														0.08 6									
32														0.03 4									
32. 2														0.14 6									
33														0.00 4									
33. 2														0.05 6									
34														0.00 2									
34. 2														0.00 8									
Ho	0.74	0.80 8	0.77 6	0.792	0.78	0.9 2	0.79 2	0.85 6	0.85 6	0.74 8	0.7 96	0.7	0.7 84	0.84	0.72 4	0.81 2	0.5 88	-	0.80 8	0.84 8	0.83 6	0.8 48	0.796
He	0.72 06	0.82 79	0.78 12	0.781 1	0.79 22	0.9 183	0.78 29	0.86	0.86 59	0.72 37	0.7 988	0.6 963	0.7 973	0.82 96	0.75 17	0.78 22	0.5 736	-	0.84 52	0.84 89	0.80 58	0.8 731	0.768 6
p	0.58 01	0.52 18	0.86 53	0.115 3	0.98 15	0.2 514	0.86 19	0.89 81	0.51 89	0.65 47	0.6 321	0.5 13	0.2 718	0.57 24	0.61 2	0.27 9	0.8 431	-	0.93 74	0.16 78	0.67 47	0.5 142	0.706 1

SD	0.00 04	0.00 04	0.00 04	0.000 3	0.00 01	0.0 004	0.00 03	0.00 03	0.00 05	0.00 04	0.0 004	0.0 005	0.0 004	0.00 02	0.00 05	0.00 03	0.0 003	-	0.00 03	0.00 04	0.00 03	0.0 003	0.000 4
PD	0.86 7	0.95 25	0.91 76	0.906 7	0.92 48	0.9 829	0.91 6	0.96 34	0.96 34	0.86 75	0.9 328	0.8 552	0.9 234	0.94 24	0.90 23	0.91 69	0.7 506	0.31 59	0.95 58	0.95 4	0.93 36	0.9 672	0.905 7
PIC	0.66 73	0.80 74	0.74 77	0.744 4	0.76	0.9 105	0.74 71	0.84 27	0.84 97	0.67 42	0.7 736	0.6 507	0.7 652	0.80 7	0.71 37	0.74 87	0.5 096	0.28 08	0.82 38	0.82 91	0.77 83	0.8 58	0.730 7
M P	0.13 3	0.04 75	0.08 24	0.093 3	0.07 52	0.0 171	0.08 4	0.03 66	0.03 66	0.13 25	0.0 672	0.1 448	0.0 766	0.05 76	0.09 77	0.08 31	0.2 494	0.68 41	0.04 42	0.04 6	0.06 64	0.0 328	0.094 3
PE	0.49 28	0.61 4	0.55 53	0.584 3	0.56 25	0.8 364	0.58 43	0.70 67	0.70 67	0.50 64	0.5 916	0.4 283	0.5 697	0.67 53	0.46 64	0.62 15	0.2 767	0	0.61 4	0.69 09	0.66 75	0.6 909	0.591 6
TPI	1.92	2.6	2.23	2.4	2.27	6.2 5	2.4	3.47	3.47	1.98	2.4 5	1.6 7	2.3 1	3.13	1.81	2.66	1.2 1	0.5	2.6	3.29	3.05	3.2 9	2.45

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Table 3: Allele frequencies and forensic statistical parameters for the 22 STR loci in the Brunei combined (Malay and Chinese) population

All ele	D3S 1358	D1S 1656	D2S 441	D10S 1248	D13 S317	Pen ta E	D16 S539	D18 S51	D2S 1338	CSF 1PO	Pen ta D	TH 01	vW A	D21 S11	D7S 820	D5S 818	TP OX	DYS 391	D8S 1179	D12 S391	D19 S433	FG A	D22S 1045
5						0.0 45																	
6							0.00 1				0.0 02	0.1 13											
7					0.00 1					0.00 4	0.0 16	0.2 95				0.00 2	0.01 8						
8			0.00 1		0.26 9	0.0 03	0.00 7			0.00 4	0.0 42	0.0 98				0.16 5	0.01	0.5 53					
9					0.11 1	0.0 16	0.21 9	0.00 1		0.03 1	0.4 01	0.3 7				0.05 8	0.05 5	0.0 97	0.04 4	0.00 2			
9.1			0.01 7																				
9.3												0.0 4											
10		0.00	0.21		0.13	0.0	0.14	0.00		0.25	0.1	0.0				0.16	0.24	0.0	0.78	0.10			

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		2	7		4	44	7	3		2	25	84			7		19	8	9				
10. 1			0.00 1															0					
11		0.09 3	0.31 2		0.28 6	0.1 7	0.30 3	0.00 6		0.28 4	0.0 79				0.38	0.30 1	0.3 13	0.16 4	0.10 9		0.00 2		0.237
11. 3			0.11 2																				
12	0.00 1	0.03 2	0.16 5	0.085	0.15 9	0.1 09	0.19 7	0.05 9		0.36 7	0.2 03				0.19 9	0.22 7	0.0 17	0.00 3	0.11		0.03 3		0.002
12. 2			0.00 1																		0.00 1		
13	0.00 1	0.1	0.02 2	0.26	0.03 1	0.0 63	0.10 5	0.11 4		0.04 6	0.0 99				0.02 3	0.14 1	0.0 01		0.22 9		0.26 7		0.006
13. 2																					0.03 8		
13. 3								0.00 1															
14	0.02 5	0.11 2	0.14 2	0.276	0.00 9	0.0 85	0.02 1	0.19 8		0.01 2	0.0 25		0.2 15		0.00 5	0.00 8			0.17 6		0.21 5		0.033
14. 2																					0.13 3		
15	0.32	0.27	0.01	0.22		0.0	0.00	0.25			0.0		0.0			0.00			0.16	0.00	0.07	0.0	0.355

	1	1				97	1	2			08		67			1			1	9	7	01	
15. 2								0.00 1													0.19 3		
15. 3		0.00 4																					
16	0.29 9	0.2	0.00 1	0.128		0.0 8		0.13 2	0.01 9		0.0 01		0.1 4						0.08 6	0.01 2	0.00 9	0.0 02	0.169
16. 2																					0.02 5		
16. 3		0.00 7																					
17	0.28 7	0.06		0.029		0.0 85		0.08 5	0.08 7				0.2 3						0.01 5	0.08 2	0.00 4	0.0 01	0.176
17. 2																					0.00 4		
17. 3		0.07 4																					
17. 4						0.0 01																	
18	0.06 2	0.01 7		0.002		0.0 61		0.04 5	0.07 3				0.2 41						0.00 3	0.19 4		0.0 28	0.015
18.		0.02																		0.00			

3		4																		1			
19	0.00 4	0.00 1				0.0 36		0.04 4	0.18 6				0.0 92							0.18 6		0.0 68	0.007
19. 3		0.00 1																				0.0 01	
20						0.0 47		0.01 9	0.09 9				0.0 15							0.18 8		0.0 45	
20. 3		0.00 2																					
21						0.0 29		0.01 5	0.03 5				0.0 01							0.11 2		0.1 8	
21. 2																						0.0 03	
22						0.0 17		0.01 4	0.06 8											0.10 8		0.1 94	
22. 2																						0.0 07	
23						0.0 04		0.00 6	0.17 8											0.06 2		0.1 75	
23. 2																						0.0 04	
24						0.0		0.00	0.15											0.02		0.1	

						04		5	7											3		37	
24. 2																						0.0 06	
25						0.0 05		0.00 1	0.07 4					0.00 3						0.01 8		0.0 75	
25. 2																						0.0 03	
26									0.02											0.00 4		0.0 46	
26. 2																						0.0 07	
27									0.00 3					0.00 2						0.00 1		0.0 13	
28														0.04 7								0.0 04	
28. 2														0.00 2									
29														0.22 9								0.0 01	
30														0.25 2									
30.														0.01									

2														4									
30. 3														0.00 1									
31														0.12 9									
31. 2														0.07 8									
32														0.04 2									
32. 2														0.12 4									
33														0.00 3									
33. 2														0.06 3									
34														0.00 2									
34. 2														0.00 8									
35. 2														0.00 2									
Ho	0.73	0.82	0.78	0.762	0.76	0.9	0.78	0.85	0.87	0.74	0.7	0.7	0.8	0.83	0.72	0.8	0.5	-	0.82	0.83	0.82	0.8	0.758

	66	77	42	4	83	03	81	15	53	46	366	327		76	48		683		18	76	77	396	4
He	0.72 15	0.84 51	0.79 58	0.784	0.79 01	0.9 133	0.78 93	0.85 23	0.87 49	0.71 77	0.7 639	0.7 456	0.8 113	0.83 91	0.75 66	0.77 8	0.5 867	-	0.84 8	0.85 76	0.81 89	0.8 662	0.756 7
p	0.96 12	0.71 89	0.37 76	0.038 6	0.69 73	0.8 127	0.41 7	0.97 21	0.47 92	0.31 23	0.1 525	0.5 248	0.3 937	0.55 79	0.32 48	0.23 29	0.9 047	-	0.58 68	0.26 32	0.87 09	0.5 142	0.745 8
SD	0.00 02	0.00 02	0.00 04	0.000 2	0.00 04	0.0 002	0.00 04	0.00 01	0.00 04	0.00 03	0.0 003	0.0 004	0.0 005	0.00 03	0.00 05	0.00 04	0.0 002	-	0.00 03	0.00 03	0.00 03	0.0 004	0.000 3
PD	0.86 64	0.95 98	0.92 88	0.917 4	0.92 41	0.9 842	0.92 26	0.96 24	0.96 91	0.86 13	0.9 147	0.8 937	0.9 354	0.95 2	0.90 71	0.91 25	0.7 625	0.34 96	0.95 81	0.96 23	0.94 12	0.9 661	0.899 8
PIC	0.66 81	0.82 72	0.76 59	0.749 2	0.75 76	0.9 058	0.75 66	0.83 52	0.86 11	0.66 63	0.7 358	0.7 064	0.7 833	0.81 88	0.72 13	0.74 22	0.5 176	0.31 37	0.82 86	0.84	0.79 43	0.8 506	0.718 2
M P	0.13 36	0.04 02	0.07 12	0.082 6	0.07 59	0.0 158	0.07 74	0.03 76	0.03 09	0.13 87	0.0 853	0.1 063	0.0 646	0.04 8	0.09 29	0.08 75	0.2 375	0.65 04	0.04 19	0.03 77	0.05 88	0.0 339	0.100 2
PE	0.49 05	0.65 15	0.57 36	0.527 7	0.54 16	0.8 055	0.58 08	0.69 78	0.74 52	0.50 05	0.4 872	0.4 806	0.5 954	0.66 68	0.46 76	0.59 9	0.2 546	0	0.64 01	0.67 06	0.65 15	0.6 745	0.527 7
TPI	1.91	2.9	2.34	2.09	2.16	5.2 6	2.38	3.37	4.01	1.96	1.9	1.8 7	2.4 8	3.04	1.82	2.5	1.1 6	0.5	2.81	3.08	2.9	3.1 2	2.09

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