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VIRGINIA DEPARTMENT OF FORENSIC SCIENCE
VALIDATION OF CASEWORK DIRECT WITH POWERQUANT

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PURPOSE

This study was designed to test the Promega Casework Direct System in conjunction with the Promega PowerQuant® System for use as a male DNA screening tool for samples from sexual assault kits.

MATERIALS AND METHODS

Mock sexual assault samples were created to test the performance of the Casework Direct System (CD; Promega Corp., Madison, WI) with the Promega PowerQuant® System (PQ) for sexual assault casework sample screening. Vaginal and buccal swabs were collected from female donors and submerged into a series of seminal dilutions to create four sets of mixtures (Table 1). The dilution series was, 1:10,000, 1:50,000, 1:75,000 and 1:100,000 of seminal fluid, and each dilution was tested in triplicate. Semen was diluted using Type I water. The swabs were inverted for approximately 30 seconds in ~150 µL of semen dilution. All swabs were allowed to dry for at least 1 hour prior to sampling for DNA extraction.

Mixture Set	Donor and Sample Type	
1	Male 1	Female 1-vaginal swabs
2	Male 2	Female 2-vaginal swabs
3	Male 3	Female 3-buccal swabs
4	Male 4	Female 2-buccal swabs
5	Male 2	Female 4-buccal swabs

Table 1. Seminal fluid donors and female vaginal and buccal swab donors used for each mixture set.

The tip of each of four swabs (approximately 1/8 of the swab) was cut and placed into a microcentrifuge tube for CD extraction, as described in the Virginia Department of Forensic Science (VDFS) Procedures Manual.¹ The CD lysate for each sample was quantified using the PowerQuant® System, following the manufacturer's recommendations, with the exception that the PQ Dilution Buffer (used to prepare the standards) was also used for the no-template control.² Automated serial dilution of the male standard and reaction plate setup was performed using a robotic method developed specifically for the PQ system on the Biomek® NX^P Automation Workstation (Beckman Coulter, Fullerton, CA). The quantitation for each CD

¹ Forensic Biology Procedures Manual. Extraction of DNA. Virginia Department of Forensic Science. Issued December 23, 2019.

² PowerQuant® System Technical Manual. Promega. Revised 1/2020.

lysate was performed in duplicate, with an emphasis on extremely dilute semen samples with so little male DNA that the Y-DNA quantitation would be extremely low or undetectable. Amplification and detection were performed using the QuantStudio™ 5 Real-Time PCR System (QS5; Thermo Fisher Scientific, Waltham, MA), which was calibrated for the following dyes: FAM for the autosomal target (84-base-pair amplicon), CAL Fluor® Gold 540 for the male targets (81bp and 136bp), TMR for the internal positive control (IPC) (435bp), Quasar® 670 for the degradation target (294bp), and CXR for the passive reference dye. The raw data were collected with QuantStudio™ Design and Analysis Software (Thermo Fisher Scientific) ver. 1.4.3 or 1.5.1, and analyzed using the PowerQuant® Analysis Tool (Promega) ver. 1.0.0.0.

The remainder (outer layer) of each of the same previously sampled four swabs was differentially extracted and purified using the DNA IQ™ System (Promega Corp.) on the Biomek® NX^P Automation Workstation, as described in the VDFS Procedures Manual.¹ Sperm fractions were quantitated in duplicate using the PQ System on the QS5 as described above, then amplified for autosomal STRs using the PowerPlex® Fusion (Fusion) (Promega Corp.) kit and a robotic setup, and Y-STRs using the AmpFISTR™ Yfiler™ (Yfiler) (Applied Biosystems [AB], Foster City, CA) kit, following the VDFS Procedures Manuals.^{3,4} Prior to Y-STR half-reaction amplification, sperm fraction DNA extracts with concentrations below the target amount (0.3ng male/5µL) were concentrated and resolubilized in Type I water. Non-sperm fractions were quantitated but not typed, due to the large excess of female DNA expected. Amplification was performed using a GeneAmp™ 9700 Thermal Cycler (Thermo Fisher Scientific).

All samples were separated and detected on the 3500xL Genetic Analyzer (Thermo Fisher Scientific) using Data Collection Software v3.1, according to the VDFS Procedures Manuals.^{5,6} Fusion samples and allelic ladders were prepared by mixing 9.5 µL Hi-Di formamide (AB), 0.5 µL WEN ILS size standard (Promega), and 2 µL sample or 1 µL allelic ladder (Promega). Yfiler samples and allelic ladders were prepared by mixing 8.5 µL Hi-Di formamide (AB), 0.5 µL GeneScan™ 600 LIZ® size standard (AB), and 1 or 2 µL sample, or 2 µL allelic ladder (AB). The sample plate was heated at 95° C for 3 minutes and snap-cooled at 0° C for three minutes. The amplification products were separated using the manufacturer's recommended settings, including a 24 s or 12 s, 1.2 kV injection, 1210-1800 s, 15 kV separation, 36 cm (length), 50 µm i.d. capillary, and POP-4 polymer (ABI). Analysis of electropherogram data was completed using the GeneMapper® ID-X v1.4 analysis software (ABI) for Fusion and Yfiler data, as directed in the VDFS Procedure Manual.⁷

The CD kit assessed with PowerQuant® was also tested on different substrates with various contaminants to assess whether or not these commonly encountered adulterants would interfere with the CD lysis of cells and/or the subsequent detection of DNA by qPCR in the lysates. Four different combinations of substrate/contaminant were tested (carpet/soil, bed sheet/water-soluble lubricant, denim/baby oil, underpants/Nonoxynol-9) in order to simulate

³ Forensic Biology Procedures Manual. PowerPlex® Fusion Amplification and Long Term Storage. Virginia Department of Forensic Science. Issued December 23, 2019.

⁴ Forensic Biology Procedures Manual. AmpFISTR™ Yfiler™ Amplification and Long Term Storage. Virginia Department of Forensic Science. Issued December 13, 2019.

⁵ Forensic Biology Procedures Manual. CE for PowerPlex® Fusion. Virginia Department of Forensic Science. Issued December 30, 2019.

⁶ Forensic Biology Procedures Manual. CE for AmpFISTR™ Yfiler™. Virginia Department of Forensic Science. Issued December 13, 2019.

⁷ Forensic Biology Procedures Manual. Analysis of CE Results Using GeneMapper® ID-X. Virginia Department of Forensic Science. Issued December 13, 2019.

commonly encountered substrates and contaminants. The contaminant was applied to the substrate and allowed to dry. One hundred microliters of a female buccal cell suspension, created from two buccal swabs, and 300 µL male semen (1:10,000 or 1:50,000 dilution), was pipetted onto each 1 cm² substrate/contaminant and allowed to dry. Stained substrates without contaminants were also created to serve as controls. For each combination of seminal fluid dilution and substrate, approximately 1/8 of the sample was cut for Casework Direct testing and the remainder of the cutting was used for differential extraction. Carpet and bed sheet samples were extracted and purified using the DNA IQ™ System. The denim and underpants samples were organically extracted and purified/concentrated using a Microcon® DNA Fast Flow filter (MilliporeSigma, Burlington, MA), according to the Virginia Department of Forensic Science Procedures Manual.¹ DNA quantitation and typing were performed as described above.

Artifacts that affected interpretation were observed in the Yfiler data from mixture 2; as a result, that data was not used. Fusion data from four samples and Yfiler data from an additional two samples were also not used to draw conclusions due to non-donor peaks that could affect allele counts: four of the samples exhibited a single extra peak in the stutter position but well above the stutter threshold, one sample exhibited several non-donor alleles, and possible alleles below the limit of detection were noted in the last sample.

RESULTS AND DISCUSSION

Mixtures 1-4 (swabs)

A portion was removed from four swabs when preparing the CD extracts and combined for each sample to simulate the maximum number of swabs typically received with casework samples. The range of semen dilutions was chosen such that the amount of male DNA in the CD extract was at the limit of detection for the Y-chromosome target for the PQ/QS5 system. The resulting sperm fraction Fusion profiles were evaluated to determine the extent of the male contributor present. This was determined by tallying the number of alleles in the mixture that could be unambiguously attributed to the male. Currently, casework samples screened with Casework Direct that yield no Y-chromosome DNA quantitation value, displayed as 'N/A' with the Plexor® HY System (Promega Corp.), are not processed with Fusion, since the remainder of the sample will most likely not generate an informative male Fusion profile that can be interpreted as a non-elimination.⁸ This is due to the number of male alleles indistinguishable from female and the number of allelic and locus dropouts. A partial or full Yfiler profile may, however, be obtained from the samples. The Yfiler profile may be of value if there is a suspect in the case. The manufacturer reports that the same levels of sensitivity are obtained with the PowerQuant® System and Plexor® HY System.⁹

In samples for which male DNA was detected in both quantitations of the CD lysate, the resulting Fusion profiles were suitable for a non-elimination of the male contributor in eleven of 27 (41%) mock sexual assault sample sperm fractions (Table 2). Nine of these samples were prepared with 1:10,000 seminal fluid and two were prepared with 1:50,000 seminal fluid. The

⁸ Virginia Department of Forensic Science. Validation of Casework Direct. 2017.

⁹ Ewing, M.M. et al. The PowerQuant™ System: A New Quantification Assay for Determining DNA Concentration and Quality. Promega Corporation Web site. <https://www.promega.com/resources/profiles-in-dna/2014/the-powerquant-system-a-new-quantification-assay-for-determining-dna-concentration-and-quality/> Updated 2014. Accessed March 15, 2021.

remaining 16 samples (59%) did not yield a sufficient amount of the male contributor for a non-elimination.

If the Y-chromosome DNA target is not detected with PowerQuant[®], then no value is displayed for the [Y] concentration. No male DNA was detected in one or both of the quantitation assays for 21 of the CD lysates (Table 2). These lysates were from samples prepared with seminal fluid in the 1:50,000-1-100,000 range. Nineteen of the sperm fractions (90.5%) from these samples yielded Fusion profiles that exceeded the amount of dropout tolerated for a conclusion of not eliminated for the male contributor. Two (9.5%) of the sperm fractions, however, gave a sufficient Fusion profile for a non-elimination of the male contributor, following the VDFS Procedures Manual.¹⁰ Both of these samples were vaginal swabs with 1:50,000 semen. The first sample showed 3.6 pg/μL male DNA in the first quantitation and no male DNA detected in the second quantitation of the CD lysate, and the second sample had no male DNA detected in either quantitation of the CD lysate. Figure 1 shows the completeness of the male Fusion profile detected for each [Y] DNA measurement in the corresponding CD lysates. Completeness was measured by determining the number of unambiguous (unshared with the female) male alleles observed.

A partial or full Yfiler profile may be obtained from samples that do not yield sufficient Fusion results to include a male contributor. The Yfiler profiles from the sperm fractions were evaluated to determine which of these met the criteria for non-elimination, according to the VDFS procedures.¹¹ It was possible to include the male contributor with Yfiler in 18 of the 21 (86%) samples where the Fusion criteria for inclusion were not met (Table 2). In the subset of these samples where no male DNA was detected in one or both quantitations of the CD lysate, inclusion of the male with Yfiler was also successful in 86% (6/7) of samples. Additionally, the male contributor could be included in the Yfiler profiles from the two vaginal swab samples with a 1:50,000 dilution of semen, where Fusion testing also included the male, but may not have been conducted in actual casework, given that no male DNA was detected in one or both quantitations of the CD lysate.

¹⁰ Forensic Biology Procedures Manual. Interpretation of PowerPlex[®] Fusion Data. Virginia Department of Forensic Science. Issued December 23, 2019.

¹¹ Forensic Biology Procedures Manual. Interpretation of AmpFℓSTR[™] Yfiler[™] CE Data. Virginia Department of Forensic Science. Issued December 13, 2019.




Sample	CD [Y] (ng/μL)		Fusion				Yfiler # loci
	1	2	auto. alleles diff.	loci w/ alleles	auto. locus	auto. allelic	
			from female	diff. from female ¹	dropout of male ²	dropout of male ²	
male 1 (1:10,000) + fem 1 vag 1	0.0069	0.0229	26/26	19/19	0	0	17
male 1 (1:10,000) + fem 1 vag 2	0.0120	0.0020	26	19	0	0	17
male 1 (1:10,000) + fem 1 vag 3	0.0090	0.0108	26	19	0	0	17
male 1 (1:50,000) + fem 1 vag 1		0.0036	21	18	0	5	11
male 1 (1:50,000) + fem 1 vag 2			25	19	0	1	12
male 1 (1:50,000) + fem 1 vag 3	0.0002	0.0025	16	12	0	10	7
male 1 (1:75,000) + fem 1 vag 1		0.0015	4	4	7	9	16
male 1 (1:75,000) + fem 1 vag 2	0.0015	0.0002	4	4	7	10	11
male 1 (1:75,000) + fem 1 vag 3	0.0029	0.0024	9	8	2	13	10
male 1 (1:100,000) + fem 1 vag 1		0.0011	11	10	2	11	12
male 1 (1:100,000) + fem 1 vag 2			6	6	6	9	16
male 1 (1:100,000) + fem 1 vag 3			7	7	5	10	12
male 2 (1:10,000) + fem 2 vag 1	0.0010	0.0007	20/32	15/19	2	8	
male 2 (1:10,000) + fem 2 vag 2	0.0029	0.0012	32	19	0	0	
male 2 (1:10,000) + fem 2 vag 3	0.0026	0.0009	1	1	13	6	
male 2 (1:50,000) + fem 2 vag 1			2	2	12	7	
male 2 (1:50,000) + fem 2 vag 2			2	2	12	8	
male 2 (1:50,000) + fem 2 vag 3			2	2	12	8	
male 2 (1:75,000) + fem 2 vag 1			7	7	7	12	
male 2 (1:75,000) + fem 2 vag 2		0.0010	3	3	11	9	
male 2 (1:75,000) + fem 2 vag 3	0.0015		1	1	13	7	
male 2 (1:100,000) + fem 2 vag 1			2	2	12	8	
male 2 (1:100,000) + fem 2 vag 2			0	0	14	6	
male 2 (1:100,000) + fem 2 vag 3			0	0	14	6	
male 3 (1:10,000) + fem 3 buc 1	0.0046	0.0064	28/28	17/17	0	0	14
male 3 (1:10,000) + fem 3 buc 2	0.0045	0.0083					17
male 3 (1:10,000) + fem 3 buc 3	0.0024	0.0036	28	17	0	0	16
male 3 (1:50,000) + fem 3 buc 1	0.0012	0.0015					10
male 3 (1:50,000) + fem 3 buc 2	0.0006	0.0004	23	15	1	3	15
male 3 (1:50,000) + fem 3 buc 3	0.0014	0.0017	20	13	2	4	5
male 3 (1:75,000) + fem 3 buc 1	0.0005	0.0002	14	12	2	10	4
male 3 (1:75,000) + fem 3 buc 2	0.0016		5	4	5	7	4
male 3 (1:75,000) + fem 3 buc 3		0.0006	3	3	2	5	0
male 3 (1:100,000) + fem 3 buc 1	0.0006	0.0007	8	7	5	10	1
male 3 (1:100,000) + fem 3 buc 2	0.0007	0.0010	8	7	4	12	2
male 3 (1:100,000) + fem 3 buc 3	0.0018	0.0035	7	7	0	11	6
male 4 (1:10,000) + fem 2 buc 1	0.0061	0.0042	30/30	19/19	0	0	17
male 4 (1:10,000) + fem 2 buc 2	0.0058	0.0042	29	19	0	1	7
male 4 (1:10,000) + fem 2 buc 3	0.0078	0.0095	30	19	0	0	16
male 4 (1:50,000) + fem 2 buc 1	0.0021	0.0016					13
male 4 (1:50,000) + fem 2 buc 2	0.0056	0.0043	23	17	0	7	7
male 4 (1:50,000) + fem 2 buc 3	0.0041	0.0021	27	17	0	3	10
male 4 (1:75,000) + fem 2 buc 1	0.0008	0.0005	10	9	5	11	10
male 4 (1:75,000) + fem 2 buc 2	0.0012	0.0017	1	1	11	9	13
male 4 (1:75,000) + fem 2 buc 3	0.0021	0.0010	12	10	3	12	5
male 4 (1:100,000) + fem 2 buc 1	0.0002		8	8	5	13	15
male 4 (1:100,000) + fem 2 buc 2	0.0014	0.0015	18	14	1	10	11
male 4 (1:100,000) + fem 2 buc 3	0.0022	0.0020	11	9	5	9	12

Table 2. Casework Direct, autosomal and Y-DNA STR analysis of mock sexual assault sample sperm fractions. For each mixture, the first row also includes the total possible number of male alleles and loci different from the female.

¹ includes loci used for statistics only

² does not include loci with no results or where male alleles may be masked

Key:

-  = no inclusion/stat possible
-  = data not used (QC purposes)
-  = no [Y] for one or both CD replicates/male included-Fusion

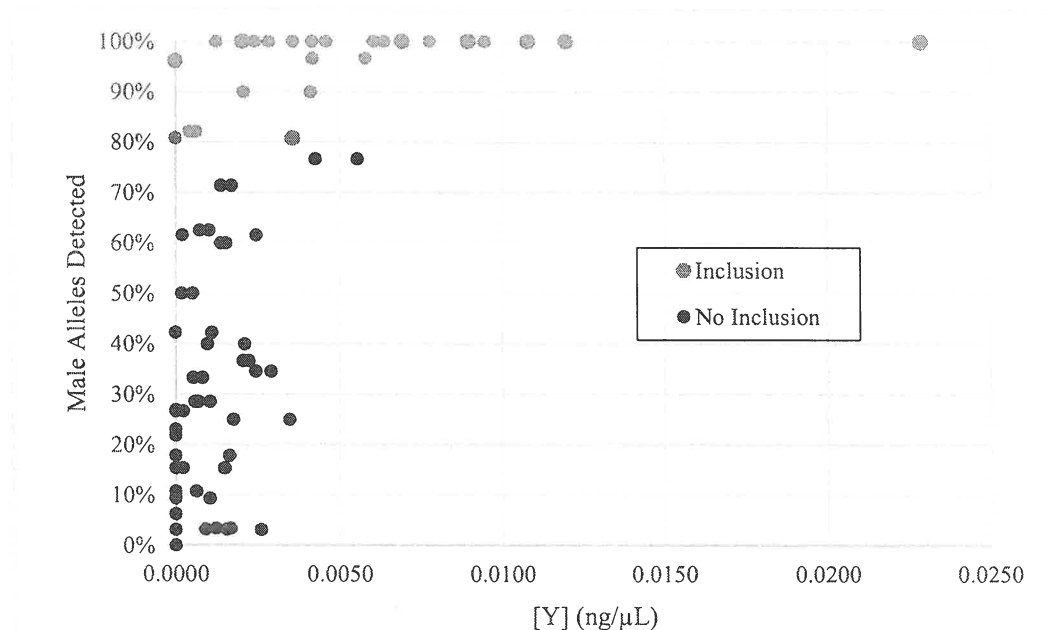


Figure 1. Correlation of Casework Direct Y-DNA quantitation values and male alleles detected by PowerPlex® Fusion typing of the mock casework sample sperm fractions. CD lysates were quantitated in duplicate. Both duplicate measurements are plotted on the graph. If the Y-chromosome DNA target was not detected, a zero is shown for the Y concentration.

Mixture 5 (substrates/contaminants)

Table 3 shows the CD lysate Y-chromosome quantitation results from the substrates and contaminants tested, compared with those from the vaginal and buccal swabs series at the same dilutions of seminal fluid. It is important to note, however, that the volume of seminal fluid used

Sample Set Type	Average CD Y Quant (ng/μL)	
	1:10,000 semen	1:50,000 semen
Mixtures 1-4 vaginal/buccal swabs	0.0058±0.0049	0.0014±0.0016
Mixture 5 substrates only	0.0013±0.0005	0.0002±0.0001
Mixture 5 substrates + contaminants	0.0008±0.0008	0.0002±0.0004

Table 3. Average CD Y-chromosome DNA quantitation values plus standard deviations obtained from swabs and other substrates tested with and without the addition of various contaminants. Volume of seminal fluid used to prepare Mixture 5 samples was approximately half that used in each Mixture 1-4 sample.

to prepare the mixtures on the substrates/contaminants (300μL) was approximately half that used for each sample (600μL) in mixtures 1-4, which consisted of four swabs. Even when this is taken into account, the quantitation data demonstrate lower DNA yields for the different substrates tested and substrates with contaminants when compared with swabs that were tested at

the same dilution. This may reflect the characteristics of the contaminants and substrates, but may also reflect different sampling (removal of swab tip for CD versus a corner of a square shaped sample cutting). Due to the large surface area of the carpet fibers, only a portion of each stained carpet cutting was used for testing. This may have contributed to a decrease in DNA yield in these samples, since it was difficult to visualize the semen stain during sampling, even with the aid of an alternate light source. Oily contaminants have been demonstrated to show a reduction in DNA yield for DNA IQ™ System purification.¹² These inhibitors may also have suppressing effects on efficient Casework Direct lysate generation or interfere with detection by PQ. The data show that the CE STR results from these DNA IQ™ or organic/Microcon® extracted and purified samples mirror the quantitation data obtained from the Casework Direct cuttings; however, the data are limited. None of the samples resulted in a Fusion profile from the sperm fraction that was sufficient for a non-elimination of the male contributor when the female was used as an assumed known (Table 4). These profiles did not appear to be mixtures, so a general interpretation for single source samples may also be appropriate, depending on the case specifics, such as potential for carryover from the non-sperm fraction, [Auto]/[Y] and where the item was recovered. If the single source approach were used, the carpet plus soil sample (1:10,000 semen) would be suitable for comparisons, but would still exceed the amount of dropout allowed for a non-elimination of the male contributor (data not shown).

Sample		CD [Y] (ng/μL)		Fusion				Yfiler
		1	2	auto. alleles diff. from female (32 poss.)	loci w/ alleles diff. from female (20 poss.) ¹	auto. locus dropout of male ²	auto. allelic dropout of male ²	# loci
DNA IQ	carpet-male 2 (1:10,000) + fem 4 buc	0.0009	0.0004	1	1	0	2	0
	carpet+soil-male 2 (1:10,000) + fem 4 buc	0.0003	0.0005	5	4	0	6	0
	sheet-male 2 (1:10,000) + fem 4 buc	0.0013	0.0019	22	17	0	11	0
	sheet+lubricant-male 2 (1:10,000) + fem 4 buc	0.0019	0.0004	26	17	0	6	1
	carpet-male 2 (1:50,000) + fem 4 buc	0.0002	0.0003	1	1	0	2	0
	carpet+soil-male 2 (1:50,000) + fem 4 buc			0	0	0	1	0
	sheet-male 2 (1:50,000) + fem 4 buc	0.0001	0.0002	0	0	0	1	0
	sheet+lubricant-male 2 (1:50,000) + fem 4 buc							0
Organic Microcon	denim-male 2 (1:10,000) + fem 4 buc	0.0020	0.0018	1	1	0	1	2
	denim+baby oil-male 2 (1:10,000) + fem 4 buc	0.0006	0.0022	0	0	0	0	0
	UP-male 2 (1:10,000) + fem 4 buc	0.0010	0.0012	18	15	0	12	
	UP+Nonoxynol-9-male 2 (1:10,000) + fem 4 buc	0.0006	0.0001	13	10	0	8	11
	denim-male 2 (1:50,000) + fem 4 buc		0.0001	0	0	0	0	0
	denim+baby oil-male 2 (1:50,000) + fem 4 buc	0.0011	0.0007	7	7	0	9	
	UP-male 2 (1:50,000) + fem 4 buc	0.0002	0.0005	2	2	0	3	2
	UP+Nonoxynol-9-male 2 (1:50,000) + fem 4 buc			2	2	0	1	3

Table 4. Casework Direct, autosomal and Y-DNA STR analysis of mock sexual assault sample sperm fractions from various substrates and contaminants.

¹ includes loci used for statistics only

² does not include loci with no results or where male alleles may be masked

Key:

- ☐ = no inclusion/stat possible
☐ = data not used (QC purposes)

The percentage of unambiguous Fusion alleles detected from the male contributor in the sperm fraction for each substrate/contaminant, along with the average Y-chromosome DNA quantitation value measured in the CD lysate, is shown in Figure 2.

¹² The Impact of Contaminants on DNA Extracted Using the DNA IQ™ System. Vanessa M. Covert, Virginia Commonwealth University Masters' Thesis.

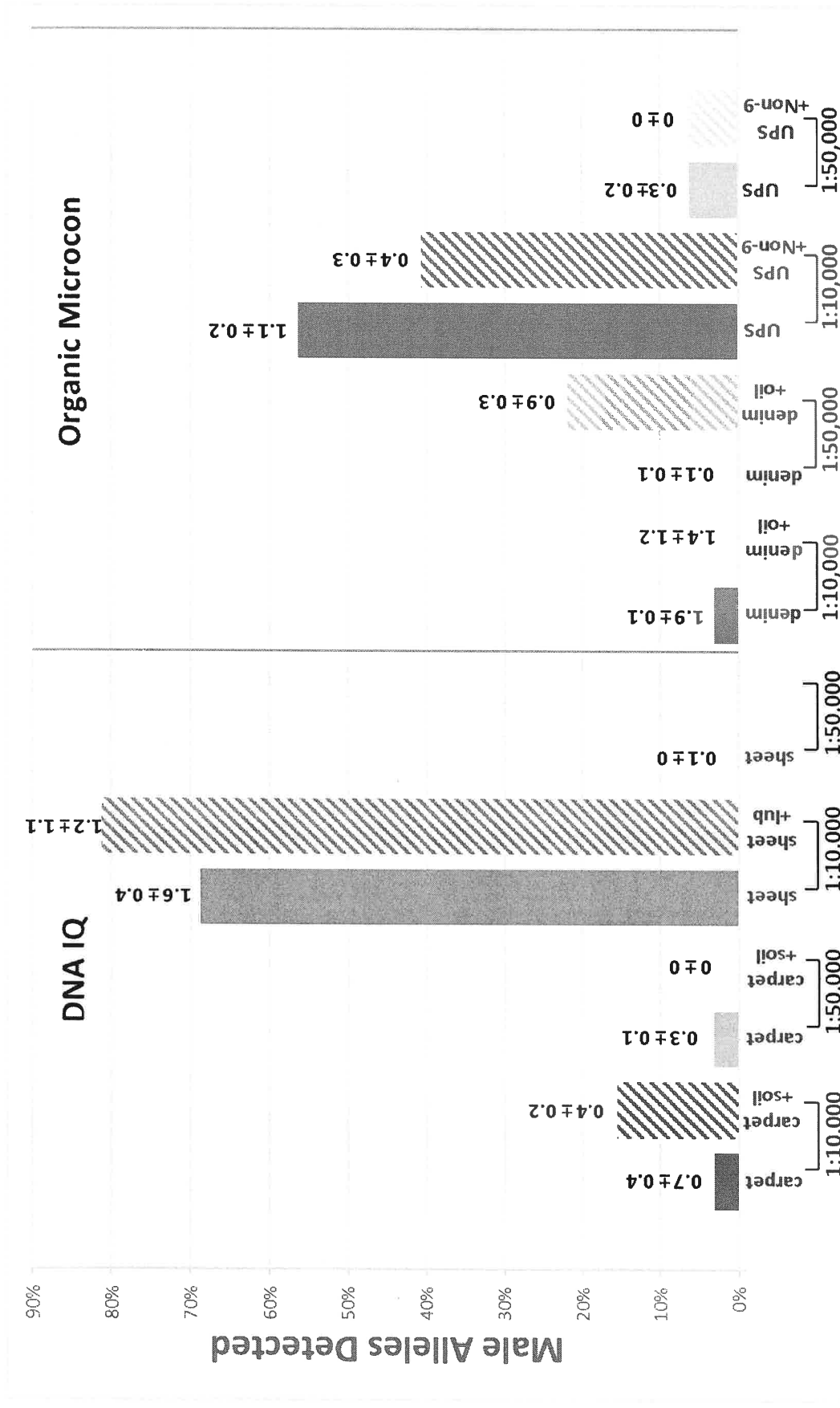


Figure 2. Percentage of unambiguous Fusion alleles detected from the male contributor in sperm fraction for each substrate/contaminant. Solid bars indicate sexual assault sample on substrate versus bars with diagonal stripes which, indicate sexual assault sample on substrate that also includes the indicated contaminant. Seminal fluid dilution is shown along the X-axis. Number above each bar is the average Y-chromosome DNA quantitation value plus standard deviation measured in the corresponding CD lysate (pg/ μ L). If the Y-chromosome DNA target was not detected, a zero is shown for average Y concentration. Sheet+lub 1:50,000 results not included (QC purposes). Lub=lubricant, UPS=underpants, Non-9=Nonoxynol-9.

All but one of the samples in this set produced Yfiler results at less than the minimum number of required loci (four) to include the male contributor (Table 4). The underpants plus Nonoxynol-9 (1:10,000 semen) yielded a partial Yfiler profile with 11 loci (0.6 pg/μL and 0.1 pg/μL [Y] measured in the CD lysate).

CONCLUSIONS

The Casework Direct Kit is a simple and sensitive screening tool for detecting male DNA on evidentiary samples and has been particularly useful for the rapid screening of sexual assault evidence. Furthermore, it has previously been shown to be more sensitive than the traditional sperm search of Christmas Tree stained slides created from mock sexual assault sample smears and acid phosphatase presumptive testing.

Based upon this study, if no Y-chromosome DNA is detected in the CD lysate with PowerQuant[®], the remainder of the sample will typically not yield a male Fusion profile that can be interpreted as a non-elimination. This is due to the number of male alleles indistinguishable from female and the number of allelic and locus dropout events. A partial or full Yfiler profile may, however, be obtained from the samples. The Yfiler profile may be of value, particularly if there is a suspect in the case. Generally, the lower the value of the Y-chromosome DNA quantitation in the CD lysate, the greater the degree of allelic and locus dropout observed for the male contributor. Thus, the CD system used in conjunction with PowerQuant[®] and the QuantStudio[™] 5 is suitable for casework sample screening for the presence of a male contributor. The performance of the CD system in conjunction with the PowerQuant and QuantStudio 5 systems was similar to that reported for the CD system used in conjunction with the Plexor[®] HY and Stratagene systems.