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The Plexor® HY System: Not Solely a Quantitation Technique

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A multiplex quantitation system that determines the amount of total human and male DNA in a sample would streamline the decision process when amplifying forensic mixtures of female and male DNA, as well as preserve potential evidentiary DNA and help eliminate the need to screen for body fluids.

INTRODUCTION

More than four years ago the Centre of Forensic Sciences developed and validated an in-house CFS-HumRT assay to quantitate total human DNA in casework samples. Since that time, we have been interested in introducing into casework a multiplex quantitation system that would determine the amounts of total human and male DNA in a sample. Such a system could also eliminate the need to screen for body fluids for certain samples. A multiplex quantitation system would also streamline the decision process when amplifying forensic mixtures of female and male DNA as well as preserve potential evidentiary DNA. Depending on the absolute quantity and ratio of male to female DNA, autosomal STR and/or Y-STR amplification is performed. To be useful, multiplex systems must be accurate, give reproducible results and be capable of detecting male DNA in the presence of large quantities of female DNA. We examined the Plexor® HY System with these criteria in mind.

PLEXOR® HY TECHNOLOGY

The Plexor® HY System[a–g] is a four-dye multiplex real-time PCR system that allows simultaneous quantification of total autosomal and male DNA. The components and methodology have been described previously (1–3). In contrast to other technologies, accumulation of amplification product is measured by a decrease in fluorescent signal, which is directly proportional to the amount of PCR product (Figure 1, Panel A). Furthermore, a dissociation stage is added to the end of the PCR cycling to generate a melt curve. The melt temperature serves as a quality check of reaction specificity, as a homogeneous product creates a well defined melting curve (Figure 1, Panel B).

Amplification results are imported into the Plexor® Analysis Software (forensic release), which generates amplification curves. A normalization tool is incorporated into the Forensic Report function of the Plexor® HY software (Figure 2), and the parameters can be adjusted for the amount and volume of DNA to be amplified, depending on each laboratory's specific guidelines. The normalization function determines: 1) the amount of template to add, 2) dilution factor, if necessary, 3) which samples are below the threshold quantity, and 4) which samples may require further purification or dilution. The user can choose to express the results either in picogram or nanogram quantities.

EXPERIMENTAL SETUP AND RESULTS

We quantified total autosomal DNA from nonprobative differentially extracted casework samples using our CFS-HumRT QPCR (TaqMan® MGB Assay; 4) and an Applied Biosystems 7900HT real-time PCR system. Epithelial total DNA (1 ng) was amplified using the AmpFlSTR® Profiler Plus™ kit, and DNA from the spermatozoal fraction was amplified using the PowerPlex® Y System[h,i]. If less than 1 ng of DNA was recovered from the spermatozoal fraction, the entire amount of DNA was amplified with PowerPlex® Y. If no male DNA was detected in the spermatozoal fraction, we estimated the ratio of male to female DNA to target approximately 1 ng of male DNA for amplification (Table 1). If no male DNA was evident, we amplified up

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to 500 ng of DNA from the epithelial fraction. For this study we selected samples that generated partial or complete PowerPlex® Y profiles.

We quantified 2µl of genomic DNA extracted from epithelial and sperm fractions using the Plexor® HY System and an Applied Biosystems 7500 instrument. We quantitated samples in duplicate using serial dilutions of the supplied male genomic DNA standard (50 ng/µl to 0.0032 ng/µl). Table 2 lists DNA concentrations from epithelial and spermatozoal fractions and the average concentration for each sample. The Plexor® HY System provided reproducible data with good correlation between replicates, even at low DNA concentrations (as observed with the male-specific quantities).

The Forensic Report, a function of the Plexor® Analysis Software, provided a ratio of total human:Y DNA (Table 1), which is useful in determining further processing steps for the sample and the threshold for successful amplification of male DNA in the presence of a large excess of female DNA.

We used the calculated DNA quantities to re-evaluate the amount of male DNA previously amplified with PowerPlex® Y and assess the profiles generated to estimate relative DNA amounts (Table 1). A wide range of male DNA amounts (approximately 8 pg to 5,380 pg) generated full Y-STR profiles.

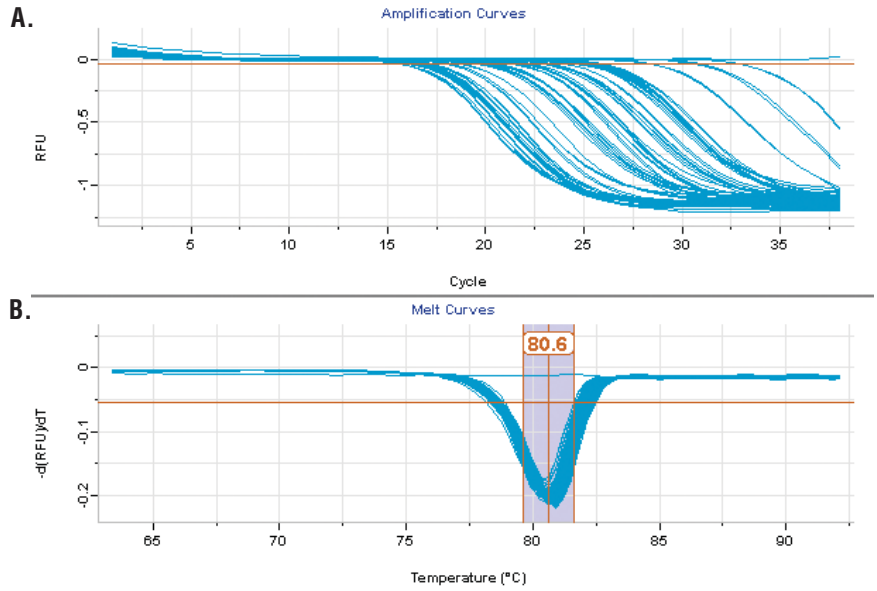

Figure 1. Results of the Plexor® HY System. Panel A. Typical amplification curves for casework samples. **Panel B.** Typical melt curves.

ADVANTAGES OF A MULTIPLEX DNA QUANTITATION SYSTEM

Accurate quantification of male and total DNA will help enable two major paradigm shifts in DNA analysis in sexual assault cases at the CFS. First, a combined human- and male-specific quantitative assay allows more effective use of Y-chromosome-specific DNA analysis. Second, we could eliminate serological prescreening for semen on Sexual Assault Examination Kit (SAEK) swabs, moving to a "direct-to-DNA" approach in which we extract and quantify DNA from all internal swabs (and some external swabs in a case-dependent manner).

Sample Name	Location	Sample Type	[Auto]	[Y]	[Auto]/[Y]	Auto STR Dilution Status	Auto STR Volume	Auto STR Quantity	Auto STR Dilution Factor	Y STR Dilution Status	Y STR Volume	Y STR Quantity	Y STR Dilution Factor	IPC Status	Curves Status
405-1SP	A4	Unknown	1.93E1	1.67E1	1.2									OK	OK
405-1SP	B4	Unknown	1.62E1	1.69E1	1.0									OK	OK
405-1SP	Average		1.78E1	1.68E1	1.1	Overconc.	N/A	N/A	106.31	Overconc.	N/A	N/A	83.95	OK	OK
208-2EP	A5	Unknown	6.11E0	3.02E-2	202.6									OK	OK
208-2EP	B5	Unknown	5.91E0	3.33E-2	177.2									OK	OK
208-2EP	Average		6.01E0	3.17E-2	189.3	Overconc.	N/A	N/A	35.98	In Range	31.5	1.00	N/A	OK	OK
202-1SP	A6	Unknown	1.46E1	7.31E0	2.0									OK	OK
202-1SP	B6	Unknown	1.39E1	6.04E0	2.3									OK	OK
202-1SP	Average		1.43E1	6.67E0	2.1	Overconc.	N/A	N/A	85.58	Overconc.	N/A	N/A	33.36	OK	OK
215-1SP	A7	Unknown	4.87E-1	2.70E-4	1800.2									OK	OK
215-1SP	B7	Unknown	4.77E-1	2.04E-4	2338.2									OK	OK
215-1SP	Average		4.82E-1	2.37E-4	2031.6	In Range	2.1	1.00	N/A	Underconc.	N/A	N/A	N/A	OK	OK
116-3SP	A8	Unknown	4.54E0	3.03E0	1.5									OK	OK
116-3SP	B8	Unknown	4.36E0	3.30E0	1.3									OK	OK
116-3SP	Average		4.45E0	3.16E0	1.4	Overconc.	N/A	N/A	26.65	Overconc.	N/A	N/A	15.81	OK	OK
514-2EP	A9	Unknown	5.84E-1	1.35E-1	4.3									OK	OK
514-2EP	B9	Unknown	5.36E-1	1.18E-1	4.6									OK	Check ..
514-2EP	Average		5.60E-1	1.27E-1	4.4	In Range	1.8	1.00	N/A	In Range	7.9	1.00	N/A	OK	Check ..

Figure 2. Forensic report with a normalization tool to assist in the setup of autosomal and Y-STR amplifications. EP = epithelial fraction; SP = sperm fraction.

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Table 1. Correlation of Plexor® HY Data and Data Generated With our In-House Quantification System and the Subsequent Y-STR Amplification Product.

	CFS Data			Plexor® HY Quantification			
Sample	[Total DNA] CFS HumRT (pg/µl)	Quantity of Total DNA Amplified in PowerPlex® Y (ng)	Y-STR Profile Generated with PowerPlex® Y (Y = Full Profile)	Average [Autosomal] (pg/µl)	Average [Y] (pg/µl)	Ratio [Autosomal]:[Y]	Amount of DNA Amplified That Generated a Profile (pg)
405-1SP	6,700	1	Y	17,600	16,600	1:0.94	2,480
517-1EP	131,700	500	Y	142,000	58.3	1:0.0004	221
517-1SP	872	19	N/1 peak	1,110	0	–	–
202-1SP	6,350	1	Y	14,200	6,630	1:0.47	1,044
226-1EP	80,000	100	Y	116,00	6.15	1:0.00005	7.7
228-1EP	76,100	100	Y	183,000	17.5	1:0.0001	23
215-1EP	165,000	500	Y	238,000	4.87	1:0.00002	15
215-1SP	340	1	N	482	0.241	1:0.0005	0.7
238-1EP	142,300	500	2 peaks	156,000	0.292	1:0.000002	1
116-3SP	586	1	Y	4,430	3,150	1:0.71	5,380
516-1SP	1,985	1	Y	5,910	5,630	1:0.95	2,836
260-1EP	72,500	500	2 peaks	94,400	0.52	1:0.000006	3.6
514-1SP	1,400	1	Y	2,260	3,450	1:1.53	2,464
212-1SP	1,800	1	Y	1,810	4,610	1:2.55	2,561

MORE EFFECTIVE APPLICATION OF Y-STRS

Use of Y-STR analysis in sexual assault cases involves primarily two scenarios: 1) developing a Y-STR profile when previous attempts to generate a male DNA profile with autosomal STRs were unsuccessful and 2) addressing an assumption used to interpret autosomal STR mixtures to determine the number of male contributors. For instance, an examiner might interpret minor peaks in an autosomal STR analysis based on an assumption of a single male contributor; Y-STR testing can be used to support this position.

In the absence of a validated total human and male-specific DNA multiplex quantification assay, autosomal STR amplification is our primary analysis method in most cases. If the case history, body fluid identification and/or autosomal STR analysis results warrant Y-STR analysis, we use autosomal analysis results to estimate the amount of DNA for subsequent PowerPlex® Y amplification. If we observe minor alleles, we use the ratio of female to male peak heights to estimate the quantity of male DNA. If we observe no foreign (male) DNA where serological or case history indicate male DNA would be present, we qualitatively assess how much total DNA to add to the Y-STR reaction, which can often be the entire remaining DNA quantity. We assess the quality of the resulting Y-STR profile postamplification to determine whether sufficient male DNA was targeted for reliable interpretation. Clearly, knowing the quantity of male DNA would be of value to obtain reliable high-quality results, assist in determining which DNA-typing system to use and allow an alternative

Table 2. Reproducibility (as Reported by the Plexor® Analysis Software).

	Autosomal Component (pg/µl)				Male Component (pg/µl)			
Sample	Replicate 1	Replicate 2	Average	Std. Deviation	Replicate 1	Replicate 2	Average	Std. Deviation
405-1SP	19,200	16,100	17,600	2192.03	16,600	16,700	16,600	70.71
517-1EP	143,000	142,000	142,000	707.11	61.4	55.1	58.3	4.45
517-1SP	1,070	1,140	1,110	49.50	0	0	0	0.00
202-1SP	14,600	13,900	14,200	494.97	7,260	6,000	6,630	890.95
226-1EP	121,000	111,000	116,000	7071.07	5.68	6.63	6.15	0.67
228-1EP	176,000	190,000	183,000	9899.49	18	16.4	17.2	1.13
215-1EP	234,000	242,000	238,000	5656.85	5.36	4.38	4.87	0.69
215-1SP	486	477	482	6.36	0.275	0.208	0.241	0.05
238-1EP	156,000	157,000	156,000	707.11	0.244	0.339	0.292	0.07
116-3SP	4,530	4,340	4,430	134.35	3,010	3,280	3,150	190.92
516-1SP	5,790	6,020	5,910	162.63	5,130	6,130	5,630	707.11
260-1EP	93,500	95,300	94,400	1272.79	0.730	0.309	0.520	0.30
514-1SP	2,300	2,210	2,260	63.64	3,440	3,470	3,450	21.21
212-1SP	1,760	1,860	1,810	70.71	4,530	4,690	4,610	113.14

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approach to treating items, such as internal swabs, where we expect a male/female DNA mixture.

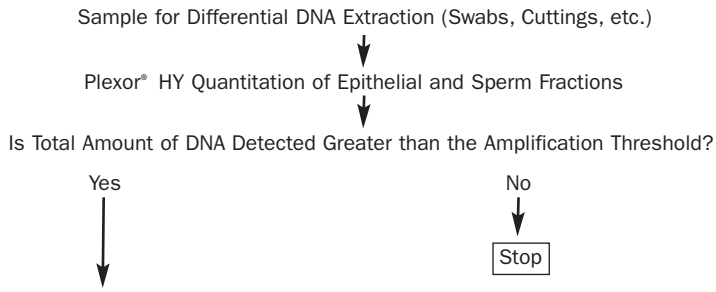
Historically, most forensic labs, including the CFS, have used primarily autosomal STRs. Use of a single technology allowed linear progression through DNA analysis. In sexual assault cases, the decision to proceed through DNA analysis was based on serological testing (i.e., is semen present?) and total DNA quantity. Y-STR analysis offers examiners another DNA-typing method with complementary strengths and weaknesses. Knowing each method's limitations allows us to decide which method is likely to provide greater probative value, especially when DNA is limited.

DIRECT-TO-DNA APPROACH

At present, our laboratory examination of oral, vaginal and rectal SAEK swabs involves prescreening for semen. We are committed to eliminating this prescreening in most cases in favor of a "direct-to-DNA" approach. This approach, which involves an initial differential extraction for all internal SAEK swabs, requires both male and total human DNA quantitation. This allows us to decide how to best proceed when processing each swab.

We base decisions about the appropriate processing of internal swabs on DNA quantification (Figure 3). The decisions depend on at least two factors: 1) the ratio of male:total DNA in the epithelial and sperm fractions and 2) the absolute quantities of male and total human DNA.

If male DNA is not detected or if the total amount of DNA is below the threshold at which amplification will be attempted, no further processing is performed. If male DNA is detected and the total amount of DNA in either fraction is above the amplification threshold, ratios will be considered



If Ratio of Male DNA:Total DNA is:	Then:
>1:5	Proceed with autosomal STR analysis
Between 1:5 and 1:25	Proceed with autosomal STR and Y-STR analysis
<1:25	Proceed with Y-STR analysis if total amount of male DNA is greater than threshold

Figure 3. A rudimentary example of a decision tree used to determine the appropriate processing of internal swabs based on DNA quantification.

(Figure 3). These decision parameters are necessarily simplified; decisions must also take into account the specific case history and all possible quantification results, which are beyond the scope of this discussion.

The advantages of this direct-to-DNA approach are multifold:

1. Reducing case turnaround times by eliminating serological prescreening of swabs
2. Increasing cost-effectiveness
3. Maximizing sample retention by eliminating destructive serological tests
4. Allowing upfront decisions regarding the type of DNA analysis to be performed, resulting in effective use of a sample
5. Obtaining higher quality Y-STR results due to a more accurate and systematic approach to achieve target levels of DNA, thereby potentially increasing the success rate and data quality

Knowing that male DNA is present may also assist investigations in less obvious ways. One example is the investigation of possible drug-facilitated sexual assaults, where the perpetrator's identity and even the very existence of an assault are uncertain. Prescreening of internal swabs for the presence of male DNA can assist in determining whether an investigation will be pursued. Consequently expensive toxicology tests and other investigative resources can be directed to those cases in which the identification of a possible perpetrator based on DNA analysis is most likely.

CONCLUSIONS

Clearly, implementing a multiplex system that quantitates total human and male DNA in one reaction can greatly enhance the efficiency and quality of DNA analysis. The Plexor® HY System has proven to be effective, accurate and sufficiently sensitive to provide this tool to the forensic DNA analysis community.

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