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Sensitivity and Heterozygous Balance of Globalfiler IQC™ PCR Amplification and Verifiler Plus™ Kits

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Abstract

The Combined DNA Index System (CODIS) core loci was expanded from the existing 13 to 20 STRs, meaning a new kit will needed for many laboratories. A validation study was performed in accordance with SGWDAM guidelines Scientific Working Group on DNA. This main objective of this study was to measure the sensitivity and the heterozygous allele balance of Globalfiler IQC™ kit and Verifiler Plus™ kit in order to ensure that the kits would produce reliable profile. DNA samples ranging from 4 ng to 0.016 ng were amplified in 25 ul reaction volume to define the sensitivity and heterozygous balance of two systems. The data suggested that the Verifiler Plus™ kit is more sensitive than the Globalfiler IQC™ kit. However, the data of heterozygous balance also show that the Globalfiler IQC™ kit appeared to be more balanced peak heights than the Verifiler Plus™ kit. This performance demonstrated that both amplification kit can produce robust and reliable results in forensic DNA identification.

Keywords: internal quality control system; 6-dye multiplex assay; sensitivity; heterozygous balance.

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1. Introduction

Short Tandem Repeat (STR) Typing has proven to be an extremely discriminating method for human identification. A significant discrimination power of DNA typing increased by combining DNA profiling results at several independent STR loci. Many multiplex STR has been developed to deliver enhancing sensitivity for forensic sample which reflected from the expansion of the CODIS core loci to 20 STR [1-2].

The Globalfiler IQC™ PCR amplification kit is a multiplex STR 6-dye assay which co-amplify the 21 polymorphic STR markers D13S317, D7S820, D5S818, CSF1PO, D1S1656, D12S391, D2S441, D10S1248, D18S51, FGA, D21S11, D8S1179, vWA, D16S539, THO1, D3S1358, AMEL, D2S1338, D19S433, DYS391, TPOX, D22S1045, SE33, a Y-specific insertion/deletion locus (Yindel) and an internal quality control (IQC) system. This system consists of two synthetic sequences (74 bp and 435 bp) which can help determine degraded samples or PCR inhibitors. This study aims to compare the new STR kit with the Verifiler Plus™ kit in term of sensitivity and heterozygous balance.

The Verifiler Plus™ is six-dye STR multiplex assay and also contains two internal quality controls. This approach was also served to verify the quality of the kit to be helped in routine analysis for a new DNA profiling kit. The information from our study can be used to choose a suitable system for human identification with forensic samples.

2. Material and Methods

2.1 DNA samples

1224 Genomic DNA (InnoGenomics Technologies) was performed and analyzed with the following inputs: 4, 2, 1, 0.5, 0.25, 0.125, 0.063, 0.031 and 0.016 ng DNA. Quantifiler HP DNA quantification™ Kit (Thermo Fisher Scientific, Waltham, MA) [3] was used to quantify DNA concentration in triplication.

2.2 STR amplification

Amplification was performed using the Globalfiler IQC™ Kit (Thermo Fisher Scientific, Waltham, MA) [4] and the Verifiler Plus™ Kit (Thermo Fisher Scientific, Waltham, MA) [5] in 25 µl reaction volumes, 29 amplification cycles following the manufacturer's recommendation. DNA samples were amplified in triplicate for each kit using a ProFlex™ Thermal cycler (Thermo Fisher Scientific, Waltham, MA).

2.3 Capillary electrophoresis and Data Analysis

Amplified PCR products were detected on 3500xl Genetic Analyzer according to the manufacturer protocols using Data Collection Software v4.0. All profiles were analyzed using GeneMapper ID-X 1.6 software [6].

3. Results and Discussion

3.1 Sensitivity study

Sensitivity test was conducted to determine the lowest amount of input DNA that can be successfully amplified. Full 1224 Genomic DNA has 41 alleles (37 autosomal STR alleles, plus X and Y at Amelogenin, the Y indel, and an 11 allele at DYS391) when amplified using Globalfiler IQC™ kit and 44 alleles (41 autosomal STR alleles, plus X and Y at Amelogenin and the Y indel) when amplified using Verifiler Plus™ kit. Data were collected from triplicated amplification series ranging from 4 ng to 0.016 ng input DNA (Fig 1). Samples were amplified using Globalfiler IQC™ kit (Thermo Fisher Scientific, Waltham, MA) and the Verifiler Plus™ kit (Thermo Fisher Scientific, Waltham, MA), all amplified products were analyzed on the same 3500xL instrument. Full STR profiles were obtained reproducibility at 0.25 ng from both kits. Partial profiles were produced at their analytical threshold with a DNA input of 0.063 ng and 0.031 for each kit respectively.

In addition, complete profiles were obtained reproducibility at 0.125 ng input DNA using Globalfiler IQC™ kit and 0.063 ng using Verifiler Plus™ kit (Fig.1). This is concordance with previous studies in the developmental validation of Verifiler Plus™ PCR amplification kit [7-8]. Previous data showed that full STR profiles were obtained from all the samples ranging from 1 ng to 0.063 ng of total DNA input in triplication on 3500xL instrument. The validation of the Globalfiler IQC™ kit has not been published elsewhere, which is the original GlobalFiler™ kit to include the IQC system. The added enhancement of an IQC system is a sensitive indicator that allows for the peak-height ratio of the IQC Large to the IQC Small marker to be used to distinguish between normal reactions. However, previous studies reported that the Globalfiler IQC™ kit and Investigator 24plex QS® kit produced full STR profiles with 0.125 ng in comparison to the Powerplex Fusion® for DNA input 0.5 ng [9-10].

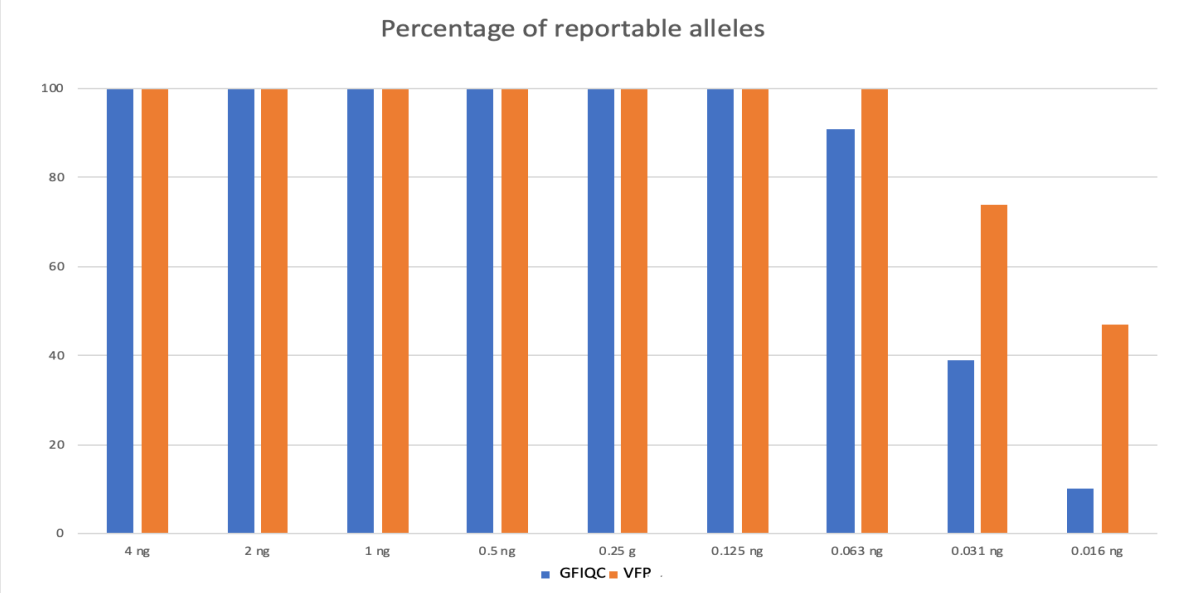

Figure 1: Percentage of reportable alleles detected in sensitivity test of input DNA ranging from 4 ng to 0.016 ng using Global IQC™ kit (GFIQC) and Verifiler Plus ™ kit (VFP).

The percentage of reportable alleles were showed in Figure 1, Verfiler Plus™ kit showed higher reportable alleles when compared to Globalfiler IQC™ kit with DNA input of 0.016 ng. These data suggested that Verifiler Plus™ PCR Amplification kit was more sensitive than Globalfiler IQC™ Amplification kit at the low level of DNA input. The results from both studies by Jasmine and his colleagues (2019) [11] founded that the Verifiler Plus™ has high sensitivity comparing to the PowerPlex Fusion® kit and Investigator® 24plex QS kit.

Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated from negative control samples (Table 1). With a maximum LOQ of 80 RFU, the lowest analytical threshold of 200 RFU was set for the sensitivity comparison for both kits.

3.2 Peak Height and Peak Height Ratio

The peak balance ratio is determined by dividing the peak height of an allele with lower RFU by the peak height of an allele with a higher RFU value. Heterozygous balance allele should be more than 60% according to the recommendations of ENFSI [12] in order to get the true homozygous allele at the low input DNA. Peak height ratio comparison was done with input DNA ranging from 0.016 ng to 4 ng at the lowest analytical threshold 200 RFU. Figure 2 and Figure 3 showed the peak height and peak height ratio at different input DNA when amplified by using Globalfiler IQC™ kit and Verifiler Plus™ kit respectively. These data demonstrated that heterozygous peaks of Globalfiler IQC™ were very well balanced at 0.5 ng input DNA when Verifiler Plus™ were balanced at 1 ng input DNA.

Furthermore, 0.125 ng input DNA, peak height ratio decreased from 99% to 46% with Globalfiler IQC™ kit and 99% to 31% with the Verifiler Plus™ kit. In general, the heterozygous balance was decreased when low input DNA. The balance peak is important when analyzed forensic sample mixed stain [13-17]. The locus imbalances may be confused between a single donor profile and a mixed profile.

Table 1: Limit of quantification value for the Globalfiler IQC™ kit and Veriifiler Plus™ kit. The Limit of quantification was calculated as mean + 10*standard deviation.

Dye channel	LOD		LOQ	
	GFIQC	VFP	GFIQC	VFP
Blue	8.53	7.42	19.67	17.2
Green	14.35	9.63	31.51	21.74
Yellow	8.52	7.15	19.23	16.61
Red	11.34	10.96	24.91	24.32
Purple	12.12	11.57	26.57	25.91
Orange	9.6	28.16	22.63	80.35

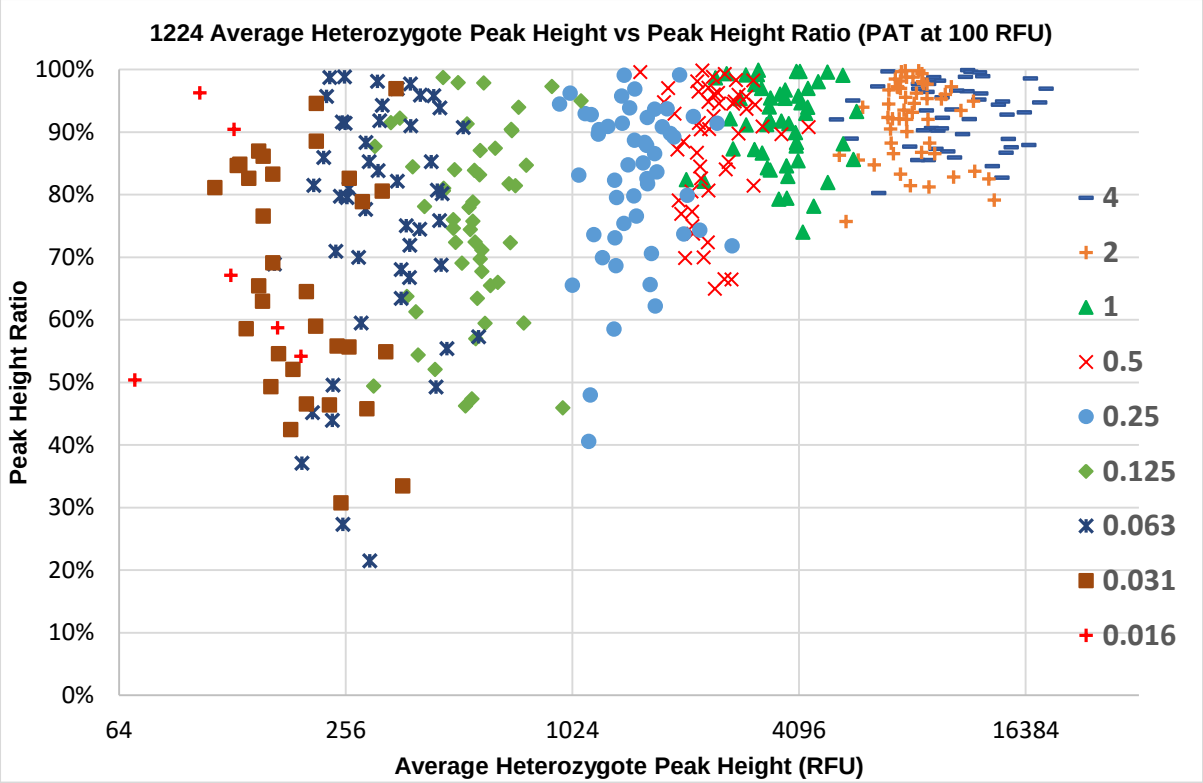

Figure 2: Heterozygous Peak Height and Peak Height Ratio for replicated in the DNA dilution series using Globalfiler IQC™ kit.

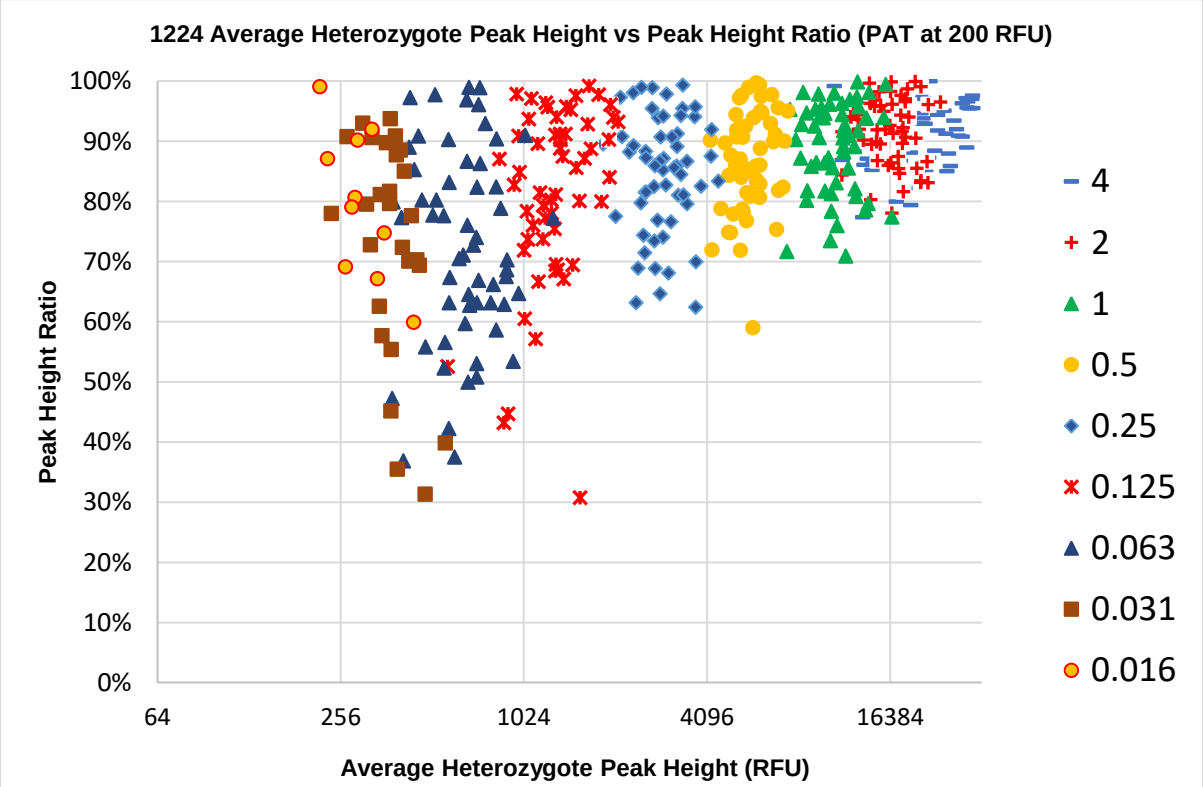

Figure 3: Heterozygous Peak Height and Peak Height Ratio for replicated in the DNA dilution series using Verifiler Plus™ kit.

4. Conclusion

This study is the first evaluate the sensitivity of Globalfiler IQC™ kit compared to Verifiler Plus™ kit in term of minimum input DNA to generate the full and balanced STR profile. The combination of 23 STR loci and 2 gender discrimination markers (Amelogenin and Y indel), the Verifiler Plus™ kit is the most highly discriminated system currently. In addition, 2 IQC markers are included in the primer mix that can help the inference of sample degradation or inhibition. However, another STR marker of choice in forensic analysis that highly discriminated and has the IQC system is the Globalfiler IQC™ amplification kit. Moreover, this kit has the three additional highly recommended CODIS core loci (SE33, DY391, Amelogenin). Moreover, Vranes and his colleagues (2019) [18] and Wang and his colleagues (2020) [19] reported the development and evaluation of the Investigator 26plex QS kit, which co-amplify 23 autosomal STR loci (TH01, D3S1358, Penta D, D6S1043, D21S11, TPOX, D1S1656, D12S391, Penta E, D10S1248, D22S1045, D19S433, D8S1179, D2S1338, D2S441, D18S51, vWA, FGA, D16S539, CSF1PO, D13S317, D5S818, and D7S820), one Y chromosome STR (DYS391), two internal quality control markers (Quality Sensor QS1 and QS2) along with Amelogenin. Unfortunately, our experiment does not study the performance of Investigator® 26plex QS kit. Furthermore, all three kits with IQC system can also be used for direct amplification of blood or buccal cells on FTA cards [4-5, 20-21].

In this study, we compare the sensitivity and heterozygous balance peak of two commercial STR kits (Globalfiler IQC™ kit and Verifiler Plus™ kit) with very low template DNA. The main results demonstrated that the minimum DNA input to generate a full and balanced STR profile is 0.063 ng for Verifiler Plus™ amplification kit. This study also showed that the Verifiler Plus™ kit is more sensitive than the Globalfiler IQC™ kit. The Globalfiler IQC™ kit was found to generate the balanced STR profiles at the minimum input DNA of 0.5 ng whereas the Verifiler Plus ™ kit was 1 ng. Overall, this study demonstrated that the Verifiler Plus™ kit and the Globalfiler IQC™ kit are robust and extremely useful STR assay for forensic human identification.

Acknowledgements

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5. Conflict of interest

None.

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Supplementary Table 1: Peak heights for each allele detected in the 1224 dilution series using Globalfiler IQCTM amplification kit (n = 3 replicates). A complete profile consists of 41 alleles (shown in green boxes). The surviving sister alleles are shown in yellow boxes. Alleles drop-out are shown in red boxes.

DNA (ng)	Rep	D3S1358		vWA		D16S539		CSF1PO	TPOX	Yindel	AMEL		D8S1179		D21S11		D18S51	DYS391	D2S441	D19S433		TH01	
		15	19	11	16	11	12	12	8	2	X	Y	13	16	29	31.2	14	11	14	14	15.2	6	**8**
4	1	10115	8831	12093	11132	10264	9468	14958	16606	17529	15594	13521	18344	17377	11420	9811	16135	7892	31870	14302	13495	11522	11508
	2	9609	10061	12616	12006	10905	9473	14523	16796	17820	16005	14010	18893	18312	11662	11254	16495	7612	30814	15628	13894	12681	12545
	3	9682	8774	11751	10534	9535	9709	13449	15699	17596	15542	12856	16771	17016	10957	10583	15479	8211	29507	14612	13865	11762	11716
2	1	8566	8242	8942	7282	7683	7655	12814	12495	11800	15110	11952	12242	11617	7881	7259	14366	7730	22049	8649	9400	7972	7873
	2	8058	9288	8287	8182	10020	8137	13872	14612	12033	14358	11842	10675	11426	8500	7917	16570	6851	22387	9189	8758	8354	7525
	3	7330	7444	7833	6777	7030	5959	11588	10585	11371	13072	10946	10550	10259	6988	7226	14180	6268	20391	8055	7531	6961	7556
1	1	3579	3288	3528	3367	3209	3309	5726	5338	4833	5389	5338	5279	6167	3107	2832	8532	3477	9671	4186	4455	3208	3518
	2	2947	2974	2924	2552	2793	2930	4689	4174	3405	5376	4404	4113	3479	2443	2477	6517	2913	8880	3835	3708	3680	4029
	3	4265	4038	3585	3289	3663	3088	6898	6155	5487	6036	5630	5718	5038	3738	3137	8140	3091	11044	3757	4398	3940	5043
0.5	1	2247	2484	2038	2467	1898	2145	3613	2721	2124	3071	3126	2166	3261	1870	1814	4031	1811	6032	2608	2103	1734	2193
	2	2052	2429	2454	2881	2441	1830	4188	3904	2827	3884	3482	3046	3228	2725	1972	4810	1469	5395	2546	2450	1858	2404
	3	2439	2410	1845	1985	2081	1815	3960	3149	2514	4560	4139	2597	2616	2404	1680	4674	2096	5862	2078	3125	2812	2764
0.25	1	813	1241	1538	1124	1416	1356	2022	1912	1426	1985	1967	1308	1993	1089	1586	2206	1235	2842	1209	1604	1546	742
	2	2018	1800	1561	1323	1773	1464	3335	2551	1835	2371	2594	2232	2064	1458	1200	3378	2068	4052	1462	1789	1890	1771
	3	1719	1510	992	1348	1032	993	2243	2205	2119	3172	2278	1872	1565	1409	1501	2888	1787	3528	1717	1315	1527	1479

0.125	1	688	480	419	517	564	515	934	636	657	892	917	625	544	469	630	1453	702	1539	424	568	517	417
	2	636	529	354	419	469	463	988	650	587	1053	1109	673	746	751	492	875	591	1714	798	650	766	626
	3	492	624	482	636	282	518	1172	805	350	1326	609	724	413	583	304	1215	415	949	603	590	753	357
0.063	1	309	273	304	455	271		467	385	456	736	422	397	414	289		873	317	654	234	224	290	235
	2	442	318	289	230	208	242	446	540	343	468	399	319	425	312	331	460		630	431	450	241	263
	3	358	347	286	341	320	273	414	289	371	412	514	287		201	283	1385	260	1184	501	552	280	441
0.031	1		258					350			355	344		545			255		419	318	251		
	2										318		209	221				240	368		313	246	
	3	401			245	221		251	222								339			262			276
0.016	1												254				365		228				
	2											281							246				
	3																						

Supplementary Table 1: (cont.) Peak heights for each allele detected in the 1224 dilution series using Globalfiler IQCTM amplification kit (n = 3 replicates). A complete profile consists of 41 alleles (shown in green boxes). The surviving sister alleles are shown in yellow boxes. Alleles drop-out are shown in red boxes.

DNA (ng)	Rep	FGA		D22S1045	D5S818		D13S317		D7S820		SE33		D10S1248		D1S1656		D12S391		D2S1338	
		23	24	16	10	12	10	13	10	11	14	25.2	13	14	15	17	18	19	17	23
4	1	16855	15696	20586	9141	8813	10793	10100	9223	8325	9752	8341	6727	6542	9129	8978	5970	5308	12395	12335
	2	17811	15658	21140	9162	8949	11286	11425	9236	8630	10294	9324	7073	7049	9405	9288	5551	5843	14502	12260
	3	15177	14079	18993	8396	8664	10081	9094	8595	7536	9143	7820	7412	5949	8619	8418	5367	4938	12706	12217
2	1	10033	9559	14763	8052	7693	8543	8523	7371	7581	7535	6818	7290	6886	9689	8548	5631	4858	10085	8725
	2	11547	9559	14667	7613	7395	8687	8887	7849	7865	8249	7590	8326	6934	8869	9086	6352	5432	10229	9819

	3	8648	8820	13727	7349	6782	8073	8537	7556	7699	7664	6761	6246	5867	7748	7513	6221	4710	8729	8671
1	1	4115	4425	5774	3338	2910	4402	4268	4241	3369	3522	3310	3196	3200	3474	4191	2066	2516	4117	4130
	2	4289	3763	5087	3165	3192	3787	4287	4042	3205	3166	3089	2642	2624	3038	3506	2258	1861	3848	3671
	3	4868	4890	7107	3725	3578	4661	4569	3992	4170	3798	4224	3843	3526	4030	4043	2584	2805	4821	3568
0.5	1	2893	2776	3431	2223	2180	1885	2695	2353	2040	2176	2256	2268	2272	2323	2393	1746	1842	1894	2353
	2	2902	3035	4128	2527	2338	3426	2790	2983	2678	2137	2363	2815	2660	2387	2486	2258	1737	3434	3121
	3	3073	2879	4426	2295	2421	1930	2971	2322	2123	2583	2443	2849	2394	2470	1822	1556	1549	2837	2692
0.25	1	1215	1337	1940	1591	1411	1818	1573	1496	1190	1450	1014	1136	1267	1145	1267	976	922	1416	1403
	2	1863	1692	2994	1687	1557	1907	2566	1454	1709	1662	1326	2335	1721	1841	2304	1194	1108	1378	1952
	3	2099	1305	2388	1068	1149	1967	1766	1697	1499	654	1612	977	1670	1753	1642	1165	968	1455	1330
0.125	1	516	505	719	449	755	956	569	478	613	456	543	372	343	704	477	354	324	597	683
	2	762	716	1102	690	491	710	838	515	614	287	327	427	618	365	467	408	202	703	446
	3	428	563	854	338	730	783	517	742	671	583	422	489	300	657	476	291	456	589	814
0.063	1	613	340	611	442	471	318	387	463	345	361			329		397	318		235	232
	2	327	229	436	354	211	400	364	303	330	429	292	277	221		233	257	254	518	393
	3	402	498	517	245	268	386	377	254	327	309	315	545	375	490				295	598
0.031	1	356	287	310		218			228	202			240		269		236			249
	2			268															382	
	3	288	238	324	202		233	424	337			259								

0.016	1						214		227		
	2										
	3										

Supplementary Table 2: Peak heights for each allele detected in the 1224 dilution series using Verifiler Plus TMamplification kit (n = 3 replicates). A complete profile consists of 44 alleles (shown in green boxes). The surviving sister alleles are shown in yellow boxes. Alleles drop-out are shown in red boxes.

DNA (ng)	Rep	D3S1358		vWA		D16S539		CSF1PO	D6S1043		Yindel	AMEL		D8S1179		D21S11		D18S51	D5S818		D2S441	D19S433	
		15	19	11	16	11	12	12	11	21.3	2	X	Y	13	16	29	31.2	14	10	12	14	14	15.2
4	1	30992	30250	22269	18321	24506	22499	25698	19093	15272	31189	31374	29965	27478	25678	23420	20442	32072	15611	14861	31633	24173	22820
	2	29678	26945	21347	16948	22652	19588	23519	15467	13376	30585	30520	29141	24041	25280	21670	20326	30594	14417	14713	30388	22293	23155
	3	28824	27766	20304	17887	23208	19780	24227	15015	11621	29923	29998	28689	27261	23183	20897	20420	29755	14147	13666	29962	21024	21661
2	1	18592	16262	14085	12701	16072	14398	20414	11816	12559	19727	18837	16097	14885	14268	14053	14008	26944	13009	11858	28975	13852	14379
	2	23236	20130	15249	16056	18797	17347	25020	15696	12601	27512	24409	23560	17267	18303	18053	18695	29621	17410	14969	31315	16979	16325
	3	19492	18324	15068	13487	18678	14579	21866	13528	12448	21354	21640	18501	16983	15733	15569	14939	27356	14884	14413	32002	16478	15161
1	1	11595	11165	9343	9941	9645	9440	20576	9591	8873	13035	13707	9726	9631	7878	10035	9485	20386	10854	12176	22681	10574	10167
	2	9721	8344	8962	8490	7621	8438	15342	6265	8742	12829	10714	9259	7764	8904	7507	7878	18419	11427	9782	22075	9012	11027
	3	12802	12815	12918	12582	13823	11351	22415	12023	8834	16609	15465	12318	9894	11901	11181	12063	26131	13866	14131	27351	10827	12270
0.5	1	6781	6628	5771	6499	6255	6097	12465	5228	4584	6636	6296	5094	5595	5654	6052	6384	13296	7752	7366	13864	5647	6011
	2	7297	4305	5251	4960	5087	5509	10711	5639	4909	5959	6107	4975	5091	4013	6386	5484	10030	7991	6582	11763	5998	4722
	3	598	509	532	517	605	473	10442	494	3559	5661	5709	5279	4420	398	496	445	10422	4853	577	9328	5609	419

		4	4	3	6	9	8		7					6	0	0			7		8
0.25	1	3924	3125	3647	2484	3460	3085	5656	2708	3177	3485	3513	3018	2749	3153	3489	3331	6522	2894	4637	6516	2220	2486
	2	2419	2135	3028	2747	2744	2921	4700	2988	2135	2651	2830	2308	1794	2314	2844	2320	6178	3290	2723	5801	2369	2324
	3	2256	3490	3048	2983	2418	2738	5821	2054	2262	3210	3851	3623	2762	2411	3447	2952	5022	3271	3773	4456	3543	4292
0.125	1	1110	1422	1326	1052	1216	1751	2358	912	1074	1268	1425	815	1203	1078	1202	1256	2482	1469	1285	2423	1514	1737
	2	1443	1171	911	793	1285	778	1975	756	398	1707	2402	740	962	983	1079	1571	2100	1030	1099	2368	938	1032
	3	1601	1724	1051	1360	1242	1364	2480	917	1375	1008	2050	1851	1470	1341	2084	1959	3608	1288	1412	2779	1125	1402
0.063	1	244	515	674	681	836	525	972	518	530	710	740	732	580	451	340	425	884	603	223	1227	979	619
	2	683	1085	620	715	962	759	1628	677	656	558	953	485	520	737	744	715	976	1233	659	1154	540	759
	3	452	715	591	812	883	558	1608	484	413	1116	746	1061	456	406	1197	775	1141	874	585	1252	441	485
0.031	1	283	257		275	363		612	536	377	203	403		277	348		653	670	475	344	899	344	383
	2	325		374		250		676			345		386	351		383	493	923	386	362	462	293	315
	3		266			581	207	735			229	382	449		261		219	433		535	284	423	265
0.016	1	412		238				502			267	232		378		315	218	459	312	339	344	377	
	2	280				256	317	485		424		366					348	588	276			384	
	3							526										655		289	605	381	200

Supplementary Table 2: (cont.) Peak heights for each allele detected in the 1224 dilution series using Verifiler Plus TMamplification kit (n = 3 replicates). A complete profile consists of 44 alleles (shown in green boxes). The surviving sister alleles are shown in yellow boxes. Alleles drop-out are shown in red boxes.

DNA (ng)	Rep	FGA		D10S1248		D22S1045	D1S1656		D13S317		D7S820		Penta E		Penta D		TH01		D12S391		D2S1338		TPOX
		23	24	13	14	16	15	17	10	13	10	11	17	20	9	12	6	8	18	19	17	23	8
4	1	22188	22182	15899	13861	31633	30920	30004	24364	21548	16072	15777	13253	11707	27578	30990	23897	27825	16564	16705	18474	16013	31203
	2	21617	20092	14723	12828	30943	30028	29015	22001	18644	15821	13813	12213	10611	26184	28792	23869	27136	14544	14820	18986	16469	29304
	3	22168	20602	14453	12444	30531	29528	28689	21294	20162	15663	14538	10786	10698	25995	28258	23851	26138	15431	13152	16305	17846	28212
2	1	19061	16134	13297	12454	25805	18944	20928	17715	15306	13118	12385	11874	10874	16308	16594	16541	16524	13174	12442	12171	12648	23978
	2	22572	18832	17522	15996	31207	22354	21489	23853	19825	18827	18589	15936	13830	18769	22573	19846	19834	18728	17169	18987	17027	30780
	3	19362	17554	15498	14246	30997	20787	20592	18666	18118	15568	14000	12354	10429	19939	16273	17600	18024	12564	11819	14942	15213	27557
1	1	13244	11805	12027	10895	18217	12291	11919	12010	11024	10625	10840	10289	9743	9077	9521	10052	9109	9684	7768	8697	8070	19974
	2	11796	9245	11024	12887	20355	11730	11077	12583	11443	10061	8701	9580	10199	9532	9968	11579	9416	8418	8581	10982	9583	19351
	3	16115	15147	13296	14167	25374	15918	15834	18721	14493	12636	13196	9478	12471	13419	12309	12001	15266	13992	11313	14049	13184	25899
0.5	1	7772	6997	6423	7143	13147	6875	6390	6346	7755	6233	5317	4805	6259	6529	5431	5108	5633	5133	6012	5623	4382	13883
	2	6624	7330	5963	5947	10017	6371	6037	7194	6558	5970	7925	6519	5447	5346	4913	5661	6580	6146	4420	6873	7192	14762
	3	5547	6689	5978	6295	9930	6095	6132	5457	6763	4425	5247	5528	4139	5427	4929	5396	5270	4885	5385	5479	4715	11462
0.25	1	4877	4068	3034	2857	5074	4066	4422	3428	3129	3648	3083	4435	3104	2205	2764	2942	1858	2661	3070	2559	2977	5322
	2	2955	3646	2768	2643	5205	3428	3406	3188	2451	3007	2480	2991	3296	2157	2099	2485	2510	1972	1766	2880	1985	6562
	3	394	450	2905	216	6875	383	3674	327	346	2700	272	338	2504	3284	226	2330	317	2712	353	3788	3073	5902

		3	4		1		7		1	8		9	0			1		3		8			
0.125	1	1538	1501	1357	1276	2802	1272	1432	1805	1602	1446	1385	1423	1284	533	1232	1042	1279	1686	1672	1389	1734	2168
	2	1662	1116	1044	864	2326	1006	1364	1473	1112	1177	923	1408	1644	1103	1071	1232	1188	1258	563	1548	1060	2339
	3	2132	1792	1248	949	3339	2016	1937	2169	2022	1196	860	1426	1497	897	1217	1640	2050	1542	1073	1785	1826	1821
0.063	1	1072	724	610	551	988	752	912	794	738	493	825	822	531	737	386	409	722	490	631	459	355	1154
	2	989	1087	449	898	963	649	980	791	683	440	428	610	824	790	651	734	1069	527	423	876	792	1194
	3	761	579	332	883	1601	616	1050	1445	1116	346	818	498	938	472	588	626	350	700	472	530	637	1279
0.031	1		550	269	485	714	286		410	335	366		550	382	210	269	414	330	271	372	591		789
	2	741	233			432	389			496	342	310		449	684		417	366		200	265		726
	3	355	506	445	257	906	370	407	809	323	517	234	428	379		271	271			301	311	383	485
0.016	1			307	277	387		342		202					306	409	217	249					285
	2	277		557	334	287	457		219	221	230		467				353				210		524
	3		308	406	273	503					214		312	247			260			320		483	479