Forensic Science International: Genetics 84 (2026) 103516

Contents lists available at ScienceDirect

Forensic Science International: Genetics

journal homepage: www.elsevier.com/locate/fsigen

Who packed the drugs? Improved DNA recovery and its impact on findings given activity-level propositions

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ARTICLE INFO

Keywords:
Indirect transfer
DNA extraction
Activity-level

ABSTRACT

The interpretation of forensic DNA findings given activity-level propositions depends on the processes governing DNA transfer, persistence, and recovery, as well as on the laboratory methods used to recover DNA. This study investigated the effects of transfer type, substrate characteristics, and DNA extraction strategy on DNA yield, STR profiling performance, and resulting likelihood ratios given activity-level propositions. Direct and indirect transfer experiments were performed using personal bags (backpacks and purses) and zip-lock bags. Three analytical approaches were compared: Chelex extraction, a co-extraction protocol combining the QIAamp DNA Mini Kit and mirVana™ miRNA Isolation Kit, and Direct PCR. Consistent with established transfer principles, direct transfer produced substantially higher DNA quantities than indirect transfer across all methods. The proportion of DNA transferred from bags to zip-lock bags was slightly influenced by bag type and inner surface characteristics. Extraction approach noticeably affected DNA yield and profiling success. The co-extraction protocol generated higher DNA quantities and more robust STR profiles compared with Chelex and Direct PCR. Direct PCR provided advantages in minimizing DNA loss but showed limitations related to inhibition and overall profile quality compared with the co-extraction protocol. Differences in extraction efficiency altered the distributions of DNA quantities observed under direct and indirect transfer scenarios, thereby influencing likelihood ratios given activity-level propositions. These findings are consistent with inter-laboratory studies demonstrating that methodological variation can influence the distributions underlying evaluations of DNA findings given activities. Overall, the results demonstrate that laboratory methodology, particularly the DNA extraction approach, plays a critical role not only in DNA recovery and profiling performance but also in the evidential weight assigned when evaluating DNA findings given activity-level propositions.

1. Introduction

Understanding the mechanisms of DNA transfer, persistence, prevalence, and recovery (DNA-TPPR) is essential in forensic investigations, as it provides the basics for experts to make assessments of results given activity propositions. This requires a thorough understanding of the multiple, interrelated factors [1].

DNA transfer is the movement of DNA from one surface to another. It can occur through various pathways [1,2]. Direct (primary) transfer occurs when DNA is deposited directly onto an object, surface, or another person's skin through direct physical contact; Indirect (secondary or subsequent) transfer occurs when DNA is transferred again after its initial deposit, meaning the original source is not in direct contact with the final location where the DNA is found [1,3]. Several factors influence transfer [1,4], such as surface type, surface roughness, topography, and physicochemical properties [4,5]. In addition, contact

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https://doi.org/10.1016/j.fsigen.2026.103516
Received 9 March 2026; Received in revised form 22 April 2026; Accepted 23 April 2026
Available online 30 April 2026
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frequency, duration, and pressure are important factors: most DNA is transferred during the initial contact, while greater handling intensity or applied friction further increases DNA yield . Individual shedder status (influenced by body area, skin condition, age, and gender) further influences transfer, as high shedders deposit DNA more easily [6].

DNA persistence refers to how long DNA remains on a surface in a detectable quantity and quality (e.g. completeness, degradation). The amount of DNA generally decreases over time, but the decay rate is highly variable [4]. Environmental factors (such as temperature, UV exposure, rain, wind, humidity, and microbial activity) and successive use by multiple people can influence DNA persistence [4].

Original DNA deposits on objects can remain after others handle an item, though recent users often, but not always, dominate [1].

Handwashing greatly reduces DNA on skin, but DNA can persist on clothing after washing [1,4].

In casework, a range of DNA collection methods are currently used, including swabbing, taping, and cutting, followed by different isolation techniques such as organic extraction, Chelex, or silica-based approaches. The choice of method strongly influences the yield and quality of DNA. The ReAct project [7], which compared 23 laboratories performing the same experiments, demonstrated substantial inter-laboratory variability, with DNA yields ranging from picograms to nanograms under identical conditions. This variability underscores the impact of methodological choices and the need for greater standardisation and transparency in forensic workflows. A major limitation of extraction procedures is DNA loss: van Oorschot et al. [8] reported losses of 20–76% depending on the method, with contributing factors including tube transfers, washing steps, and DNA adsorption to plastic consumables or matrices [9]. In this study, we specifically tested Chelex and a commonly used co-extraction protocol.

Chelex has been widely used in forensic laboratories due to its rapid workflow, minimal handling steps, and avoidance of toxic organic solvents. Its simplicity and efficiency have contributed to its broad adoption. However, a major drawback is the limited capacity to remove PCR inhibitors, which can lead to amplification failure or incomplete STR profiles. In Chelex extraction, the resin binds divalent ions such as Mg^{2+}, which are essential cofactors for DNases, thereby preventing DNA degradation. The 56 °C incubation facilitates cell lysis and DNA release, while heating at 95 °C denatures proteins and inactivates nucleases. Following centrifugation, the DNA remains in the supernatant, whereas Chelex beads and cellular debris form the pellet, yielding a crude but PCR-compatible extract [10,11].

Co-extraction is frequently used to enable simultaneous DNA and RNA recovery from one sample. In our study, we employed a hybrid protocol integrating the QIAamp DNA Mini Kit with the mirVana™ miRNA Isolation Kit [12]. In the co-extraction method, samples are lysed using the mirVana™ lysis buffer, which contains strong chaotropic and denaturing agents together with the reducing agent 2-mercaptoethanol, effectively disrupting protein cross-links and releasing nucleic acids from cells. The DNA is subsequently captured on the silica membrane of a QIAamp® column in the presence of ethanol, washed to remove impurities, and finally eluted in buffer, yielding highly purified DNA suitable for downstream analyses. Fantinato et al. [3] demonstrated that this co-extraction strategy yields significantly higher DNA quantities and superior-quality profiles compared to a modified QIAamp protocol. The inclusion of a reducing agent and an extended incubation period are thought to be critical for enhancing DNA recovery from touch deposits.

Direct PCR is also a technique suggested to improve results from low-quantity samples [9,13]. It enables the amplification of genetic material directly from the sample, eliminating the need for DNA extraction and purification steps. This technique offers both advantages and limitations [1]. The literature indicates that, in addition to being a faster approach due to the elimination of the DNA extraction step, this method can improve DNA recovery from various substrates, minimizing DNA loss. It is effective for low-template samples, and lowers the risk of sample handling errors [1,6,9,13]. However, this method also has limitations, such as susceptibility to inhibitors carried by the template, or less effective cell lysis [9,13].

In forensic evaluation, sub-source likelihood ratios address the question of whose *DNA it is*, while activity-level likelihood ratios address *how it came to be there*. There is growing recognition by courts that the presence of DNA alone is insufficient to make decisions; rather, it is the circumstances of its deposition that are crucial. Activity-level assessment is used to evaluate findings given alternative propositions put forward by the defence and prosecution [6]. The ReAct project observed significant variability of DNA recovery across laboratories [7], and further showed that this variability directly affects the likelihood ratios calculated given activity-level propositions. Thus, methodological choices such as extraction procedures become central not only for producing interpretable profiles but also to support robust activity-level reporting in judicial settings.

In this study, we have re-investigated an old case scenario using new methods with the expectation of higher DNA yields. First, we examined the quality of the DNA results using different extraction and analysis strategies represented by DNA quantities and sub-source LRs. Secondly, the impact of using different methods on activity-level LRs derived from probabilistic models of DNA transfer and persistence was investigated. We compared activity-level LRs derived from (i) this study and (ii) the previous experiment [6]. For the latter, a probabilistic framework was developed which was based on a previously described Bayesian network [6] with case-specific modifications. Finally, RNA analysis was performed to investigate the potential source of the cellular material and to evaluate how this influenced detection outcomes.

2. Material and methods

This study received ethical clearance from the Data Protection Officer (DPO) (reference: 24/19717) at Oslo University Hospital. Written informed consent was obtained from all participants.

2.1. Simulated drug packaging

Twenty participants were instructed to provide a backpack or purse from regular personal use. Two zip-lock bags filled with 15 mL of sugar (as drug package imitators) were packed and placed inside each item by an investigator wearing gloves. The zip-lock bags were left for a 24-hour period. During this time, the bags were manually agitated five times to mimic typical movement and contact during transportation, thereby representing an indirect transfer scenario.

In addition, to demonstrate a direct transfer scenario, 13 participants were provided a new and empty zip-lock bag and instructed to handle the bag (open and close) three times and then return the bag in a clean envelope. All handling took place at least one hour after washing hands.

2.2. Sample collection

2.2.1. Transfer study

One sample was collected by swabbing the outer surface and lock of each of the zip-lock bags, either stored in the bag/purse (zip-lock bag 1 (S1) and zip-lock bag 2 (S2)) and from those directly handled (S3). In addition, a sample was collected from the internal surface of the personal bag (S4, approx. 20 cm^2 area).

Swabbing was performed using sterile, moistened cotton swabs. A moistened (one drop of water) Taper tip swab (MWSCS/F Taper tip Swab, Dryswab, MWE - Medical Wire) was used for S1. For S2, S3 and the interior surfaces (S4), moistened swab (MW1048/F Tubed Sterile Dryswab-Forensic, MWE-Medical Wire) were applied. Swabs were dried in ventilated evidence pouches and stored there until further processing. The tips of the swabs collected from zip-lock bag S1 were cut directly into PCR tubes for immediate analysis. Following the drying process, the remaining swabs collected from the zip-lock bag (S2) and from the inner surface of the bag (S4) were processed by cutting off the swab tips,

which were subsequently transferred to 1.5 mL Eppendorf tubes for further analysis (Fig. 1).

2.2.2. Extraction study

The objective of this study was to directly compare the efficiency of two extraction protocols: the Chelex and the Co-extraction method. A total of 20 samples were collected from the skin (forearms and neck) of 4 participants, using swabs (MW1048/F Tubed Sterile Dryswab-Forensic, MWE-Medical Wire). Two swabs per area were collected at the same time to maintain the same force placed on the swabbing process. Swabs were rubbed simultaneously with medium pressure over the designated skin area. The swab pairs were separated between the Chelex extraction and the co-extraction method.

2.3. Sample processing

2.3.1. Direct PCR analysis

DNA from swabs collected from S1 were analysed without prior extraction. The PCR reaction mix was added directly to the tube containing the swab tips. The PowerPlex Fusion 6 C System kit (Promega, Madison, WI, USA) was used for amplification, performed on a Veriti 96-Well Thermal Cycler (ThermoFisher, Waltham, MA, USA), following the manufacturer's recommendations.

2.3.2. Co-extraction of DNA and RNA

Swabs from S2-S4 underwent a co-extraction protocol using the QIAamp DNA Mini Kit (QIAGEN) in combination with the mirVana™ miRNA Isolation Kit (Invitrogen, ThermoFisher). The applied method was retrieved from the paper by Lindenbergh et al. [14] with a few exceptions described by Wang et al. [15]. The final elution volumes were 60 µL for RNA and 100 µL for DNA, respectively.

2.3.2.1. Chelex sample analysis. Half of the samples collected (Section 2.2.2) were extracted using a 5% Chelex procedure [16] (no prior incubation with water) and 200 µL of Chelex 5% (Bio-Rad, Hercules, CA, USA) was added to the Eppendorf tubes containing the swabs. Samples were incubated at 56 °C for 30 min (final minute at 1400 rpm), followed by 95 °C for 8 min. Samples were centrifuged and supernatant was transferred to new tubes for storage at −20 °C. The protocol was carried out as outlined by Fonneløp et al. [6].

2.3.2.2. DNA quantification, amplification, and analysis. Quantification was performed on samples from S2-S4 using the PowerQuant System (Promega, Madison, WI, USA). Reactions were prepared using the manufacturers' recommendations and analysed via qPCR on the 7500 Real-Time PCR System (ThermoFisher, Waltham, MA, USA). PCR amplification was carried out using the PowerPlex Fusion 6 C System (Promega, Madison, WI, USA). PCR was performed in a Veriti 96-Well Thermal Cycler (ThermoFisher, Waltham, MA, USA) as recommended by the manufacturer (1 ng template or 15 µL if 1 ng could not be reached, 25 µL reaction volume and 29 amplification cycles). Capillary electrophoresis was conducted on an Applied Biosystems 3500xL Genetic Analyser. Samples were injected at 1.2 kV for 24 s. The results were analysed using the GeneMapper ID-X Software version 1.6 (ThermoFisher, Waltham, MA, USA). The analytical threshold (AT) for alleles was set to 100 RFU.

2.4. Statistical analysis

Two analytical levels were considered. Sub-source likelihood ratios were computed to evaluate the strength of DNA evidence with respect to source attribution. Separately, activity-level likelihood ratios were computed to evaluate alternative propositions about how the DNA was deposited. A potential DNA mixture was defined when three or more alleles were observed at a locus. Results were analysed using R version 4.3.0.

2.4.1. Quantitative data processing and evaluating results given sub-source propositions

Sub-source likelihood ratios (LRs) were calculated using EuroForMix v.4.1.0 under two alternative propositions:

Hp: The DNA originates from the person of interest (POI).

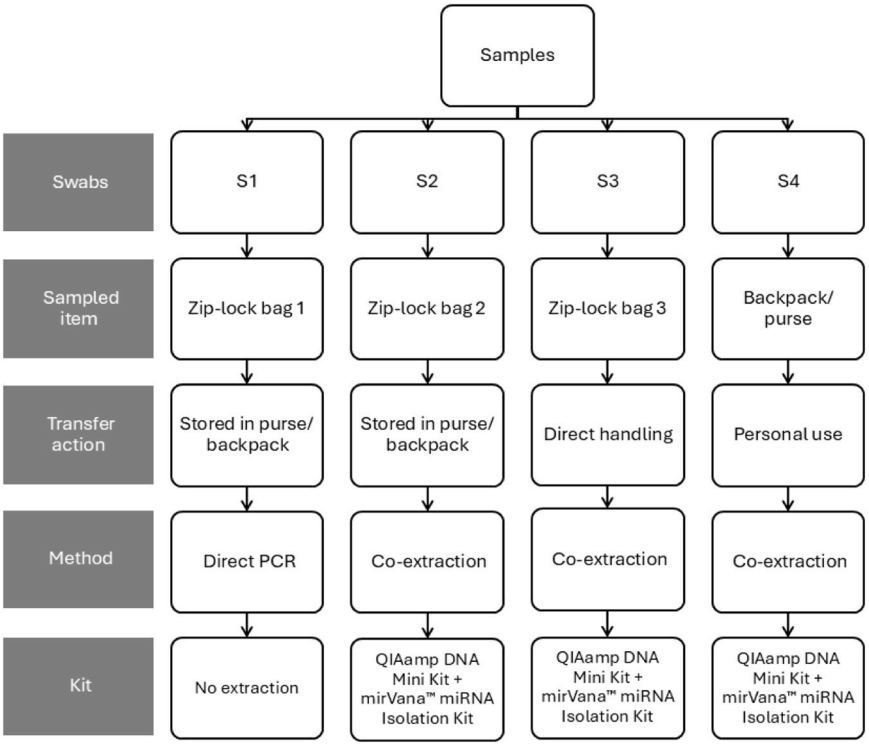

Fig. 1. Schematic representation of the swabs S1-S4 and their respective sampled item, transfer action, method and kit applied.

Hd: The DNA originates from an unknown, unrelated individual.

When more than two alleles were observed in a marker, an additional unknown contributor was included in both the numerator and the denominator.

The average RFU for a contributor was calculated as the total RFU ($RFU_{t_o t}$) divided by the number of loci (n, 23 excluding amelogenin), multiplied with the contributor's mixture proportion as shown in Eq. 1.

$$\overline{RFU}_{POI} = M_x \times \frac{RFU_{tot}}{n} \quad (1)$$

The Wilcoxon rank-sum (Mann–Whitney U) non-parametric test was used for assessing differences between two independent group distributions.

The average DNA concentration attributed to the POI ($Quant_{POI}$) or unknown contributors was calculated as the total DNA quantity multiplied by the respective mixture proportion.

The percentage of transfer from the personal bag to the zip-lock bag was calculated for each bag. The transfer proportion (Tx) was calculated by dividing the DNA quantity detected on the zip-lock bag (S2) by the total DNA quantity detected on the zip-lock bag and in the purse (S2+S4) as shown in Eq. 2. Samples collected from zip-lock bag 1 (S1) were not quantified and were therefore excluded from the calculation of transfer percentages.

$$Tx = \frac{Q_{S2}}{Q_{S2} + Q_{S4}} \quad (2)$$

2.4.2. Evaluating the biological findings given activity level propositions

2.4.2.1. Case example. To evaluate the potential impact of improved DNA extraction efficiency on the interpretation of biological findings at activity level, we re-investigated the case example described in Fonneløp et al. [6]. In this scenario, a large depot of drugs was discovered at a hideout. The drugs were stored in zip-lock bags placed inside a gym bag. The suspect (Person A) denied any knowledge of the drugs but recognized the gym bag as previously belonging to him, claiming that it had been lost or stolen two weeks earlier.

The competing activity level propositions were defined as:

Hp: The suspect packed the drugs.

Hd: The suspect had no involvement with the drugs, but was the previous owner of the gym bag, which was stolen two weeks prior. An unknown individual packed the drugs.

List of variables:

We follow the notation used by Gill et al. [17], summarised here:

- t is the probability of direct transfer, persistence and recovery of DNA from the POI (under Hp only).
- t' is the probability of direct transfer, persistence and recovery of DNA from an unknown contributor (under Hd only).
- b is the probability of background DNA, representing DNA present on the items from unknown sources and unrelated activities. Because the experimental design did not generate dedicated background-only samples, background was modelled using the simplified approach of Gill et al. [17], with a scaled function anchored at $b(0) = 0.05$. This value reflects a low but non-zero probability of detecting DNA from unknown contributors, consistent with previous studies. The same background function was applied across both extraction methods.
- s is the probability of indirect transfer, persistence and recovery.
- Suffixes are applied to described probabilities of an event given a particular contributor, e.g., t_A refers to the probability of direct transfer, persistence and recovery of DNA from contributor A.

Additional notation used in the equations:

- k: number of zero observations (non-detects)
- n: total number of observations in the group
- μ, σ: parameters of the fitted lognormal distribution of POIconc
- x: DNA concentration threshold used when calculating exceedance probabilities

2.4.2.2. Data. In brief, DNA quantity for the person of interest (POI) was represented by POI_{conc} (ng/μL) as the quantitative quality metric. This mirrored the approach of §3.5.1 in Fonneløp et al. [6], where RFU values were used, but with POI_{conc} replacing RFU. The POI_{conc} values were also extracted from the previous experiment [6]. Furthermore, samples were divided into **direct** groups (used to inform functions t and t') and **indirect** groups (used to inform functions s and b), following the framework of Fonneløp et al. [6].

In the experimental setup, the bags were packed by an investigator wearing gloves. Consequently, the study design did not generate observations of direct transfer from an unknown contributor, and the probability of direct transfer from an unknown individual (t') could not be estimated from the experimental data.

For the activity-level evaluation we therefore adopted the proxy $t' = t$, following the approach described by Gill et al. [17], which reflects a casework scenario in which an unknown individual may have handled the item directly. This assumption was used solely to enable comparison of likelihood ratios across extraction methods. If glove use were assumed in the case scenario, t'would approach zero and the resulting activity-level likelihood ratios would be substantially lower.

2.4.2.3. Distribution fitting. For each group, a lognormal distribution was fitted to positive POI_{conc} values using the *fitdist* function from the **fitdistrplus** R package. This yielded group-specific parameters (μ, σ) for direct and indirect transfer.

2.4.2.4. Zero-inflation (non-detects). To incorporate the observed proportion of samples that did not have a measurable quantity (non-detects), parametric tail probabilities were multiplied by (1−p0) where p0 represents the non-detect rate within each group. To stabilize estimates and avoid boundary artefacts, Jeffreys prior smoothing was applied:

$$p_0 = \frac{k + 0.5}{n + 1} \quad (3)$$

where k is the number of zero values in n samples.

Probability functions

For a threshold x (ng/μL), the following probability functions were defined:

Direct transfer (POI):

$$t_A(x) = (1 - p_{0,\text{direct}}) \left[1 - \Phi\left(\frac{\ln(x) - \mu_{\text{dir}}}{\sigma_{\text{dir}}} \right) \right] \quad (4)$$

Unknown direct (assumed same as POI, following Gill et al. [17]):

$$t'(x) = t_A(x) \quad (5)$$

Indirect transfer (from bag):

$$s(x) = (1 - p_{0,\text{indirect}}) \left[1 - \Phi\left(\frac{\ln(x) - \mu_{\text{ind}}}{\sigma_{\text{ind}}} \right) \right] \quad (6)$$

Background: scaled to match *b(0) = 0.05:*

$$b(x) = \min\left\{ 1, \frac{s(x) \cdot 0.05}{s(0)} \right\} \quad (7)$$

Probability of unknown under H_d:

$$\Pr(U_d | x_U) = t'(x_U) + b(x_U)\,[1 - t'(x_U)\,] \quad (8)$$

Likelihood ratios (activity level):

Let $U_d$denote the event that DNA from an unknown contributor is detected at or above the threshold x_U. Further, let $t_A(x)$denote the probability that DNA from contributor A(the POI) exceeds threshold $x$through direct transfer, persistence and recovery. Likelihood ratios

(LR) were defined for the POI threshold x_A and the unknown threshold x_U, according to Gill et al. [17]:

- Only POI observed:

$$LR = \frac{[s(x_A)(1 - t_A(x_A)) + t_A(x_A)]\,[1 - b(x_U)]}{s(x_A)\,[1 - \Pr(U_d|x_U)]} \quad (9)$$

Under H_p, secondary transfer is explicitly allowed, as the items were transported in the suspect's bag; Eq. 9 therefore combines direct and secondary transfer probabilities.

- POI and unknown observed:

$$LR = \frac{[s(x_A)(1 - t_A(x_A)) + t_A(x_A)]\,b(x_U)}{s(x_A)\,\Pr(U_d|x_U)} \quad (10)$$

3. Results

3.1. DNA recovery and sub-source Likelihood Ratio in transfer scenarios

3.1.1. Direct and Indirect transfer

DNA was detected in the samples collected after direct (S3) and indirect (S2) handling (storage in personal bags/backpacks). Quantities were higher from the direct transfer experiment compared to those obtained from indirect transfer experiments (Fig. 2a, supplementary table S2 and S4)

On average, the highest sub-source Log_{10} LR values supporting the detection of the POI were observed in the direct experiment (average direct = 27.53) (Fig. 2b).

3.1.2. Proportion of DNA transferred from interior of personal bags to zip-lock bags

POI was strongly supported as a contributor (LR>100) in 33/40 samples, collected both from the zip-lock bags stored in the personal bags (S2, 15/20) (average $Conc_{POI}$= 0.006 ng/μL); and from the samples collected from the interior of the personal bags (S4, 18/20) (average $Conc_{POI}$= 0.06 ng/μL) (Fig. 3, supplementary table S2 and S3).

Samples collected from the zip-lock bag (S2) showed lower peak heights attributed to the POI (average RFU_{POI} = 677) and also a lower sub-source LR to support the proposition that the POI was a contributor to the sample (average Log_{10} LR_{POI} = 18.8), in comparison with samples collected from the interior of the bag (S4) (average RFU_{POI} = 4353; average Log_{10} LR_{POI} = 28.04) (Fig. 4).

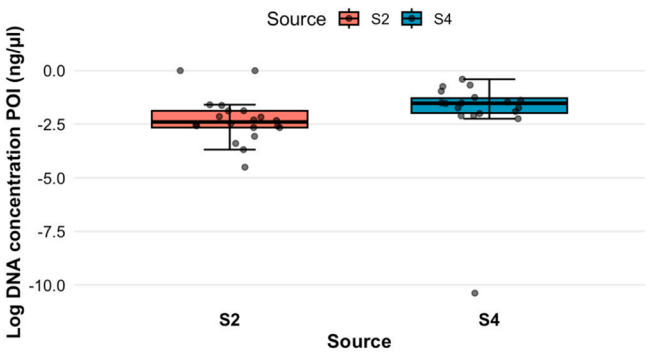

Fig. 3. Boxplots of samples S2 (zip-lock bag, co-extraction, coral) and S4 (bag interior, co-extraction, blue): sub-source Log_{10} calculated with EFM - transformed DNA concentration (ng/μL) matching the POI.

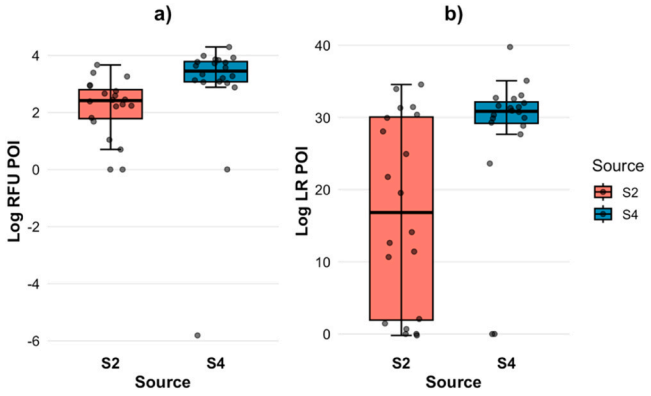

Fig. 4. Boxplots of samples S2 (zip-lock bag, co-extraction, coral) and S4 (bag interior, co-extraction, blue): (a) Log_{10}RFU values matching the POI; (b) sub-source Log_{10}LR values matching the POI, calculated with EFM.

The number of contributors detected in the zip-lock bag samples ranged between 0 and 3 contributors (average = 1.4), while bag interior had up to 4 contributors (average = 2.6).

3.1.3. Percentage of transfer per bag

Participants provided a total of 20 bags, consisting of 10 purses and

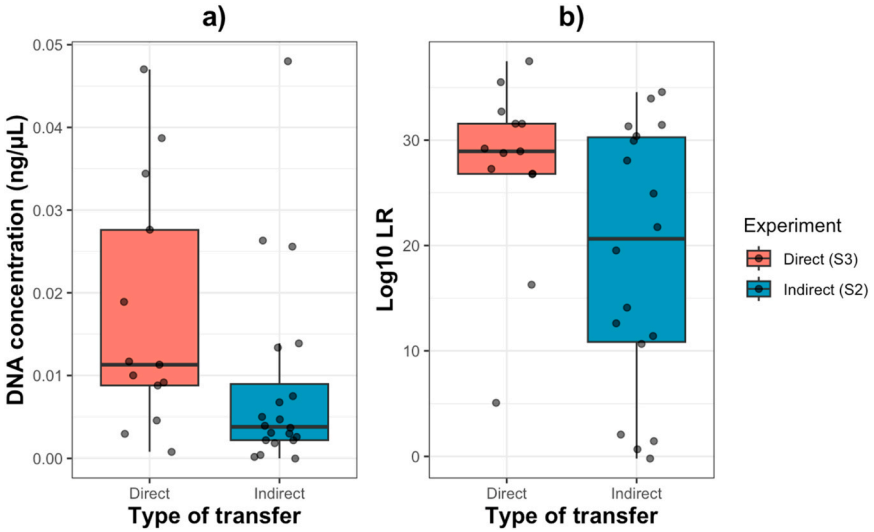

Fig. 2. Boxplots of direct (coral) and indirect (blue) transfer analysed with co-extraction (ng/μL in 100 μL): (a) DNA concentration for Direct (S3) and Indirect (S2) transfer; (b) sub-source Log_{10} LR values, calculated with EFM.

10 backpacks. Comparable DNA quantities were detected in samples from purses (average DNA concentration = 0.04 ng/μL) and backpacks (average DNA concentration = 0.09 ng/μL). A Wilcoxon rank-sum test showed no significant difference in DNA quantification between the two bag types ($p = 0.578$).

The proportion of DNA transferred from the inner surface of the bag to the zip-lock bag was calculated as described in Section 2.4. A higher percentage of DNA transfer to the zip-lock bags was observed from the purses than from the backpacks provided by the participants (Fig. 5a).

We classified the bags according to their interior, porous or non-porous. Nine of the twenty bags were categorized as non-porous, with two of them being purses and the other seven being backpacks. Of the twenty bags, eleven were found to be porous, with eight being purses and three being backpacks.

A slightly higher percentage of transfer was obtained from the non-porous surface to the zip-lock bag than from the porous surface (Wilcoxon rank-sum test showed no significant difference in DNA transfer between the two types of surfaces, $p = 0.71$) (Fig. 5b, supplementary table S6).

Between the two types of bags, backpack and purses, all backpacks were of a bigger size, comparing with the purses. The interiors of the backpacks included both porous and non-porous material but were more frequently made of waterproof (non-porous) linings compared to purses, which typically had fabric linings.

3.1.4. mRNA results

The presence of body fluids was assessed based on mRNA profiling results. All RT-negative reactions showed no expression of housekeeping genes, and all negative controls processed alongside the samples yielded negative results.

In total, 40 samples (20 from inside personal bags and 20 from zip-lock bags) were analysed. Most samples (82.5%, N = 33) displayed peaks above the analytical threshold for the two housekeeping genes, indicating presence of cellular material, while 17.5% (N = 7) were negative (one or both the housekeeping genes were not detected). In 7 samples the marker for female or male was detected; 10% (N = 4) had female marker (XIST) and 7.5% (N = 3) had male marker (RPS4Y1). Only one sample showed presence (peaks in > 50% of markers) of blood (HBB, CD93). Twenty percent (N = 7) of the samples showed a sporadic detection of one of the body fluids. Two samples showed sporadic detection of saliva (STATH), one sample showed sporadic detection of blood (CD93) and mucosa (MUC4), one sample displayed sporadic detection of blood (HBB), and one sample exhibited sporadic detection of blood (HBB), saliva (STATH) and menstrual blood (MMP11).

3.2. Comparison of methods

3.2.1. Extraction test

An extraction test was performed to specifically compare the DNA-results after extraction with Chelex 5% and the co-extraction including the 2 h incubation with the mirVana™ miRNA Isolation Kit (Invitrogen, ThermoFisher). The results demonstrate higher DNA concentrations and better-quality sub-source profiles ($Log_{10}LR$) obtained from the samples extracted with co-extraction (average DNA concentration=0.038 ng/μL; average of $Log_{10}LR = 31.76$), (Fig. 6, supplementary table S8) compared to the Chelex 5% extraction (average DNA concentration=0.0065 ng/μL; average $Log_{10}LR=21.97$) (Fig. 6). The Wilcoxon rank-sum test (two-sided) showed that the differences in DNA concentration were significant (W= 17, $p = 0.01$).

3.2.2. Direct PCR (S1)

For samples collected from the zip-lock bags analysed with Direct PCR (S1), we were able to obtain a DNA profile aligning with the POI in 17 out of 20 samples (LR>100).

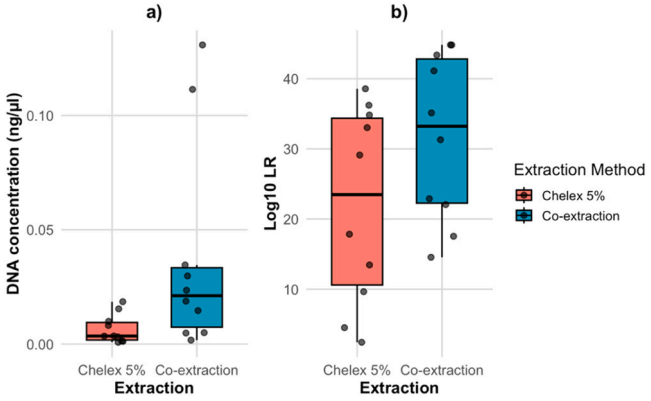

Fig. 6. Boxplots showing DNA concentrations—Chelex 5% (ng/μL in 200 μL) and co-extraction (ng/μL in 100 μL)—and sub-source $Log_{10}LR$ values, calculated with EFM, for Chelex 5% (blue) and co-extraction (coral).

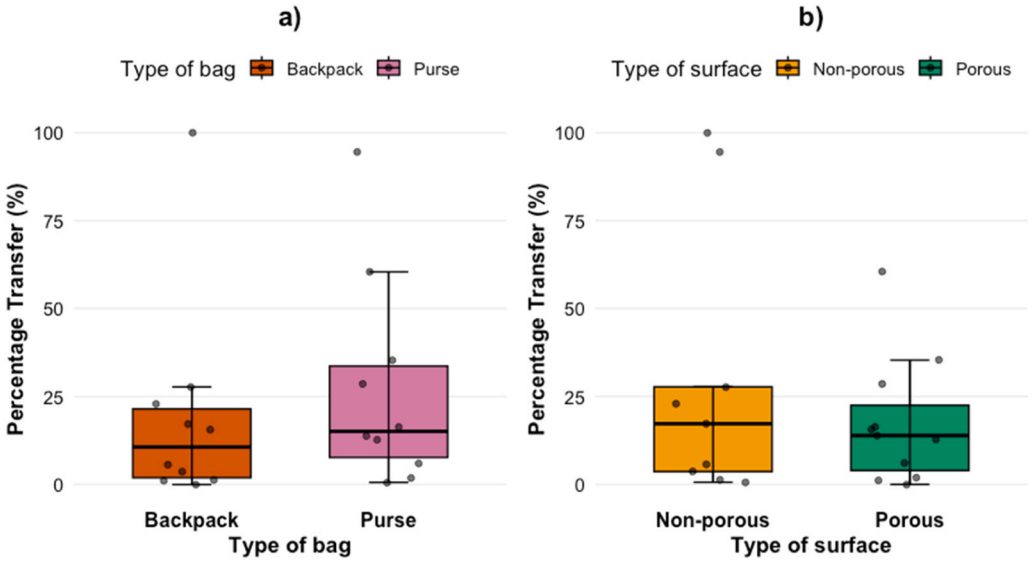

Fig. 5. Boxplots showing the percentage of DNA transfer from bag interiors (S4) to corresponding zip-lock bags (S2), stratified by (a) bag type: backpack (orange) and purse (pink), and (b) surface type: non-porous (yellow) and porous (green).

Compared to the co-extraction profiles (S2), the results from S1 were of lower quality, with more background noise and less prominent peaks (Fig. 7, supplementary table S1).

3.2.3. Indirect transfer experiments (all methods)

DNA profile quality (RFU and sub-source LR) in samples collected after indirect transfer was compared across the three (extraction) methods: Chelex extraction from the previous study (2020 data) [6], Direct PCR (S1), and the co-extraction protocol (S2). Variation in Log_{10} RFU and sub-source Log_{10} LR values matching the person of interest (POI) was observed across methods. Both direct PCR and co-extraction outperformed Chelex with respect to DNA recovery and profile quality (Fig. 7, supplementary table S7). Mean Log_{10} RFU values were 0.94 for Chelex (2020 data), 2.08 for Direct PCR (S1), and 2.17 for co-extraction (S2). Despite similar RFU levels for S1 and S2, profile quality differed between the two methods. S1 profiles exhibited greater variability and noise, whereas S2 profiles were more consistent. This difference is reflected in the sub-source Log_{10} LR values supporting the proposition of POI presence: the mean values were 5.53 (Chelex, 2020), 17.2 (Direct PCR, S1), and 18.8 (co-extraction, S2). Although peak heights were comparable between S1 and S2, S2 produced higher and more stable LR values.

DNA concentrations differed significantly between Chelex and co-extraction (Wilcoxon rank-sum test, $p < 0.001$), demonstrating that extraction method affects DNA yield. The total DNA quantity recovered, accounting for the higher elution volume in the Chelex protocol, also differed significantly (Wilcoxon rank-sum test, $p = 0.014$), confirming the impact of extraction strategy on DNA recovery and subsequent interpretation.

3.3. Evaluating the biological findings given activity-level propositions

For both Chelex and co-extraction, exceedance probability (i.e. the probability that the DNA quantity exceeds a given threshold x) decreases as the threshold x increases (Fig. 8). Direct transfer probabilities are consistently higher than indirect transfer probabilities, reflecting stronger signals from direct samples. The background contribution remains low across thresholds, with b(x) staying close to 0.05 at the lowest thresholds and decreasing further with increasing x.

Comparison between methods showed that co-extraction yields generally higher probabilities for both direct and indirect transfer at low quantities, with the direct curve remaining close to 1 for a wider range of POI_{conc} values compared to Chelex. This reflects the improved recovery efficiency with the co-extraction method.

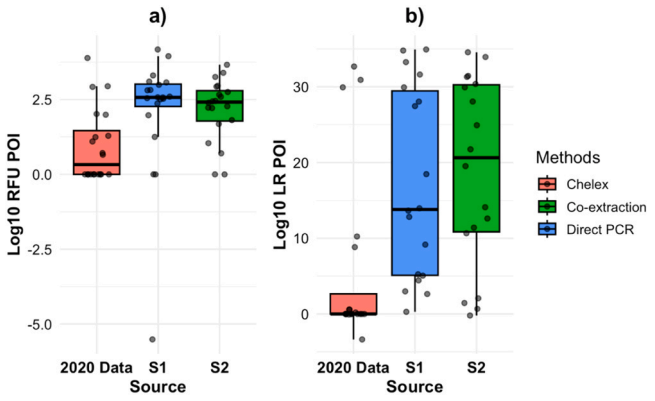

Fig. 7. Boxplots illustrating (a) Log_{10}RFU and (b) sub-source Log_{10}LR values, calculated with EFM, associated with the person of interest (POI) from all indirect transfer samples. Data are shown for three sample preparation methods: Chelex 5% extraction - 2020 Data (Experiment 1, coral), direct PCR - S1 (Experiment 2, blue), and co-extraction - S2 (Experiment 2, green).

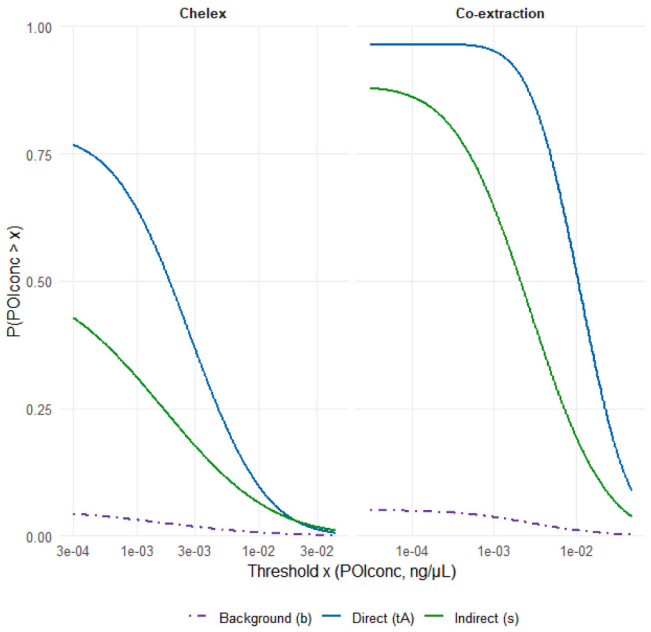

Fig. 8. Estimated exceedance probabilities $P(POIconc > x)$for direct transfer (tA), indirect transfer (s), and background (b) as functions of the DNA concentration threshold x(ng/µL), shown separately for Chelex and co-extraction.

3.3.1. Likelihood ratio calculations given activity-level propositions

Two DNA-results scenarios were considered when evaluating activity-level likelihood ratios:

(i) only DNA from the person of interest (POI) was detected in the sample, and

(ii) DNA from both the POI and an additional unknown contributor was detected.

These scenarios represent different evidential observations, not alternative propositions.

The propositions remain:

Hp: the suspect packed the drugs (direct transfer expected)

Hd: the suspect did not pack the drugs; an unknown person did (indirect transfer expected).

Likelihood ratios (LRs) were calculated according to the probability framework described in Section 2.5. The LR given activity level propositions compare the probability of the results given the alternative propositions Hp (the suspect packed the drugs) and Hd (the suspect had no involvement, and an unknown individual packed the drugs).

3.3.1.1. Chelex. When only the POI was observed, the LR was between 8–12, indicating weak support for Hp. When both the POI and an unknown were observed, the LR was less than one, at about 0.11–0.16, which provides weak support for the proposition that an unknown individual packed the item.

3.3.1.2. Co-extraction. The LR for the POI-only scenario was substantially higher, between 32–95, reflecting a larger separation between direct and indirect probabilities with this method. For mixtures including an unknown, the LR remained below 1 (≈0.06–0.16), again providing weak support for the proposition that an unknown packed the item (Table 1).

4. Discussion

4.1. DNA transfer

DNA quantities were consistently higher in direct transfer experiments compared to indirect transfer scenarios, regardless of extraction

Table 1
Likelihood ratios (LR) given activity level propositions, for two DNA result scenarios (single profile matching; POI and unknown observed) across selected POIconc thresholds, stratified by extraction method (Chelex and Co-extraction).

POI DNA detected in sample (ng/μL)	Chelex LR POI detected	Chelex LR POI and Unknown detected	Co-extraction LR POI detected	Co-extraction LR POI and Unknown detected
0	8.3	0.1	31.6	0.1
0.0012	11.6	0.2	44.4	0.1
0.0015	12.0	0.2	47.8	0.1
0.0081	11.7	0.2	84.1	0.2
0.0112	10.9	0.1	90.7	0.2
0.0353	7.6	0.1	95.2	0.2
0.0412	7.2	0.1	92.9	0.2

method (Fig. 2). This observation is consistent with established forensic principles and findings from the ReAct project, which demonstrated that direct physical contact typically results in greater DNA deposition compared to indirect transfer [1,6,7,18,19].

Although initial DNA quantities detected from backpacks and purses were comparable, our findings revealed a higher percentage of DNA transfer from purses compared to backpacks during indirect transfer scenarios. Backpacks generally had larger interior surface areas. Although shaking was conducted during storage to allow for friction and movement of the zip-lock bags, it is possible that the zip-lock bags did not come in contact with the whole inner surface, thereby reducing transfer to occur from the inside of the backpack compared to the smaller sized purses.

Supporting this observation, Fonneløp et al. [6] showed that the inner surface characteristics of bags affect DNA retention and subsequent transfer. It is hypothesized that purses, which often contain frequently handled personal items, accumulate more DNA from their owners, facilitating greater indirect transfer to objects stored within. This is further demonstrated by the increased DNA transfer percentages from purses in our findings. Although samples collected from the interior of the purses produced a lower DNA yield, they transferred a greater amount of DNA to the zip-lock bags. This highlights the need to consider specific material and usage characteristics within broad item categories when evaluating DNA transfer in forensic contexts [6].

A higher percentage of DNA transfer from non-porous surfaces to zip-lock bags was observed (Fig. 5). This is consistent with DNA transfer principles described by H. Arsenault et al. [4], where substrate characteristics strongly influence preservation and transfer efficiency: porous materials (e.g., cardboard, cotton, paper) absorb and retain DNA, hindering recovery, while non-porous surfaces (e.g., glass, plastic, metal) allow easier transfer. Recovery from porous substrates via swabbing may be limited, reducing detectable DNA despite higher retention [1,4]. Together, these findings illustrate that substrate properties affect both sub-source detection probabilities and the shape of indirect-transfer distributions that feed into activity-level evaluations.

4.2. Extraction strategies and profiling performance

4.2.1. Chelex

In forensic workflows, the choice of DNA extraction method is critical, as forensic samples are often limited in quantity and degraded. In this study, we compared a resin-based extraction method, Chelex 5%, with a co-extraction approach combining two different kits, QIAamp DNA mini kit with the mirVana™ miRNA isolation kit. The co-extraction method demonstrated superior efficiency in terms of both DNA yield and quality, consistently outperforming Chelex across both direct and indirect transfer experiments. While Chelex has been demonstrated as a viable method for extraction of forensic samples, its performance can be limited in the presence of inhibitors [3,10,11].

To support this interpretation, we also performed a controlled extraction comparison within the present study using paired swabs collected from the same skin areas. Under these conditions, the co-extraction protocol consistently produced higher DNA concentrations and higher sub-source LR values than Chelex, confirming that the observed differences are attributable to extraction efficiency rather than differences between experiments.

The difference likely reflects the underlying lysis chemistry. While Chelex relies on heat treatment and ion chelation to release DNA, the mirVana™ lysis buffer used in the co-extraction protocol contains strong chaotropic agents and the reducing agent 2-mercaptoethanol, which more effectively disrupt cellular structures, including keratinized cells often encountered in touch DNA. The extended incubation period further enhances cell disruption and DNA release. Moreover, DNA captured on silica columns in the QIAamp® kit undergoes rigorous washing, producing cleaner extracts with fewer inhibitors, and is recovered in a stable, low-salt buffer. In contrast, Chelex yields crude, less stable lysates that may compromise downstream analyses and storage. Together, these factors explain the superior DNA recovery and profiling success observed with the co-extraction method in challenging trace samples [3,11,13].

Similar findings have been reported by Fantinato et al. [3], who showed that combining the QIAamp DNA mini kit with the mirVana™ miRNA isolation kit, consistently yield higher DNA quantities compared to a slightly modified QIAamp DNA mini kit methods. The use of tailored extraction protocols, specifically the composition of lysis buffers and incubation conditions (temperature and duration) as demonstrated here, holds significant potential for improving DNA recovery from touch deposits, particularly from corneocytes, which require harsh lysis conditions for effective disruption, as reported in the literature [3].

vanOorschot et al. [1], confirmed that DNA recovery efficiency varies substantially depending on the collection and extraction methodologies employed, which in turn directly influences observed transfer rates. Inter-laboratory studies, including the ReAct project, have also highlighted considerable variation in median DNA yields across laboratories due to differences in sampling techniques, extraction methods, and PCR strategies [1,4,7].

4.2.2. Direct PCR

Direct PCR has been described as advantageous in forensic genetics, particularly for low-template or trace samples. By omitting extraction, the method minimizes DNA loss, reduces processing time, and lowers the risk of sample handling errors. It has also been reported to improve success rates for certain substrates and reference samples, making it a practical alternative in specific forensic workflows [9,13]. To further improve DNA recovery from the zip-lock bags, Direct PCR was applied to samples collected from the second zip-lock bag. When comparing the genotyping results from Direct PCR (S1) with those from Chelex (2020 data), Direct PCR produced stronger profiles, with more alleles matching the donor and higher peak heights. This likely reflects limitations of Chelex, which often suffers from inefficient lysis of certain cell types and dilution in large extraction volumes, resulting in reduced DNA recovery and weaker amplification [9,18]. PCR inhibition may also contribute, given Chelex's limited capacity for inhibitor removal. These findings are consistent with the observations presented in Section 4.3.

In contrast, the QIAamp DNA mini kit with the mirVana™ miRNA isolation kit combination (S2, S4) outperformed Direct PCR (S1). Although Direct PCR is designed to minimize DNA loss by omitting extraction, the extraction method produced somewhat higher peaks, less noise and overall better-quality results. This likely reflects the balance between DNA yield and purity: while Direct PCR introduces all available template into the reaction, it also carries inhibitors and cellular debris that can compromise amplification efficiency and profile quality. In contrast, the extraction protocol combines efficient cell lysis with effective inhibitor removal, producing cleaner extracts that support more robust amplification. The enhanced performance is therefore best explained by improved lysis and reduced inhibition, which outweigh the

moderate DNA loss associated with silica-based purification.

Commercial STR amplification kits have now incorporated advanced buffer-polymerase formulations designed to mitigate the effects of common PCR inhibitors [9]. While these kits can tolerate a range of inhibitory substances (e.g. hematin, metal ions, rust, soil) they do not physically remove these inhibitors, which can still lead to reduced amplification efficiency and partial or no genotyping results [9,13]. Direct PCR typically initiates cell lysis during the denaturation step of the first PCR cycle, which may not be equally effective for all cell types [13]. Moreover, a key limitation is that the entire sample is consumed during amplification, leaving no extract for re-analysis [9,13].

A key consideration in Direct PCR is swab cut size. Templeton et al. [20] demonstrated that smaller fragments (~2 mm^2) are known to reduce inhibition and enhance DNA release. We performed an initial pilot study (data not shown) to find the optimal cut size. In our study, we tested three fragment sizes; small, medium, and large, approximately 1, 2, 3 mm^2 wide respectively, to evaluate their performance, and small cuts consistently provided the best results. It is important to consider, that perhaps the designated "small" swab cuts may have been oversized, contributing excess material that reduced reagent availability and introduced inhibitors, thereby compromising PCR efficiency. This supports the importance of controlling swab size as a parameter in direct amplification strategies.

Although some studies consider Direct PCR advantageous for challenging samples due to its reduced DNA loss, its limitations, such as residual inhibitors and less efficient cell lysis must be considered when choosing the appropriate method [21,22]. Our studies indicate that an efficient extraction protocol such as the combination of the QIAamp DNA mini kit with the mirVana™ miRNA isolation kit can provide better results.

4.3. Evaluating the biological findings given activity level propositions

Changing the extraction method altered the distribution of POI concentrations for both direct and indirect transfer. With co-extraction, higher POI concentrations and fewer non-detects were observed, resulting in a steeper direct transfer curve compared to Chelex. Since the activity-level likelihood ratio depends primarily on the separation between the direct and indirect distributions, this shift translated into higher LRs when only the POI was observed. Conversely, when both the POI and an unknown were observed, applying a conservative rule for the unknown kept the LR below one for both methods, indicating support for the proposition that an unknown individual packed the items.

These findings are consistent with results from the ReAct project [7], which demonstrated that variation in laboratory protocols, including extraction efficiency, has a measurable impact on DNA recovery and the resulting distributions of direct and indirect transfer. ReAct emphasized that the critical factor for activity-level evaluations is not absolute DNA yield but the relative separation between distributions. Our data provide a concrete example: a change in method widened this separation and increased the LR in scenarios where only the POI was detected.

The implications are twofold. First, laboratory methods must be reported explicitly in activity-level evaluations, as changes in extraction protocol can alter the evidential weight. Second, comparisons of LRs across studies or laboratories should only be made when thresholds, treatment of non-detects, and background scaling are harmonized. These recommendations mirror the conclusions of ReAct, which called for greater transparency and standardization to ensure comparability of activity-level assessments across laboratories.

Limitations of the present study include relatively few positive counts, reliance on a lognormal distributional assumption, and sensitivity to the estimated proportion of non-detects. The ReAct project further highlighted substantial inter-laboratory variation, underscoring the need for larger and multi-laboratory datasets to strengthen the generality of conclusions regarding methodological impact on activity-level LRs.

In the experimental setup, the bags were packed by an investigator wearing gloves, meaning that direct transfer from an unknown contributor did not occur in the generated data. Under such conditions, the probability of direct transfer from an unknown contributor (t') would be expected to be very low and comparable to secondary transfer (s), which would result in substantially lower activity-level likelihood ratios because the observations become almost equally compatible with indirect or background transfer mechanisms.

In casework, however, the handling history of an item is typically unknown and glove use cannot be assumed. To maintain general applicability, the activity-level model therefore adopts the standard assumption $t'(x) = t(x)$, representing a scenario in which an unknown individual may have handled the item directly.

The purpose of the case example in this study is to illustrate how different extraction methods influence activity-level likelihood ratios. Using the assumption $t' = t$allows the comparison to focus on the impact of extraction efficiency rather than on the experimental constraint that gloves were used. If glove use were assumed in a real case scenario, the resulting activity-level likelihood ratios would be expected to decrease substantially.

5. Conclusion

Our results show that DNA transfer and recovery are affected by contact type, substrate, and item characteristics, with direct transfer and non-porous handled items producing the highest yields. Extraction approach also plays a critical role: a co-extraction protocol using QIAamp and mirVana™ kits outperformed Chelex, while Direct PCR provided a rapid but less efficient alternative. These findings emphasize the need to carefully consider extraction methods, substrate, and item type to ensure reliable interpretation of forensic DNA and activity-level evidence.

Crucially, methodological changes at the sub-source level propagated to the activity-level evaluation. Transparent reporting and cross-laboratory standardization are essential to ensure that such methodological effects are properly accounted for in the evaluation of forensic evidence.

We therefore recommend that laboratories explicitly validate and document the impact of their chosen extraction method on transfer/persistence functions used in activity-level evaluations, particularly when revisiting historical cases.

CRediT authorship contribution statement

Sara Faria: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Ane Elida Fonneløp:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Francisco Pina-Martins:** Supervision, Methodology, Conceptualization. **Ana Clara Ribeiro:** Supervision, Methodology. **Peter Gill:** Writing – review & editing, Methodology, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The author is an Editorial Board Member for this journal and was not involved in the editorial review or the decision to publish this article.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fsigen.2026.103516.

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