Predicting the success of STR typing using the Plexor® HY System and extraction of mock cases using the Differex™ System

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The Plexor® HY System (Plexor), a novel triplex q-PCR assay, was developed to simultaneously quantitate total autosomal and male DNA in a sample and utilizes an internal PCR control (IPC). The triplex makes use of a multicopy target on human chromosome *17* to quantitate total autosomal DNA and a multicopy sequence on the human *Y* chromosome to quantitate male DNA. The Plexor technology correlates the reduction in sample fluorescence to a directly proportional increase of input DNA; the more genomic DNA in the sample, the smaller the Ct value when the signal drops.

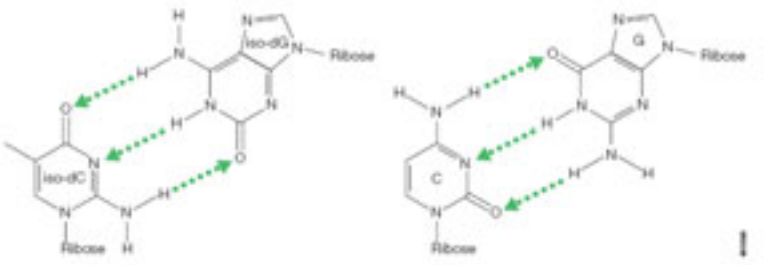
Studies were undertaken to assess the predictive value of Plexor for STR typing success. Not only were sensitivity studies performed using single source male DNA samples, but also mixture studies where the total autosomal DNA quantity remained constant while the male DNA quantity decreased. The observed and reproducible lower limit of detection was ~8.0 pg/µL. Mock casework samples were created using the Differex™ System (Differex), an automated differential extraction procedure that utilizes the Slicprep™ 96 Device, a 96 well spin basket device. The performance of Differex was compared to the Virginia Department of Forensic Science's semi-automated differential extraction procedure and was found to produce very similar DNA yields for the fractions and STR typing results. Both sets of mock casework samples were utilized to test the ability of Plexor to predict, using PowerPlex® 16, 16 BIO and Y, the STR typing outcome success. Most striking were mixture samples containing a large excess of female DNA, which provided only the female contributor's PowerPlex®16 profile, but Plexor quantitation data indicated that the samples contained a small amount of male DNA and thus provided PowerPlex® Y typing results. One such sample was measured to contain 3,350X more female to male DNA and only 0.008 ng/µL of male DNA. That sample produced a nearly complete PowerPlex® Y profile consisting of 92% of the alleles of the complete male profile.

Non-probative casework samples were quantitated with Plexor, the previous STR typing results evaluated and the samples typed for PowerPlex® Y. The accuracy of the Plexor quantitation data and its predictive value for STR typing was similar to that observed for the mock casework samples. Guidelines for implementation and the use of Plexor to guide downstream processing and typing decisions are being determined.

Introduction

Human DNA specific quantitation is a required and routine procedure in the analysis of forensic casework. Quantitative real-time PCR (q-PCR) has rapidly become the most widely used method for DNA quantitation in forensic laboratories. The Plexor® HY System (Plexor) is a q-PCR triplex assay comprised of an internal positive control (IPC), primers that recognize an autosomal DNA multi-copy target on chromosome 17 and primers that recognize a male DNA multi-copy target on chromosome Y (1). Plexor exploits the use of the modified bases, Methylisocytosine and Isoguanine. The modified bases hydrogen-bond to each other, but not to any other nucleotides (Figure 1, panel A). The 5' primer used in the Plexor procedure has a fluorescent reporter attached and a Methylisocytosine at the 5' terminus. During the extension step of the polymerase chain reaction (PCR) process, a Dabcyl-iso-dGTP residue is incorporated when it base-pairs with the iso-dC residue (Figure 1, panel B). The Dabcyl group is a quencher and therefore, the more rapidly the fluorescent signal is quenched, the greater the quantity of genomic DNA in the sample. Thus, the amplification plot of fluorescent signal is the inverse of the Taqman® assay q-PCR plot.

A.



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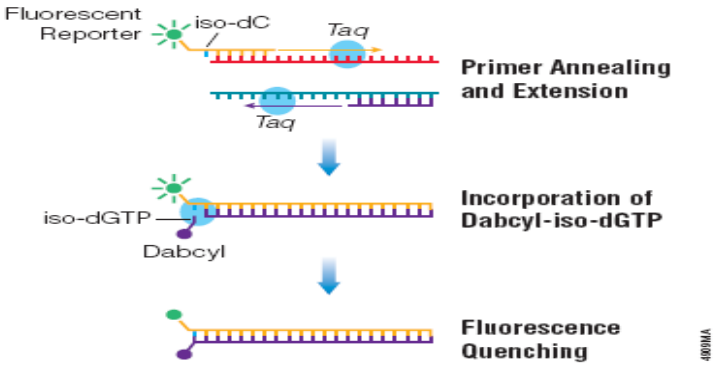

Figure 1. Plexor technology. A. Modified bases (left), Iso-dC and Dabcyl-iso-dG, base-pair with each other. Normal base-pairing (right). B. Fluorescent signal quenching during the PCR process.
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The goals of these experiments reported by the Virginia Department of Forensic Science (VDFS) were to: 1) Evaluate the sensitivity and accuracy of Plexor using male/female mixtures and single source male samples. 2) Analyze mock casework samples by comparing the performance of the Differex automated differential extraction system with the VDFS semi-automated differential extraction procedure and assess the predictive value of Plexor quantitation data by STR profiling. 3) Evaluate the predictive performance of Plexor using non-probative casework samples.

Materials and Methods

Mixture and sensitivity samples were prepared with either tissue samples extracted using phenol/chloroform followed by Microco®n purification, buccal or blood samples extracted using the DNA IQ™ System and Biomek® 2000 Automation Workstation (Biomek) as described (2) or commercially prepared cell line DNA samples, GM9947A and GM9948 (Promega Corp., Madison, WI). Samples used for the sensitivity and mixture studies were quantitated using the AluQuant™ Human DNA Quantitation System (AluQuant) as described (3). Female:male mixtures were created by mixing 1 ng/µL of female DNA with decreasing amounts of male DNA. These mixture samples were quantitated with Plexor in triplicate on two separate days.

Twelve mock sexual assault samples were provided by Promega Corp. The samples were prepared by placing approximately 1,000 (1K), 10,000 (10K) or 50,000 (50K) spermatozoa on an epithelial swab (vaginal [Vag] or buccal [Buc]). The swabs were cut into equal halves and each half processed using the VDFS semi-automated DE procedure and the Differex DE procedure. A single measurement for Plexor quantitation was performed.

Plexor was employed as described by the manufacturer for use with the Stratagene MX3005P real-time PCR instrument (1). Thermocycler plates used for the real-time PCR were purchased from Greiner Bio-One (Monroe, NC) and both the real-time PCR film and caps were utilized. The gain settings for the instrument were modified depending on whether the film or caps were used. When using the film, the gain settings were: 2X FAM, 1X C560, 1X C610 and 1X Cy5. When using the caps, the gain settings were: 4X FAM, 2X C560, 1X C610 and 1X Cy5.

STR typing using the PowerPlex® 16 and PowerPlex® Y Systems was performed as defined by the manufacturer's recommendations (4,5). Fragments were separated using a 310 Genetic Analyzer (Applied Biosystems, Foster City, CA), the raw data were collected with ABI Data Collection Software and analyzed using GeneScan and Genotyper software, versions 3.1 and 2.5, respectively. Allele calls were performed using the PowerTyper™ 16 or PowerTyper™ Y Macros (Promega Corp.). STR typing of casework samples was performed using the PowerPlex® 16 BIO System and the FMBIO gel imaging system as described (3,6). The FMBIO III+ was also utilized to analyze the PowerPlex Y typing of the same casework samples (non-probative). Conditions used were as described for

the PowerPlex® 16 BIO gels, however the JOE filter was replaced with a 555 nm filter.

Results

A sensitivity study using a single source male DNA sample was performed in duplicate. Concentration estimates generated by the Plexor assay (performed were similar to the expected DNA concentration based on the AluQuant quantitation values and the DNA dilution (Table 1).

A mixture study was performed using female and male DNA samples. Female DNA (1 ng/µL) was mixed with decreasing concentrations of male DNA (1 – 0.00375 ng/µL). These mixture samples were quantitated in triplicate on two separate days. The data demonstrate that the male DNA quantity estimates were consistent (small SD) and slightly below the estimated value (Table 2).

Genomic DNA (ng/uL)	Auto (ng/uL)	Y (ng/uL)	Auto (ng/uL)	Y (ng/uL)
0.2	0.22	0.29	0.18	0.31
0.1	0.17	0.21	0.11	0.17
0.05	0.088	0.12	0.079	0.1
0.025	0.034	0.046	0.026	0.033
0.0125	0.023	0.029	0.01	0.027
0.009375	0.018	0.025	0.029	0.024
0.00625	0.031	0.021	0.0082	0.016
0.003125	0.0087	0.0044	0.0086	0.0062
0.00156	-	0.0062	-	0.0055
0	-	0.0022	-	-

Table 1. Male DNA sensitivity series quantitation data.

Mock casework samples were created using both the automated Differex™ System (Differex) and the semi-automated VDFS differential extraction (DE) procedures using the Biomek robot. The Differex procedure utilizes the Slicprep™ 96 device (Slicprep), which is a 96 well spin-basket fashioned to fit into a 96 deep well plate. The use of the Slicprep device allows for the automation of DE process from the point of placing the sample cutting into the Slicprep device inserted into a deep well plate. The semi-automated VDFS DE procedure requires that the examiner first generates a non-sperm lysate and washed sperm pellet. These two isolates are then pipetted into a deep well plate for robotic DNA purification using the Biomek robot. The performance of the Differex and the VDFS DE procedures were compared and evaluated for DNA yields for the sperm and non-sperm fractions, which were similar (data not shown) and for STR typing. The number of alleles out of the total possible for the donor (female or male) was determined for each fraction and assessed as to whether or not the full complement (100 percent profile) of the female and male

donor alleles were observed when typed for PowerPlex® 16 and for male donor alleles when typed for PowerPlex® Y.

Expected [Autosomal] (ng/µL)	Average [Autosomal] (ng/µL)	Standard Deviation	Expected [Y] (ng/µL)	Average [Y] (ng/µL)	Standard Deviation
1	0.8733	0.1131	0.5	0.2867	0.0566
0.75	0.7050	0.1438	0.25	0.1850	0.0071
0.625	0.4967	0.1508	0.125	0.0867	0.0094
0.56	0.4267	0.1179	0.063	0.0322	0.0064
0.53	0.3467	0.0377	0.031	0.0155	0.0021
0.52	0.3683	0.1391	0.016	0.0083	0.0005
0.51	0.3017	0.0589	0.008	0.0072	0.0002
0.5	0.3467	0.0377	0.004	0.0014	0.0003
0.5	0.2883	0.0872	0.002	0.0001	0.0002

Table 2. Female:Male mixture series.

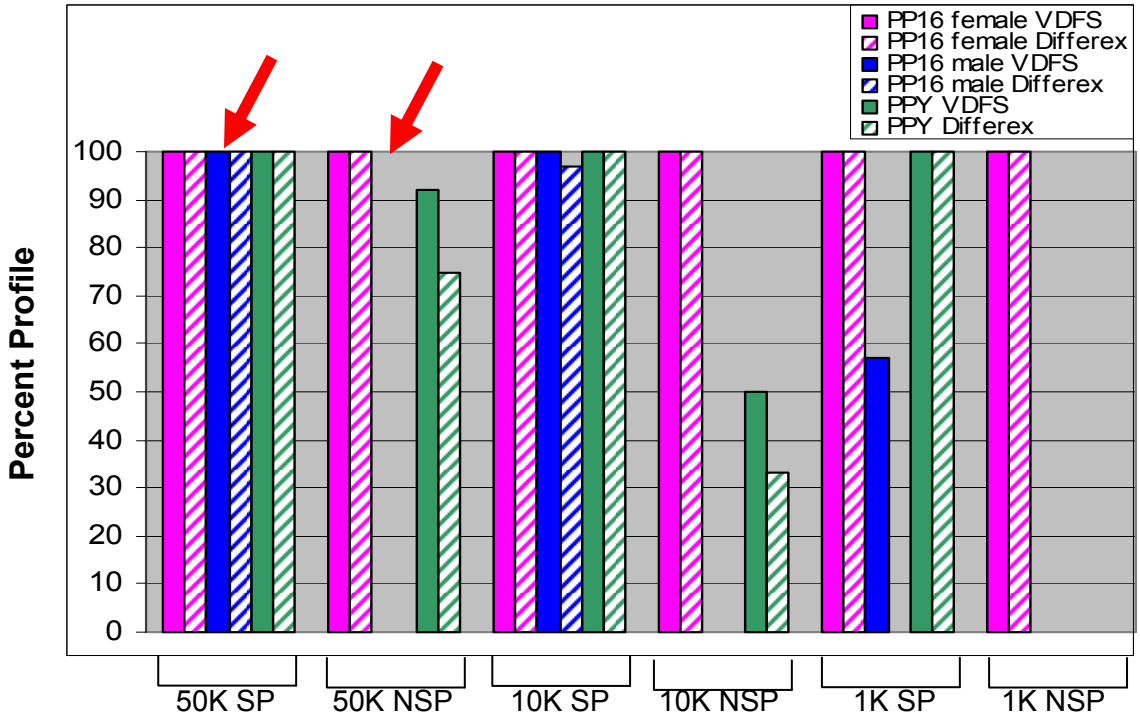

Figure 2. Vaginal Swabs plus 50K, 10K or 1K sperm cells.
Key: PP16 = PowerPlex® 16 System profile, PPY = PowerPlex® Y System profile, SP = sperm fraction, NSP = non-sperm fraction. Arrows point to the typing results for the samples shown in red type in Table 3.

The percent profile was plotted for the PowerPlex® 16 and the PowerPlex® Y typing results (Figures 2 and 3). In some samples, a greater percent profile was observed for either the Differex extracted or VDFS extracted samples. For example, the male contributor to the PowerPlex® 16 profile was not observed for the Differex extracted fraction (Figure 2, 1K sperm fraction [SP]) or far fewer alleles were observed for the male and female contributors to the PowerPlex® 16 profile for the VDFS extraction fraction (Figure 3, 1K SP). Overall, the percent typing results were similar for both the VDFS and Differex DE procedures indicating that the performances were generally equivalent.

When the Plexor quantitation data were compared with the percent typing success for the PowerPlex® 16 and the PowerPlex® Y Systems, an excellent correlation was observed between the quantitation data and the STR data (Table 3, Figures 2 and 3). Arrows point to typing results in Figure 2 for the 50K

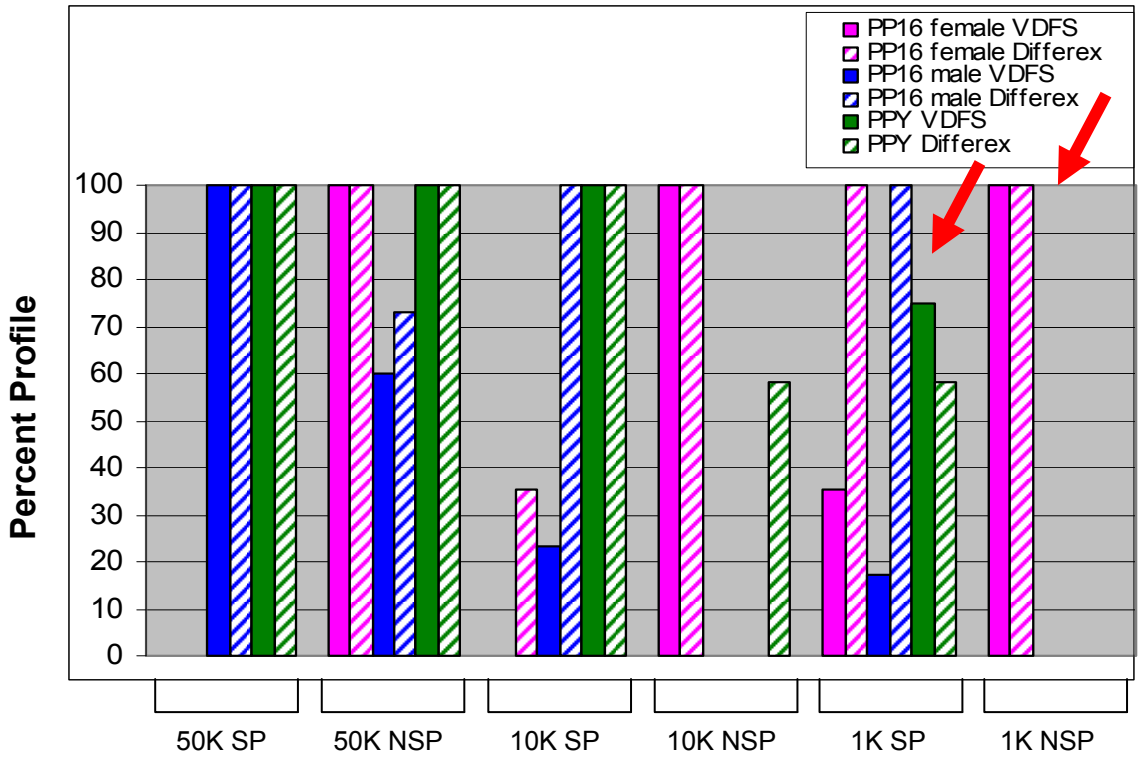

Figure 3. Buccal Swabs plus 50K, 10K or 1K sperm cells. Key: PP16 = PowerPlex® 16 System profile, PPY = PowerPlex® Y System profile, SP = sperm fraction, NSP = non-sperm fraction. Arrows point to the typing results for the samples shown in red type in Table 3.

vaginal non-sperm fraction (NSP) sample which displayed only 8 pg/µL of male DNA in the mixed sample, at a ratio of 3,350:1, autosomal to male DNA, respectively. The STR typing results corresponded to the quantitation data in that all female and no male contributor alleles were observed for the PowerPlex® 16 typing results and a partial profile was observed for the PowerPlex® Y typing

results, as expected. The SP typing results for the 50K vaginal swab displayed predictable results based on the quantitation data since the full complement of alleles for both contributors were observed with PowerPlex® 16 and a complete male profile was observed with PowerPlex® Y. Only the quantitation data for the VDFS DE procedure is displayed in Table 3. The quantitation data for the Differex extracted samples was similar except that a slightly lower yield for the non-sperm fraction samples was observed (data not shown, personal observations).

VDFS DE Samples	Plexor [Autosomal] (ng/µL)	Plexor [Y-DNA] (ng/µL)	Input Y DNA (ng)	Ratio [Auto]/[Y]
50 K Vag SP	6.090	1.990	0.75	3.06/1
50 K Vag NSP	26.800	0.008	0.02	3,350.00/1
1K Buc SP	0.036	0.008	0.02	4.50/1
1K Buc NSP	3.320	0.001	0.00	6,640.00/1
10 K Vag SP	7.200	1.060	0.75	6.79/1
10 K Vag NSP	26.300	0.002	0.01	13,150.00/1
10 K Buc SP	0.080	0.056	0.14	1.43/1
10 K Buc NSP	1.890	0.006	0.02	315.00/1
1 K Vag SP	11.670	0.060	0.15	194.50/1
1 K Vag NSP	70.170	0.001	0.00	116,950.00/1
50 K Buc SP	0.180	0.150	0.38	1.20/1
50 K Buc NSP	0.750	0.025	0.06	30.00/1

Table 3. Plexor quantitation data for the mock casework samples.

When examining the results for the buccal swab DE samples shown in Figure 3, typing results which corresponded to the Plexor data were also observed. While the data for the 1K SP fraction showed some variation due to differences in percent profile for the VDFS and Differex procedures, the STR typing outcome was generally predictable based on the quantitation data. For the 1K NSP for the buccal swabs, only 1 pg/µL of male DNA was observed, at a F:M ratio of 6,640:1 and no male alleles were observed with either PowerPlex® 16 or PowerPlex® Y typing.

Non-probative casework DNA samples were quantitated with Plexor and if male DNA was indicated, PowerPlex® Y typing performed. Table 4 displays data from six of these non-probative casework samples. PowerPlex® Y and previous PowerPlex® 16 BIO typing results were consistent with the Plexor quantitation data except for Case 2 which contained an estimated 41.4 pg/ µL of male DNA, with an autosomal:male ratio of 0.4. No PowerPlex® 16 BIO results were obtained from previous casework analysis, but a complete PowerPlex® Y profile was obtained, which provides evidence in support of Plexor accuracy.

Sample	Case Scenario	Original Typing Results	Conclusions Drawn	Plexor Quant (ng/uL) Auto	Y	Ratio	PowerPlex Y Results
Case 1 Item B4	TEG swab from rape victim analyzed. Alleles foreign to victim consistent with suspect.	6/32 alleles were foreign to victim	No conclusion drawn about inclusion or exclusion of suspect.	.202	**.00632**	31.94	Partial profile **5/12** loci
Case 2 Item H1	5 sperm heads on swabs from speculum.	SP fraction: no amp via PP16 BIO	No conclusions drawn.	.0165	**.0414**	0.4	Full profile **12/12** loci
Case 3 Item A6	Few sperm found on v/c swab from PERK kit. Unable to separate male from female profile.	13/32 alleles foreign to the victim	Alleles foreign to victim not suitable for searching in SDIS and NDIS. May be suitable for direct comparison.	.292	**.14**	2.08	Full profile **12/12** loci
Case 4 Item F2	Sperm fraction from v/c swab, P30 positive, no foreign profiles in the autosomal results.	No profile foreign to victim with autosomal typing.	No conclusion could be drawn about suspect.	1.03	**.00694**	148.35	Partial Profile 11/12 loci
Case 5 Item F1	P30 positive with some foreign alleles.	Alleles foreign to the victim were found at 12 loci.	Profile foreign to the victim was searched with no hit.	.0201	**.0022**	9.13	Partial Profile 11/12 loci
Case 6 Item B1	Sample from the underpants of rape victim. Sperm fraction was positive for P30 and did not amplify	No typing was done because of the lack of amplified product	No conclusion was drawn regarding the sample because of the failed amp.	.000954	**.00039**	2.45	Partial Profile 1/12 loci

Table 4. Non-probative casework sample analysis.

Discussion

Plexor demonstrated a reproducible limit of detection for a male DNA sample, in a mixture or single source, at approximately 8.0 pg/µL. This is similar to the reported limit of detection reported in the Plexor HY System Technical Manual, 6.4 pg/µL and also to other reported autosomal and male q-PCR assays (7).

Extraction of the mock casework samples demonstrated that the Differex and VDFS DE robotic procedures produced equivalent STR typing outcomes for the most of the sperm and non-sperm fraction samples. Some differences were observed in the percent profile produced for samples when comparing the

Differex and VDFS methods. These might be explained by inhibition during the PCR amplification or simply normal variation when purifying DNA from mixed source samples.

Comparison of the Plexor quantitation results with the STR typing results clearly demonstrated that the Plexor data provided an accurate prediction of STR typing outcomes. The success at accurately predicting STR typing results translated to non-probative casework samples. Six non-probative casework samples were typed for PowerPlex® Y after the Plexor quantitation indicated that male DNA was present in the samples. All samples showed excellent concordance between the Plexor results, the original casework typing results and the PowerPlex® Y data except for Case 4. While that sample provided a complete PowerPlex®Y profile, which is consistent with the Plexor data, no PowerPlex® 16 BIO results were obtained during original casework profiling. The original casework sample was re-amplified, but no typing results were obtained (Angie Cunnigham, personal communication).

In sum, the Plexor® HY System provided sensitive, accurate DNA quantitation with single source samples, mixtures, mock casework samples and non-probative casework samples. Moreover, the automated Differex™ System performed comparably to the VDFS semi-automated differential extraction procedure.

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