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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/).

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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

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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 µL 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.

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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.

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2.4 Capillary electrophoresis

1 µ1 PCR products were prepared in 8.7 µl formamide and 0.8 µl 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.

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2.5.3 Population study

AmpFℓSTR® 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 (Ho) and Expected Heterozygosity (He), 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 µL 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).

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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 µL) or to a quarter (6.25 µL) 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 µl 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,

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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.

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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.

The combined matching probability calculated for the 22 autosomal STR loci, was 2.11×10^{-26} in Malay; 3.36×10^{-26} in Chinese, and 8.10×10^{-27} in the pooled population; whereas the combined PD values were >0.9999999999999999999999999 and the combined power of exclusion >0.99999999.

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

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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.

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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

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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

Allele	D3S1358	D1S1656	D2S441	D10S1248	D13S317	Penta E	D16S539	D18S51	D2S1338	CSF1PO	Penta D	TH01	vWA	D21S11	D7S820	D5S818	TPOX	DYS391	D8S1179	D12S391	D19S433	FGA	D22S1045
5						0.031																	
6											0.004	0.098											
7					0.002					0.002	0.025	0.32			0.002	0.01							
8					0.247	0.002	0.012			0.004	0.018	0.143			0.178	0.004	0.527						
9					0.108	0.012	0.196	0.002		0.031	0.445	0.286			0.053	0.045	0.102	0.061	0.004				
9.1			0.01																				
9.3												0.051											

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10		0.002	0.186		0.114	0.039	0.165	0.002		0.251	0.12	0.102			0.2	0.28	0.012	0.773	0.078				
11		0.124	0.294		0.306	0.184	0.31	0.01		0.31	0.055				0.376	0.269	0.339	0.166	0.133		0.002		0.28
11.3			0.149																				
12		0.025	0.171	0.078	0.196	0.108	0.178	0.067		0.357	0.239				0.161	0.259	0.02		0.075		0.024		
12.3			0.002																				
13	0.002	0.104	0.025	0.267	0.022	0.073	0.114	0.067		0.043	0.078				0.024	0.122			0.241		0.237		0.008
13.2																					0.045		
13.3								0.002															
14	0.012	0.116	0.149	0.28	0.006	0.108	0.025	0.21		0.002	0.006		0.157		0.006	0.01			0.178		0.18		0.027
14.2																					0.116		
15	0.296	0.231	0.012	0.194		0.112		0.288			0.01		0.102			0.002			0.155	0.006	0.094	0.002	0.371

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15.2								0.002													0.263		
15.3		0.008																					
16	0.296	0.198	0.002	0.143		0.069		0.125	0.02				0.137						0.116	0.022	0.004		0.124
16.2																					0.024		
16.3		0.008																					
17	0.318	0.059		0.035		0.073		0.082	0.112				0.22						0.014	0.092	0.006		0.175
17.2																					0.006		
17.3		0.078																					
18	0.071	0.014		0.002		0.053		0.049	0.051				0.275						0.006	0.157		0.012	0.004
18.3		0.031																		0.002			
19	0.006	0.002				0.041		0.047	0.163				0.096							0.184		0.086	0.012

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19.2																						0.002	
20						0.049		0.016	0.098				0.014							0.212		0.027	
20.2																							
21						0.02		0.012	0.024											0.094		0.218	
21.2																						0.002	
22						0.02		0.002	0.102											0.125		0.212	
22.2																						0.012	
23						0.002		0.008	0.165											0.049		0.173	
23.2																						0.004	
24						0.002		0.008	0.167											0.024		0.112	
24.2																						0.002	

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25						0.004		0.002	0.071					0.006						0.024		0.057	
25.2																						0.004	
26									0.024											0.008		0.045	
26.2																						0.002	
27									0.006					0.004						0.002		0.02	
28														0.035								0.008	
28.2														0.002									
29														0.214								0.002	
30														0.245									
30.2														0.022									
31														0.165									

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31.2													0.071										
32													0.049										
32.2													0.102										
33													0.002										
33.2													0.071										
34													0.002										
34.2													0.008										
35.2													0.004										
Ho	0.7326	0.8488	0.7946	0.7287	0.7597	0.8876	0.7791	0.8488	0.8954	0.7403	0.6783	0.7636	0.814	0.8333	0.7248	0.7907	0.5543	-	0.8372	0.8295	0.8217	0.8333	0.7248
He	0.7195	0.8582	0.8067	0.7853	0.7845	0.907	0.7946	0.8382	0.879	0.7126	0.7213	0.7737	0.8151	0.8445	0.7586	0.767	0.5981	-	0.8435	0.862	0.8179	0.8542	0.739
p	0.8229	0.5217	0.4753	0.0351	0.1173	0.7497	0.6269	0.9917	0.3252	0.4567	0.006	0.9175	0.1829	0.8079	0.4634	0.0527	0.6726	-	0.4502	0.5998	0.7641	0.7676	0.4795

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SD	0.0003	0.0004	0.0003	0.0002	0.0003	0.0003	0.0005	0.0001	0.0005	0.0006	0.0001	0.0003	0.0003	0.0002	0.0005	0.0002	0.0004	-	0.0004	0.0004	0.0003	0.0003	0.0005
PD	0.8615	0.9615	0.9326	0.9208	0.9182	0.9804	0.9257	0.9561	0.968	0.8534	0.8802	0.9136	0.9353	0.9554	0.9072	0.899	0.7716	0.3706	0.9543	0.964	0.9382	0.9596	0.8853
PIC	0.665	0.8406	0.7766	0.7515	0.7486	0.8973	0.7626	0.8186	0.8645	0.6566	0.6845	0.739	0.7874	0.8243	0.7216	0.7262	0.5232	0.3331	0.8238	0.8438	0.7923	0.8345	0.6946
MP	0.1385	0.0385	0.0674	0.0792	0.0818	0.0196	0.0743	0.0439	0.032	0.1466	0.1198	0.0864	0.0647	0.0446	0.0928	0.101	0.2284	0.6294	0.0457	0.036	0.0618	0.0404	0.1147
PE	0.4882	0.6891	0.5918	0.4752	0.5216	0.7754	0.5774	0.6891	0.7834	0.4948	0.3957	0.5353	0.6211	0.6585	0.4688	0.5774	0.2341	0	0.6661	0.6509	0.6359	0.6585	0.4688
TPI	1.9	3.27	2.45	1.85	2.06	4.55	2.36	3.27	4.72	1.93	1.55	2.13	2.66	2.97	1.82	2.36	1.11	0.5	3.04	2.9	2.77	2.97	1.82

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

Allele	D3S1358	D1S1656	D2S441	D10S1248	D13S317	Penta E	D16S539	D18S51	D2S1338	CSF1PO	Penta D	TH01	vWA	D21S11	D7S820	D5S818	TPOX	DYS391	D8S1179	D12S391	D19S433	FGA	D22S1045
5						0.058																	
6							0.002					0.128											
7										0.006	0.006	0.27			0.002	0.026							
8			0.002		0.292	0.004	0.002			0.004	0.066	0.052			0.152	0.016	0.58						
9					0.114	0.02	0.242			0.03	0.356	0.456			0.064	0.066	0.092	0.018					
9.1			0.024																				
9.3												0.028											
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			0.002																				
11		0.062	0.33		0.266	0.156	0.296	0.002		0.258	0.104				0.384	0.334	0.286	0.161	0.084		0.002		0.192
11.3			0.074																				
12	0.002	0.038	0.16	0.092	0.122	0.11	0.216	0.052		0.378	0.166				0.238	0.194	0.014	0.009	0.146		0.042		0.004
12.2																					0.002		
13		0.096	0.018	0.254	0.04	0.054	0.096	0.162		0.048	0.12				0.022	0.16	0.002		0.216		0.298		0.004
13.2																					0.03		
14	0.038	0.108	0.134	0.272	0.012	0.062	0.016	0.186		0.022	0.044		0.274		0.004	0.006			0.174		0.25		0.038
14.2																					0.15		
15	0.346	0.312	0.008	0.246		0.082	0.002	0.216			0.006		0.032						0.168	0.012	0.06		0.34
15.																					0.12		

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2																					2		
15.3																							
16	0.302	0.202		0.112		0.092		0.138	0.018		0.002		0.142						0.056	0.002	0.014	0.004	0.216
16.2																					0.026		
16.3		0.006																					
17	0.256	0.062		0.022		0.098		0.088	0.062				0.24						0.016	0.072	0.002	0.002	0.178
17.2																					0.002		
17.3		0.07																					
17.4						0.002																	
18	0.054	0.02		0.002		0.07		0.04	0.096				0.206							0.232		0.044	0.026
18.3		0.016																					
19	0.00					0.0		0.04	0.21				0.0							0.18		0.0	0.002

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	2					3							88							8		5	
19.3		0.002																					
20						0.044		0.022	0.1				0.016							0.164		0.062	
20.3		0.004																					
21						0.038		0.018	0.046				0.002							0.13		0.142	
21.2																						0.004	
22						0.014		0.026	0.034											0.09		0.176	
22.2																						0.002	
23						0.006		0.004	0.192											0.076		0.178	
23.2																						0.004	
24						0.006		0.002	0.148											0.022		0.162	
24.																						0.0	

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2																						1	
25						0.006			0.078											0.012		0.094	
25.2																						0.002	
26									0.016													0.046	
26.2																						0.012	
27																						0.006	
28														0.058									
28.2														0.002									
29														0.244									
30														0.26									
30.2														0.006									
30.3														0.002									

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31													0.092									
31.2													0.086									
32													0.034									
32.2													0.146									
33													0.004									
33.2													0.056									
34													0.002									
34.2													0.008									
Ho	0.74	0.808	0.776	0.792	0.78	0.92	0.792	0.856	0.856	0.748	0.796	0.7	0.784	0.84	0.724	0.812	0.588	-	0.808	0.848	0.836	0.848	0.796
He	0.7206	0.8279	0.7812	0.7811	0.7922	0.9183	0.7829	0.86	0.8659	0.7237	0.7988	0.6963	0.7973	0.8296	0.7517	0.7822	0.5736	-	0.8452	0.8489	0.8058	0.8731	0.7686
p	0.5801	0.5218	0.8653	0.1153	0.9815	0.2514	0.8619	0.8981	0.5189	0.6547	0.6321	0.513	0.2718	0.5724	0.612	0.279	0.8431	-	0.9374	0.1678	0.6747	0.5142	0.7061

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SD	0.0004	0.0004	0.0004	0.0003	0.0001	0.0004	0.0003	0.0003	0.0005	0.0004	0.0004	0.0005	0.0004	0.0002	0.0005	0.0003	0.0003	-	0.0003	0.0004	0.0003	0.0003	0.0004
PD	0.867	0.9525	0.9176	0.9067	0.9248	0.9829	0.916	0.9634	0.9634	0.8675	0.9328	0.8552	0.9234	0.9424	0.9023	0.9169	0.7506	0.3159	0.9558	0.954	0.9336	0.9672	0.9057
PIC	0.6673	0.8074	0.7477	0.7444	0.76	0.9105	0.7471	0.8427	0.8497	0.6742	0.7736	0.6507	0.7652	0.807	0.7137	0.7487	0.5096	0.2808	0.8238	0.8291	0.7783	0.858	0.7307
MP	0.133	0.0475	0.0824	0.0933	0.0752	0.0171	0.084	0.0366	0.0366	0.1325	0.0672	0.1448	0.0766	0.0576	0.0977	0.0831	0.2494	0.6841	0.0442	0.046	0.0664	0.0328	0.0943
PE	0.4928	0.614	0.5553	0.5843	0.5625	0.8364	0.5843	0.7067	0.7067	0.5064	0.5916	0.4283	0.5697	0.6753	0.4664	0.6215	0.2767	0	0.614	0.6909	0.6675	0.6909	0.5916
TPI	1.92	2.6	2.23	2.4	2.27	6.25	2.4	3.47	3.47	1.98	2.45	1.67	2.31	3.13	1.81	2.66	1.21	0.5	2.6	3.29	3.05	3.29	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

Allele	D3S1358	D1S1656	D2S441	D10S1248	D13S317	Penta E	D16S539	D18S51	D2S1338	CSF1PO	Penta D	TH01	vWA	D21S11	D7S820	D5S818	TPOX	DYS391	D8S1179	D12S391	D19S433	FGA	D22S1045
5						0.045																	
6							0.001				0.002	0.113											
7					0.001					0.004	0.016	0.295			0.002	0.018							
8			0.001		0.269	0.003	0.007			0.004	0.042	0.098			0.165	0.01	0.553						
9					0.111	0.016	0.219	0.001		0.031	0.401	0.37			0.058	0.055	0.097	0.044	0.002				
9.1			0.017																				
9.3												0.04											
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

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	1	1				97	1	2			08		67			1			1	9	7	01	
15.2								0.001													0.193		
15.3		0.004																					
16	0.299	0.2	0.001	0.128		0.08		0.132	0.019		0.001		0.14						0.086	0.012	0.009	0.002	0.169
16.2																					0.025		
16.3		0.007																					
17	0.287	0.06		0.029		0.085		0.085	0.087				0.23						0.015	0.082	0.004	0.001	0.176
17.2																					0.004		
17.3		0.074																					
17.4						0.001																	
18	0.062	0.017		0.002		0.061		0.045	0.073				0.241						0.003	0.194		0.028	0.015
18.		0.02																		0.00			

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3		4																		1			
19	0.004	0.001				0.036		0.044	0.186				0.092							0.186		0.068	0.007
19.3		0.001																				0.001	
20						0.047		0.019	0.099				0.015							0.188		0.045	
20.3		0.002																					
21						0.029		0.015	0.035				0.001							0.112		0.18	
21.2																						0.003	
22						0.017		0.014	0.068											0.108		0.194	
22.2																						0.007	
23						0.004		0.006	0.178											0.062		0.175	
23.2																						0.004	
24						0.0		0.00	0.15											0.02		0.1	

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						04		5	7											3		37	
24.2																						0.006	
25						0.005		0.001	0.074					0.003						0.018		0.075	
25.2																						0.003	
26									0.02											0.004		0.046	
26.2																						0.007	
27									0.003					0.002						0.001		0.013	
28														0.047								0.004	
28.2														0.002									
29														0.229								0.001	
30														0.252									
30.														0.01									

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2													4										
30.3													0.001										
31													0.129										
31.2													0.078										
32													0.042										
32.2													0.124										
33													0.003										
33.2													0.063										
34													0.002										
34.2													0.008										
35.2													0.002										
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

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	66	77	42	4	83	03	81	15	53	46	366	327		76	48		683		18	76	77	396	4
He	0.7215	0.8451	0.7958	0.784	0.7901	0.9133	0.7893	0.8523	0.8749	0.7177	0.7639	0.7456	0.8113	0.8391	0.7566	0.778	0.5867	-	0.848	0.8576	0.8189	0.8662	0.7567
p	0.9612	0.7189	0.3776	0.0386	0.6973	0.8127	0.417	0.9721	0.4792	0.3123	0.1525	0.5248	0.3937	0.5579	0.3248	0.2329	0.9047	-	0.5868	0.2632	0.8709	0.5142	0.7458
SD	0.0002	0.0002	0.0004	0.0002	0.0004	0.0002	0.0004	0.0001	0.0004	0.0003	0.0003	0.0004	0.0005	0.0003	0.0005	0.0004	0.0002	-	0.0003	0.0003	0.0003	0.0004	0.0003
PD	0.8664	0.9598	0.9288	0.9174	0.9241	0.9842	0.9226	0.9624	0.9691	0.8613	0.9147	0.8937	0.9354	0.952	0.9071	0.9125	0.7625	0.3496	0.9581	0.9623	0.9412	0.9661	0.8998
PIC	0.6681	0.8272	0.7659	0.7492	0.7576	0.9058	0.7566	0.8352	0.8611	0.6663	0.7358	0.7064	0.7833	0.8188	0.7213	0.7422	0.5176	0.3137	0.8286	0.84	0.7943	0.8506	0.7182
MP	0.1336	0.0402	0.0712	0.0826	0.0759	0.0158	0.0774	0.0376	0.0309	0.1387	0.0853	0.1063	0.0646	0.048	0.0929	0.0875	0.2375	0.6504	0.0419	0.0377	0.0588	0.0339	0.1002
PE	0.4905	0.6515	0.5736	0.5277	0.5416	0.8055	0.5808	0.6978	0.7452	0.5005	0.4872	0.4806	0.5954	0.6668	0.4676	0.599	0.2546	0	0.6401	0.6706	0.6515	0.6745	0.5277
TPI	1.91	2.9	2.34	2.09	2.16	5.26	2.38	3.37	4.01	1.96	1.9	1.87	2.48	3.04	1.82	2.5	1.16	0.5	2.81	3.08	2.9	3.12	2.09

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