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TECHNICAL NOTE

CRIMINALISTICS

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The Recovery and Persistence of Salivary DNA on Human Skin

ABSTRACT: Salivary DNA is encountered in many crimes, such as sexual assaults and murders. In this study, saliva from three male donors was deposited on the skin of three female recipients. The amount of male salivary DNA remaining on the female skin was measured over a 96-h period using the Quantifiler™ Y Human Male DNA Quantification Kit. In eight of the nine experiments, a full male DNA profile matching the donor was obtained even after 96 h. In addition, the study showed that the concentration of salivary DNA varied from donor to donor and from day to day. The efficiency of two recovery methods, wet and dry swabbing and minitaping, was compared. The results indicate the tapelift method gave higher DNA recovery. This study also examined the secondary transfer of salivary DNA from skin to fabrics. Cotton and polyester give higher DNA transfer than leather.

KEYWORDS: forensic science, saliva, amylase, DNA, persistence, forensic medical examination

Saliva is a composite fluid comprised of water, electrolytes, a glycoprotein (mucin), enzymes, inorganic components, and buccal epithelial cells (1). DNA is not present in the liquid saliva but in the cellular material, such as epithelial cells and glandular cells, that are naturally sloughed from the inner lining of the mouth.

The analysis of salivary DNA on evidential material, such as skin or clothing, can provide important information to the forensic scientist. Transfer may be attributable to spitting, kissing, licking, biting, and sucking (2). Frequently in sexual assaults and murder cases, the victim has been bitten and, traditionally, bite mark analysis is the main source of evidence (3,4).

The persistence of saliva on skin can have consequences for crime scene and laboratory examinations in sexual assaults and murders. DNA in saliva has been analyzed from a variety of substrates, such as stamps, envelope flaps, cigarette butts, swabs taken from a victim of a sexual assault, drinking vessels, masks, and foodstuffs (5–8). Several techniques have been used in the past to find the best method of recovering saliva from human skin. Traditional methods involve using a single cotton swab moistened with water to recover saliva (9). This was later modified by the use of a wet swab followed by a dry swab known as the double-swab technique (10). A recent study (11) compared the single- and double-swab techniques and found no significant difference between the two methods. Forensic laboratories also extract DNA from cellular material recovered from garments submitted as evidence in part of a criminal case. The traditional method of recovering material is by swabbing, direct extraction from the bulk fabric, or scraping with a scalpel. A more rapid and simpler method has been developed by the use of minitapes (12). The postrecovery work-up of material on the swabs has also been the focus of some attention, and a modified chelex method was developed to increase the recovery of material from swabs (13).

Short tandem repeat markers on the Y-chromosome are becoming popular as a mean to detect the male component from a mixed sample in sexual assault cases (14–16) as it enables the detection of low levels of male DNA in a high background of female DNA. The use of Y human male DNA quantification proves to be a rapid method to assess the persistence of male salivary DNA on female recipient's skin, as there is no background interference from the female.

The persistence of transferred material is constantly a question of relevance to forensic scientists. Persistence studies (17–20) can be used to help the scientist assess the expected level of a given type of trace evidence remaining after a certain time period. These studies also assist in deciding after which time period we would expect the evidence to be gone and thus not worthwhile searching for the evidence in question. There have been some limited studies on the persistence of saliva. Keating and Higgs (21) reported detectable levels of amylase activity up to 30 h on breast swabs taken from victims of alleged sexual assaults. A study carried out by Sweet et al. (2) demonstrated the persistence of saliva on cadaver tissue and the ability to recover DNA for up to 48 h following application. Although a significant loss of DNA occurred between recovery after 5 min and recovery after 24 h, very little loss occurred between 24 and 48 h, demonstrating the robust nature of DNA and the stability of this molecule following dehydration of saliva. The initial loss may be attributed to degradation of the DNA or inefficient recovery due to dehydration of the nucleated cells onto the tissue of the corpse. Another study on the recovery of DNA from saliva on human skin reported that the evidence is stable on intact

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skin for at least 60 h following deposition (22). Sweet and Shutler (5) reported a case in which DNA was recovered from a bite mark on a victim's body that had been submerged in a slow-moving river for 5.5 h prior to its discovery.

When dealing with the topic of transfer and persistence, especially when small amounts of material are involved, we must be aware of the possibility of secondary transfer. Primary transfer occurs when DNA is transferred from a person to an item or another person, secondary transfer is when the DNA deposited on one item is transferred to a second item or a person. Earlier studies found that under certain conditions DNA can transfer from one individual to another and subsequently to an object (23,24). Secondary transfer of DNA during simulated strangulation has recently been reported (11).

This study represents the first examination of the loss over time of salivary DNA on the skin in living humans and presents some information on the degree to which secondary transfer of salivary DNA from skin to fabric can occur.

Materials and Methods

Collection, Application, and Recovery of Salivary DNA

All saliva samples were collected in sterile containers. To check the variability of salivary DNA from donor to donor and from day-to-day, a sample of saliva (2.5 mL) was collected from the three male donors before morning coffee, before lunch, and 1 h after lunch on four consecutive days. The saliva samples were frozen immediately after collection. Liquid saliva samples were allowed to thaw at room temperature and were vortexed to homogenize before quantitation.

Two methods of recovery of salivary DNA from skin surfaces were compared:

- The double-swab technique uses a cotton swab soaked in sterile water followed by a dry swab to collect the sample stain.
- The minitape recovery technique (12) involves using a 20 mm × 20 mm square of double-sided adhesive tape (Sellotape) mounted on one end of an acetate strip, 20 mm × 50 mm. The protective strip is left in place until ready for use. The tape is repeatedly pressed across multiple areas of the substrate to transfer cellular material from the area being examined to the tape.

The minitapes were prepared in the laboratory and were UV sterilized prior to use in a UV Stratalinker (Stratagene®, Stratalinker® 1800; Stratagene Cloning Systems, La Jolla, CA) using 1200 joules (60 sec).

To test the efficiency of recovery of both methods, 50 μL of saliva from one male donor was added to 16-labeled squares on a female recipient's leg and allowed to dry for approximately 15 min. The salivary DNA was then recovered from eight squares using the double-swab technique and eight using the minitape recovery technique. This test was run in duplicate using the same donor and same recipient 7 days after the initial experiment. Background controls were taken in each case by swabbing or minitaping the recipient's leg before the saliva was applied. These were extracted and quantified in parallel with experimental samples.

Saliva samples, which were applied to skin/fabric, were applied on the day of collection. Saliva samples were vortexed to homogenize before applying to skin/fabric. For the persistence study, a sample of saliva (2.5 mL) was collected from the three male donors, on the same day, prior to lunchtime. Three female recipients were also included in the study. A stencil template of 3 cm × 3 cm squares was made, and three sets of five squares were drawn on each of the recipients' legs, one set for each donor sample. For each set, 50 μL of donor saliva was added to each of the five-labeled squares, i.e., for recipient 1, a total of 15 samples were added, five samples from each donor. Samples were allowed to air-dry for approximately 15 min before covering with clothing. The three recipients were instructed to cover their legs with normal clothing, such as jeans, trousers, etc. The recipients were instructed not to shower during the persistence study and not to wash the areas to which the saliva was added. In each experiment, an area of skin was minitaped prior to the application of saliva to assess the level of potential background DNA.

To test the persistence of salivary DNA on skin, saliva was recovered (by the minitape technique) from the skin of the recipients at five time intervals: immediately after drying (T0), after 24 h (T24), after 48 h (T48), after 72 h (T72), and after 96 h (T96). At each of the time intervals, the salivary DNA from one square from each donor was recovered, i.e., at T0, salivary DNA was recovered from three squares from recipient 1, three squares from recipient 2, and three squares from recipient 3.

To determine the secondary transfer of salivary DNA from skin to clothing, a variety of fabrics were used: cotton, denim, leather, nylon, polyester, silk, and wool. A square, 3 cm × 3 cm, was cut from each fabric type. One female recipient was used in this experiment. Seven squares, 3 cm × 3 cm, were drawn on the recipient's arm using a stencil, and 50 μL of male saliva was added to each labeled square and allowed to dry for 15 min. Each fabric was rubbed over a labeled square on the recipient's arm 10 times. The salivary DNA was recovered using the minitape recovery technique.

Prior to use, the pieces of material were UV sterilized as described earlier.

Control negatives for samples of fabric and minitapes were extracted and quantified together with experimental samples.

Extraction of DNA

Liquid saliva, swabs, and minitapes were extracted using a modified method for saliva stains with the Qiagen® QIAamp DNA mini kit (Qiagen, Hilden, Germany).

Swabs (wet and dry combined) and minitapes were cut into small pieces and placed in a micro-centrifuge tube (for liquid saliva, 10 μL of sample was added to the tube); 180 μL of ATL buffer was added, the sample tube was then vortexed and incubated at 85°C for 10 min. Twenty microliters of Proteinase K was added (supplied with QIAamp kit), vortexed and incubated at 56°C for a minimum of 1 h. Two hundred microliters of prewarmed AL buffer was added, vortexed, and incubated for 10 min at 56°C, followed by centrifugation at 21,000 × *g* for 10 sec. Two hundred microliters of ethanol (Merck, Darmstadt, Germany) was added, vortexed, and centrifuged at 21,000 × *g* for 10 sec. Samples were carefully added to the columns in the collection tubes (QIAamp kit) and centrifuged at 6800 × *g* for 1 min. The column was removed; 500 μL of AW1 buffer was added and centrifuged at 6800 × *g* for 1 min. The column was removed; 500 μL of AW2 buffer was added and centrifuged at 21,000 × *g* for 3 min. The column was removed, and 50 μL of prewarmed water was added, incubated at room temperature for 5 min before centrifugation at 6800 × *g* for 1 min. The extract was stored at 4°C for the quantification analysis.

Quantification and Profiling of Samples

The Quantifiler kit assays were performed according to the protocol validated by Green et al. (25). The Quantifiler™ Y Human

Male DNA Quantification Kit was supplied by Applied Biosystems (), and quantification was carried out on the ABI PRISM® 7500 REAL Time PCR System (Applied Biosystems, Carlsbad, CA).

Samples were amplified for genetic profiling using the AmpF*l*STR® SGM Plus™ Amplification Kit (Applied Biosystems) and a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems), as recommended by the manufacturer (1 ng of DNA in 50 μL reaction volume, 28 cycles). Profiles were generated using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) using a 10-sec injection at 3 Kv. Sample solution was 9 μL (174 μL Hi-Di Formamide + 6 μL Genescan ROX 500 – HD Size Standard [Applied Biosystems]) and 1 μL amplified DNA. Analysis was undertaken using Genescan® analysis and GeneMapper™ ID SoftwareV3.2 (Applied Biosystems; minimum peak height of 50 rfu for heterozygotes and 200 rfu for homozygotes).

Results and Discussion

Control Negatives and Background Samples

When the samples from the control negative/background were quantified, the result in each case was negative. The limit of detection of the system was 0.0065 ng/μL in keeping with validation data published by Green et al. (25).

Calculation of DNA Concentration

The extraction method has a final resuspension volume of 50 μL. The quantification result obtained from the standard curve refers to ng/μL in this final 50-μL volume. In each case (Tables 1–3), the concentration of DNA is calculated with reference back to the amount of original saliva in question, i.e., 50 μL for the skin/fabric studies and 10 μL for liquid saliva. For example, the result from the standard curve for recipient 1/donor 1 (R1/D1) at T0 was 3.31 ng/μL. This was in a final extracted volume of 50 μL. Therefore, the amount of DNA present was 165.5 ng. The original volume of saliva deposited was 50 μL, giving a value of 3.31 ng of DNA recovered per μL of saliva deposited. The actual concentration of DNA in the liquid saliva prior to deposition was not measured.

Variability in DNA Level in Saliva Samples

The results of the variability study are given in Table 1. The results show that there can be a variation in the amount of DNA present in saliva samples taken from different donors. The mean concentration for the three donors was 11.92 ng/μL (standard deviation, SD = 4.38), 13.17 ng/μL (SD = 4.09), and 20.17 ng/μL (SD = 6.60), respectively. In the case of liquid saliva, the initial volume of saliva was only10 μL, and the amount of DNA present is divided by 10 to give the amount of DNA per μL of saliva. There is also a large variation in the quantity of DNA present in saliva samples from the same donor taken at different times of the day. Each donor had up to a two- or threefold difference in the amount of DNA present at different times of the same day. Amylase levels in saliva vary at different times of the day and generally increase after lunch (26). There is no relationship between amylase levels and the amount of DNA in saliva, but any study involving saliva should take into consideration that variations can occur in either throughout the day.

Comparison of Swabbing with Minitaping

The results of these experiments are presented in Table 2. Series 1 represents the initial test results for the 16 samples, eight minitapes, and eight swabs. Series 2 represents the duplicates run 7 days later. The mean from the minitape method was greater in each series. The range for the minitapes had higher upper and lower limits. This observation was not, however, statistically significant ($p > 0.1$). A previous study (27) compared the double-swab method with a tapelift method for the recovery of DNA from shoe insoles. Its results indicate that the tapelift method gave comparable to higher DNA recovery. The use of minitapes however excludes complications associated with water-soluble contaminants, and there is no drying or freezing required. In the light of the above, the remaining experiments were conducted using the minitape method.

The results of the recovery experiments incorporate two separate processes: the initial retrieval of material from the surface in question and then the recovery of the material from the swab/tape.

The Persistence of Salivary DNA on Human Skin

The results of persistence over a 96-h period are shown in Table 3. The average result for the three donors and three recipients is shown in Fig. 1. Each point in Fig. 1 represents the average of the nine results for the particular time, and the error bar for the standard error of the mean is also shown. Overall, as expected, the DNA concentration decreases with time. The loss of (amplifiable) DNA may be because of various factors including:

TABLE 1—*The variability of DNA concentrations (ng of DNA per μL saliva) of samples taken from three donors at different times of the day over four consecutive days.*

	Donor 1			Donor 2			Donor 3		
Day	10:00 am	11:30 am	3:00 pm	10:00 am	11:30 am	3:00 pm	10:00 am	11:30 am	3:00 pm
1	13.95	10.20	23.75	23.15	16.40	16.25	25.55	33.75	12.85
2	9.15	9.20	10.50	10.20	12.80	10.80	24.00	22.40	18.00
3	12.10	11.5	9.20	9.40	9.60	10.40	15.00	14.75	15.25
4	9.05	8.25	16.20	14.20	15.00	9.80	20.15	12.85	27.55

TABLE 2—*Comparison between swabbing skin and minitaping skin for recovery of salivary DNA (values given are ng of DNA per μL of saliva applied).*

Series Number	Average Recovery from Swabs	Average Recovery from Minitapes
1 ($n = 8$)	12.71 (SD = 4.95) (range 3.32–18.28)	15.77 (SD = 5.56) (range 5.12–23.94)
2 ($n = 8$)	6.79 (SD = 3.60) (range 3.60–13.71)	9.94 (SD = 5.93) (range 4.74–21.27)

SD, standard deviation.

TABLE 3—*Concentration of DNA (ng of DNA per μL saliva applied) recovered at different time intervals from female recipients (R1, R2, R3) using male donors (D1, D2, D3).*

	Time in Hours from Application on Skin to Sampling				
Sample	T0	T24	T48	T72	T96
R1/D1	3.31	0.61	3.39	0.39	0.59*
R1/D2	2.88	2.63	1.19	1.51	0.98*
R1/D3	17.77	6.28	0.99	4.39	0.83*
R2/D1	2.19	1.24	0.09	1.47	1.88*
R2/D2	3.97	1.19	1.93	0.24	0.61*
R2/D3	5.63	1.49	3.16	1.01	0.24*
R3/D1	1.94	0.14	0	0.01	0†
R3/D2	1.54	1.15	0.87	0.13	0.17*
R3/D3	8.39	2.01	0.16	0.03	0.28*
Average	5.29	1.86	1.31	1.02	0.62*
SD	5.15	1.81	1.27	1.40	0.57*
SEM	1.72	0.60	0.42	0.47	0.19*

*Full DNA profile of male donor obtained.
†No profile.
SD, standard deviation; SEM, standard error of mean (SD/√n); R1/D1, recipient 1/donor 1.

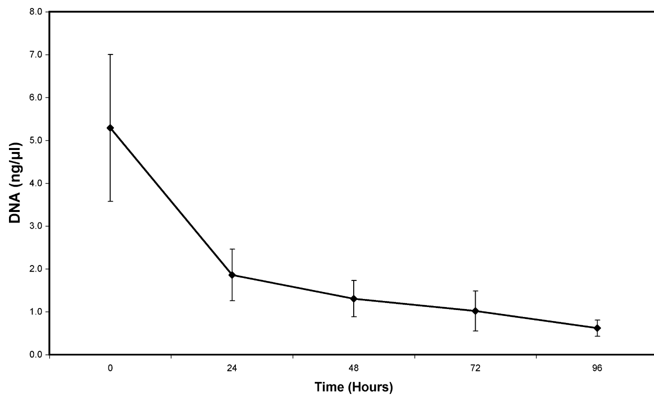

FIG. 1—*Average concentration of salivary DNA remaining on skin at five time intervals after initial application.*

- Friction from clothing in direct contact with the skin, particularly if the recipient was sweating;
- Loss owing to degradation caused by microorganisms.

The highest concentration at time interval T 96 h was 1.88 ng/μL (recipient 2, donor 1), and the lowest concentration was 0 ng/μL (recipient 3, donor 1). As can be seen from Table 3, there is a large degree of variation between experiments. This is not unexpected over a 96-h period where the recipients were expected to go about their normal lives and activities, apart from refraining from washing the area in question.

The findings mentioned are in keeping with an earlier study undertaken in this laboratory where salivary amylase activity was monitored on skin. Amylase activity was still detectable even after 96 h (28). The presence of amylase does not have any inference for the presence of DNA, but a screen for the presence of amylase could be used to indicate which area of an item was to be sampled for the presence of DNA. In a previous study, it was reported that saliva is stable on intact skin for at least 60 h following deposition on cadaveric skin. It was also suggested that DNA in dried saliva on living skin is retrievable for up to 72 h, providing the victim has not washed or it has not been removed in medical examination; however, no experimental information was published (22). It can be seen (Fig. 1) that the loss in the first 24 h is quite significant, approximately 60%. After that the rate of loss decreases. This finding is in keeping with a previous study by Sweet et al. (2), where saliva was deposited on the skin of cadavers and tested after 5 min, 24 h, and 48 h. The loss from 5 min to 24 h was in the order of 40% with no significant loss between 24 h and 48 h.

In our study, DNA profiling was carried out on all T96 samples. Eight of the nine samples profiled contained a full profile corresponding to the male donor (one sample R3/D1 failed). Six of the samples contained a profile exclusively from the donor. The reference profiles for the donors and recipients and the samples containing mixtures are presented in Table 4. The results from the other six samples are not presented in the table, as the only profile present is a full profile matching the donor. Sample D2/R2 had a minor allele, designated 15, at locus D3, which did not correspond to either donor or recipient. One explanation for this is a minor cross-contamination from donor 3 (who is a homozygous 15 at this locus), given that all the saliva samples were applied to the legs of the recipients side by side without any physical barrier between the areas where the saliva was applied. This is borne out on examination of the baseline for D2/R2 where many of the elements of donor 3 are evident but below the threshold level. Interpretation of the result for D2/R3 is more difficult. Again D2/R3 had an allele at locus D3, which did not correspond to either donor or recipient. Examination of the baseline again showed elements that correspond to donor 3. In trying to interpret mixtures, it is important to establish initially what type of mixture we are dealing with, equal contribution from each contributor or a mixture where one contributor is present in great excess to another. Care must always be exercised in the interpretation of mixtures. The approach to interpretation of D2/R3 was that the mixture for the most part could be clearly

TABLE 4—*Results of SGM plus DNA profiling from donors (D1, D2, D3), recipients (R1, R2, R3) and from two samples taken 96 h after saliva was applied to recipients.*

	SGM Plus Marker										
Sample	D3	vWA	D16	D2	AM	D8	D21	D18	D19	THO1	FGA
D1	15,18	17,18	11,12	18,19	X, Y	12,15	28,30	10,12	13,17.2	7,9.3	22,22
D2	16,17	17,17	8,13	19,20	X, Y	12,15	29,32.2	14,14	14,15	6,7	21,22
D3	15,15	16,17	11,12	16,21	X, Y	14,15	28,30	11,17	13,14	8,9.3	21,22
R1	15,16	17,17	10,12	17,19	X, X	13,14	28,31.2	14,15	12,14	7,9	22,22
R2	17,17	14,17	9,13	25,25	X, X	13,14	30,30	12,14	13,16	9.3,9.3	23,23
R3	14,16	17,19	12,12	16,17	X, X	14,14	30,31.2	17,17	13,13.2	9.3,9.3	20,23
D2/R2	[15]*, 16,17	17,17	8,13	19,20	X, Y	12,15	29,32.2	14,14	14,15	6,7	21,22
D2/R3	16,17 [14], [15]*	17,17 [19]	8,12 13	19,20	X, Y	12,14 15	29,32.2 [30] [31.2]	14,14 [17]	14,15 [13], [13.2]	6,7 [9.3]	21,22 [20]

*Allele of an unknown origin. Square brackets indicate a minor profile.

defined as having major and minor contributors. At eight of the 10 loci, donor 2 is a major contributor, and the remaining two loci (D16 and D8) can be treated as neutral (nonexclusion loci). At D16 and D8, recipient 3 is homozygote, and this contributes to an inability to assign major/minor at these loci. All the alleles in the minor contribution with the exception of locus D3 [15] are present in the profile of recipient 3. In casework situations, such as sexual assault when intimate samples are profiled, it is common to obtain mixtures, and in such situations the known profile of the victim may be subtracted to further deduce other contributing profiles. The whole area of mixture interpretation has recently been discussed (29).

In a study, which examined simulated strangulation (11), alleles of an unknown origin were also encountered. It is of note that, in our study, there is only small amount of contribution from the recipient. In a recent study on the recovery of DNA from the face of children (30), only 20% of the samples from the faces gave a full profile corresponding to the child's own profile, and at least 55% gave partial profiles. This low contribution from the skin of the recipient may be explained by the nature of the cells in question. A comparison between skin, buccal, and vaginal cells showed that the external skin epithelial cells were nonnucleated, whereas nuclei were visible in all buccal and vaginal cells examined (31).

In the experience of this laboratory, sexual assault victims frequently present themselves for medical examination up to 72 h and sometimes 96 h after the incident. This finding shows that valuable evidence can still be recovered even after such a long period of time.

Recovery of Salivary DNA from Fabrics

DNA was extracted from different fabric types, which had been in contact with saliva on skin. The highest amount of salivary DNA (1.22 ng/μL) was recovered from the cotton fabric, and the lowest amount of DNA (0.07 ng/μL) was recovered from the leather (Table 5). The absence of surface fibers on leather may explain the low DNA concentration observed. Another factor in the low concentration of DNA recovered from leather may be the presence of inhibitors on the leather. It is thought that leather contains an inhibitor of the polymerase chain reaction (PCR) probably introduced during the tanning process (27). The quantification method uses real-time PCR, and the presence of inhibitors could interfere with the quantification. Recovery of salivary DNA from fabric is important if a bitemark is made through the clothing (primary transfer). It is also important in cases where the skin samples have not yielded sufficient DNA for analysis. This may be attributable to sampling the wrong area, washing, or a long time interval between the incident and the medical examination. In such instances, the only DNA available may be that transferred to the fabric from the skin (secondary transfer).

If there is a good indication of the original location of saliva on the skin, the fabric most likely to have been in contact with the skin can be removed and extracted. An example of this is the inside of bra cups when there has been an allegation of sucking or kissing of the breasts. It is not uncommon for the victim in these cases to get dressed after the incident, especially if the incident has occurred outdoors or in the suspect's residence. The victim may then shower, and the only link to the suspect is the salivary DNA deposited on the inside of the bra. In cases of suspected oral/penile contact, it is now practice in this laboratory to minitape the inside front of suspects' underpants; the suspect frequently comes to light some days after the incident, and penile swabs are not taken. The results of this study indicate that the amount of salivary DNA recovered will be affected by the fabric composition. It should be noted that this study was limited in its scope. A more detailed examination of all the factors that affect secondary transfer is justified.

TABLE 5—*Secondary transfer of salivary DNA (values given are ng of DNA per μL of saliva applied) from human skin to various fabric types.*

Fabric Type	DNA Concentration
Cotton	1.22
Denim	0.28
Leather	0.07
Nylon	1.14
Polyester	1.15
Silk	0.59
Wool	0.44

Conclusion

The Quantifiler™ Y Human Male DNA Quantification Kit is a useful tool for detecting the presence of male DNA on females without interference from the female DNA. The minitape method is generally more effective than swabbing for the recovery of salivary DNA and is also the faster method for the collection of cellular material. Our findings show that salivary DNA will persist on skin at least up to 96 h. This has major significance for forensic science casework. This study also shows that dried salivary DNA will transfer from skin to material in contact with the skin. This also has significance for forensic science when the perpetrator's DNA has not been detected on the samples from the victim's body. This study will aid forensic medical practitioners when making decisions on how, when, and what to sample in the course of the medical examination. The data also help forensic scientists in deciding their examination strategy in relation to salivary DNA.

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