# A Comparative Study on Mixed Biological Mock Samples with a Modified Differential DNA Extraction Method for Improved Recovery of Spermic DNA from Mixed Body Fluid

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**Abstract**

**Problem:** DNA analysis is a critical aspect of forensic investigations, aiding in the identification of perpetrators and exonerating the innocent. The proportion of male and female DNA in extracted DNA determines how individuals can be identified as suspects in sexual assault cases. The differential extraction method is frequently used for the separation of male DNA from mixed body fluid in sexual assault cases. Nowadays, an efficient and improved differential extraction method is required for the separation of male DNA from mixed body fluid evidence in sexual assault cases. **Approach:** We had evaluated three lysis buffers for differential DNA extraction with mixed saliva-semenmock samples. Mock samples were processed with buffers A, C and G2 with different incubation times. During the differential extraction method, each sample was separated into four fractionsi.e.,spermic fraction, non-spermic fraction, wash fraction and re-digesting the cotton swab. **Findings:** Maximum (77.77%) of male DNA recovery with minimum (6.44%) female DNA carryover was obtained in the spermic fraction with buffer C at a 30 min of incubation time. In the non-spermic fraction, the least (2.01%) male DNA loss was found with buffer C at 30 min incubation and the recovery of female DNA was 74.54% with buffer C at 45 min, followed by73.90% at 30 min. Moreover, the recovery of male DNA with lysis buffer A was 27.11 % at 45 min and buffer G2 was 39.16% at 30 min incubation time. The loss of male DNA in wash fraction was minimum with all buffers. **Conclusion:** The current study revealed that the maximum male DNA recovery with a low quantity of female DNA carryover in the spermic fraction and the minimum male DNA loss in the non-spermic fraction was achieved with buffer C at a 30 min incubation time. Thus, the modified differential DNA extraction method with lysis buffer C at a 30 minutes incubation time is useful in the separation of male DNA from mixed body fluid in sexual assault exhibits.

**Key words:** Mock Sexual Assault Samples, Semen, Saliva, Differential Extraction Method, Spermic Fraction, Non-spermic Fraction, Wash Fraction, Re-digested Cotton Swab, DNA Recovery, DNA Quantitation.

## Introduction

The precise recovery of DNA evidence from crime scenes plays a crucial role in forensic investigations, particularly in cases involving male perpetrators(1). Most of the Forensic DNA laboratory's pending sexual assault case evidences contains spermatozoa with victim epithelial cells(2). The presence of male DNA in mixed biological samples often presents challenges due to the huge amount of female DNA present, resulting in limited sensitivity and low success rates(3,4). Some studies emphasize the significance of optimizing buffers and incubation times in the differential extraction method. It may lead to improved recovery of male perpetrator

DNA(5–7).The impact of incubation timings on DNA recovery highlights the importance of appropriately sufficient time for cell lysis and DNA release(8).Further, various methods are widely used in DNA extraction, but they may not effectively separate male and female DNA components in mixed samples, sometimes leading to lower recovery rates of male DNA(9). Some, preliminary studies using differential DNA recovery, demonstrated the use of buffers that ensured optimal conditions for DNA extraction, including a suitable pH and ionic strength. These factors contributed to efficient cell lysis and DNA recovery, particularly from male cells, thereby enhancing the identification of male perpetrators(10). In another study, the role of buffer was found to be efficient and useful for DNA extraction due to its favorable pH and its ability to facilitate the release and recovery of DNA from male cells(11). In this study, the method involves selective separation of male DNA from non-spermic or female DNA, enhancing the sensitivity and specificity of DNA profiling(12). The efficacy of the differential extraction method relies on two aspects; maximum male DNA recovery and minimum female DNA carryover in the spermic fraction(13).We are evaluating three lysis buffers with different incubation times for the development of the fast, improved and efficient differential DNA extraction method for the analysis of sexual assault case evidence.

## Materials and Methods

***Mock Sample Preparation***

Mock sexual assault samples were prepared by mixing buccal epithelial cells of female saliva 40 µl with male neat semen 2 µL (40:2 ratios). A total of 15 cotton swabs were prepared, which involved nine mock samples of saliva-semen mixed swabs and six control samples; three with saliva and three with semen. All samples were air-dried at room temperature before processing.

***Experimental Design***

This study was designed with a modified differential extraction buffer. The principle of this study was to compare three different lysis buffers with variations in their incubation periods. Each mock sample was fractionated into four portions. Spermic fraction, non-spermic fraction, wash fraction andan additional step were performed by re-digesting the cotton swab to check whether the cotton swab is responsible for male DNA loss, if any. Control samples were also prepared and used to compare the recovery proportion for evaluation of the spermic and non-spermic fractions with the set of experimental data. Hence, a total of 36 samples were obtained with all fractions.

**Methodology**

Three buffers with different compositions were used in the study. Buffer A {(Tris HCl-100mM; EDTA- 10mM; NaCl- 50mM) pH 8.2}, Buffer C {(Tris HCl-100mM; EDTA- 5mM; NaCl- 50mM) pH 8.2} and Buffer G2 (QIAGEN Buffer).

*Differential Extraction*

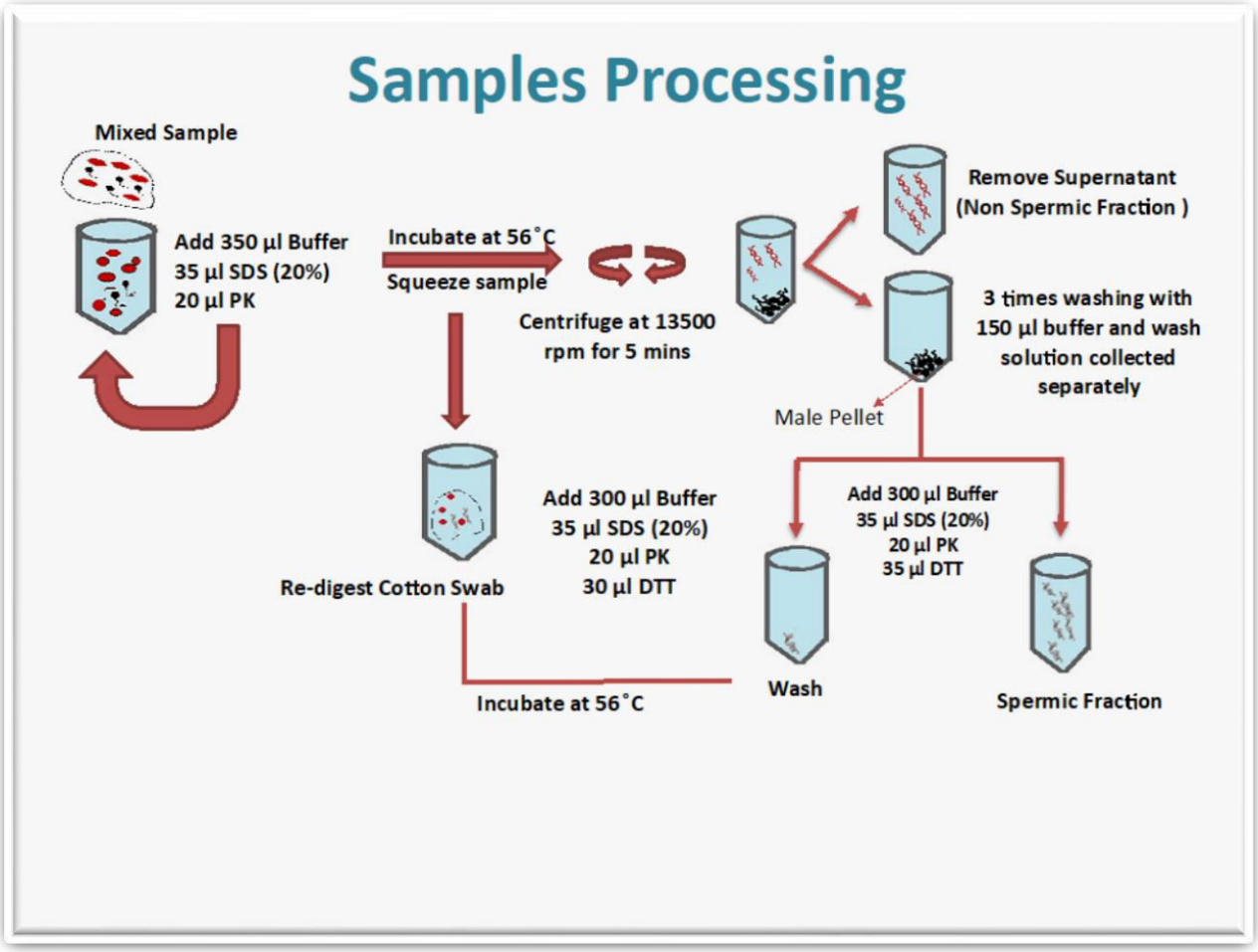


**Plate 1:** ***Illustrates the Differential extraction process and four fractions were achieved to check the efficacy of the protocol.***

Differential extraction process of mock biological mixed samples was performed. At the first step, three different sterile microcentrifuge tubes were taken separately with buffers A, C and G2. 20% sodium dodecyl sulphate (SDS) and proteinase K (PK) 20mg/ml were added as described in Plate1.Slightly mixed and incubated at 56°C for 15, 30, and 45min. After incubation, samples were transferred into a spin basket and centrifuged atfor 2 min. Spin baskets were then discarded and samples were centrifuged for 5 min at 13,500 rpm. The female supernatant was removed from each tube and placed in new microcentrifuge tubes separately. The supernatant was removed without disturbing the pellet and three washings of pellets were performed (shown in Plate1), leaving ~30 µl volume after each wash. The wash solutions were collected in new tubes of each buffer and pellet (spermic fraction) containing tubes were further lysed with three different buffers incubated at 56 °C for 1 hour (shown in Plate1).The remaining cotton swabs were re-digested with a similar process by re-adding buffers A, C and G2 with SDS, Proteinase K and DTT incubated at 56 °C for 1 hour for the lysis of retained epithelial and sperm cells in the cotton swab. The phenol-chloroform organic extraction procedure was used to purify the DNA from the spermic fraction, non-spermic fraction, wash fraction, and re-digesting the cotton swab. 40 µl of DNA were eluted. DNA quantification of all samples was performed using the PromegaPower®QuantkitqPCR(Quantitative PCR or Real time PCR) assay(14,15).

## Results

In this study, we prepared mixed mock swabs containing saliva and semen as wellas control samples. We compared the effectiveness of three different lysis buffers with various incubation times. The mixed mock samples werefractionated into four portions: spermicfraction, non-spermic fraction, wash fraction, and re-digested cotton swab. The DNAconcentration of each fraction was assessed using real-time PCR. Our analysis aimed toidentify the optimal lysis buffer and suitable incubation time for achieving a precisedifferential DNA extraction method.

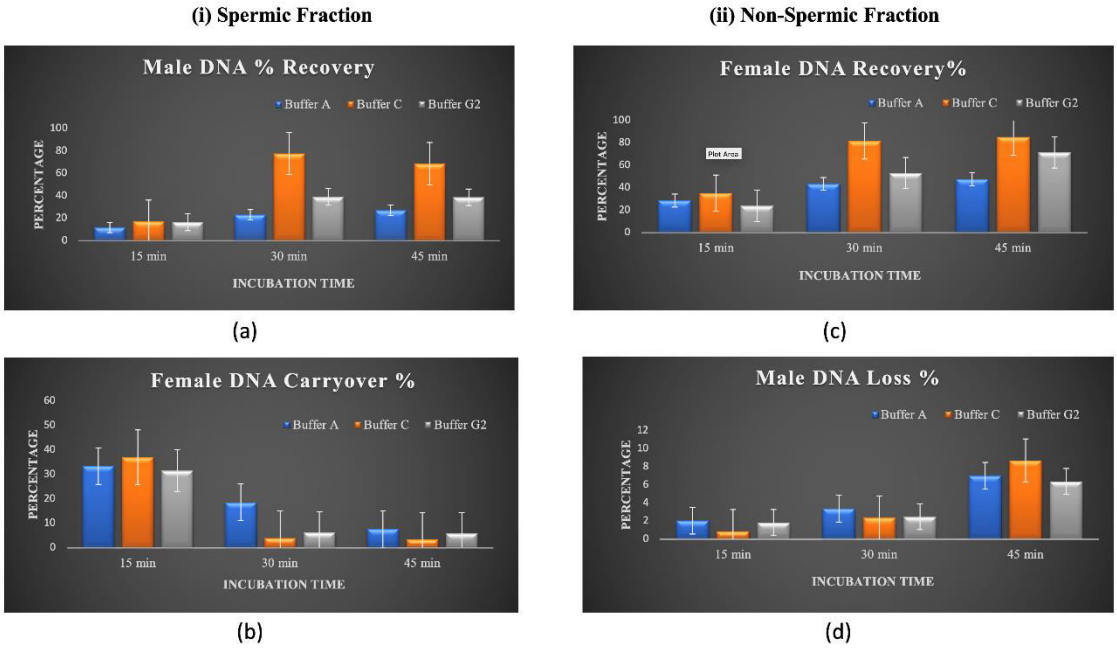


**Plate 2:** ***Illustrates the DNA recovery outcomes using various buffers andaltered incubation time intervals. (i) Spermic fraction; (a)Recovery of male DNA (b) Reduction of female DNA (acting as carryover in males DNA) in spermic fraction.(ii) Non-spermic fraction; (c) Female DNA recovery (d) Loss of male DNA in non-spermic fraction.***

***(i)Male DNA recovery and reduction of female DNA in spermic fraction:***

In the spermic fraction separated by modified differential extraction technique from a saliva-semen mixed swab mock sample, there is maximum recovery of male DNA with the leastquantity of female DNA carryover in spermic fraction, which is considered as male DNA recovery. Male DNA recovery was found to be maximum with buffer C in comparison tobuffer A and G2 (Plate2). Maximum male DNA recovery of 77.77% was observed at 30 min incubation time with this buffer C followed by 68.77% at 45 min and 17.40% at 15 min incubation time. Peak male DNA recovery with buffer A was 27.11% at 45 min, followed by 23.28% at 30 min and 11.99% at 15 min incubation time. Male DNA recovery with buffer G2 was maximum 39.16% in 30 min of incubation, followed by 38.85% at 45 min and 16.63% at 15 min of incubation time (Plate 2 a).

The maximum reduction of female DNA is considered to have the least DNA carryover. It was found to be 3.45% at 45 min with buffer C, followed by 3.95% at 30 min and 37.09% at15 min incubation time. In buffer A, female DNA carryover was 7.73% at 45 min, 18.60% at 30 min and 33.49% at 15 min. Similarly, with buffer G2 the female DNA carryover was 5.94% at 45 min, 6.35% at 30 min and 31.60% at 15 min. Buffers A and G2 showed more female DNA carryover in the spermic fraction as compared to buffer C shown inPlate 2 b.

***(ii) Female DNA recovery and male DNA loss in the non-spermic fraction***

In the non-spermic fraction, the quantity of male DNA carried is considered male DNA loss. The least quantity of male DNA loss is expected with an ideal differential buffer. The minimum quantity of male DNA loss in the non-spermic fraction was 0.88% with buffer C at 15 min incubation time, followed by 2.38% at 30 min and8.69% at 45 min. Similarly, Male DNA loss of 1.85% to 6.40% was noted with buffer G 2 in 15 to 45 min lysis time. Male DNA loss observed with incubation times 15 min, 30 min, 45 min with buffer A was 2.07*%,* 3.41% and 7.01 %, respectively.Data reveals that the least loss of male DNA in the non-spermic fraction was found in buffer C at 30 min incubation. Hence, the efficiency of buffer C at 30 minutes was concluded to be maximum and desirable(Plate 2d).

The recovery of female DNA in the non-spermic fraction was highest (85.22%) with buffer C at 45 min followed by 30 min (81.91%) and 15 min (35.27%). Similarly, with buffer A the female DNA was maximum (47.22%) at 45 min, 43.55% at 30 min and 28.70% at 15 min. Subsequently, with buffer G2, 71.55% at 45 min, 53.24% at 30 min and was minimum (24.02%) at 15 min (shown in non-spermic fraction of Plate 2c).

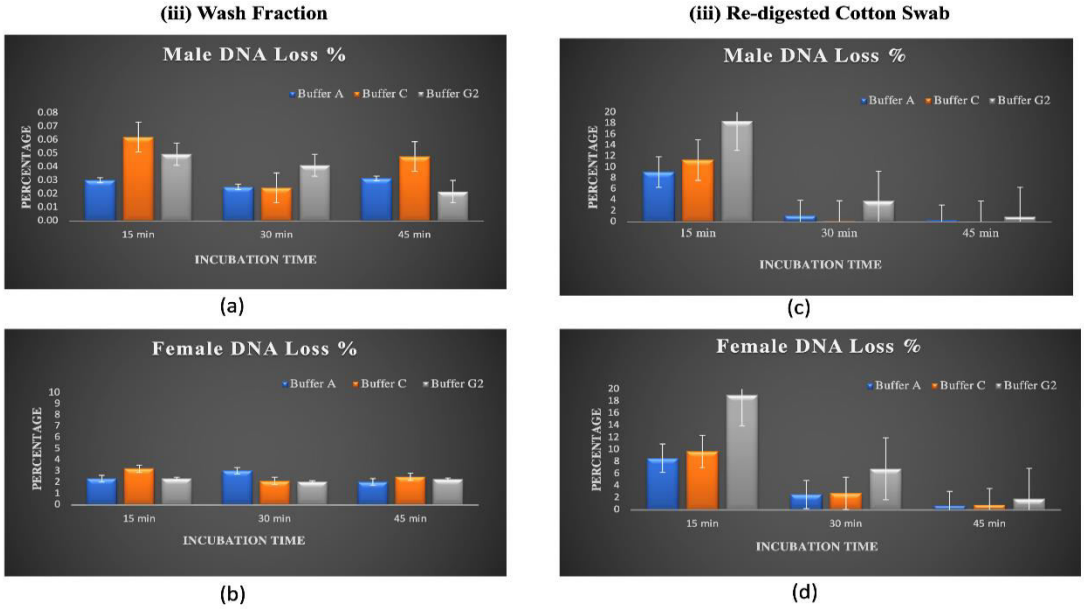


**Plate3.** ***Illustrates the male and female DNA loss in (iii) Wash fraction; (a) Male DNA loss in wash fraction, (b) Female DNA loss in wash fraction; and (iv) Re-digested cotton swabs; (c) Male DNA loss in re-digested cotton swabs, (d) Female DNA loss in re-digested cotton swabs with different buffers and altered incubation time slots.***

***(iii) Male and Female DNA loss in wash fraction***

The male pellet was washed during the separation of the spermic fraction from the non-spermic fraction and the wash solution was examined. It carried a small quantity of male as well as female DNA; hence, it is considered as male and female DNA loss. The least male DNA loss in the wash fraction was 0.02% with buffer C in 30 min incubation and the maximum DNA loss was 0.06% at 15 min (Plate 3 a whereas, the least female DNA loss was at 45 min with buffer A i.e., 2.05% and maximum at 15 min with buffer C i.e., 3.24% (Plate 3 b). Subsequently, buffer G2 indicated a minimum male DNA loss of 0.02% at 45 min and a maximum male DNA loss of 0.05% at 15 min incubation.

***(iv) Male and female DNA loss in the cotton swab***

In fraction separated from the saliva-semen mixed swab, the mock sample carried a small amount of male as well as female DNA, as noted in the case of the remaining cotton swab fraction. Plate 3depicts the least DNA loss in cotton swabs at 0.05% with buffer C in the 45 min incubation slot and it has again consistently shown the lowest DNA loss in cotton swabs with buffer C in the whole series as compared to buffers A and G2.

As found in previous experiments, the least efficiency in this regard was shown by the use of G2 buffer; hence, the efficiency of buffer C at45minutes was concluded to be maximum and desirable.

Subsequently, the loss of female DNA in the re-digested cotton swab was very minimal at 45 min in all three buffers followed by 30 min and 15 min (shown in Plate 3 d). The minimum loss was 0.87% with buffer C, 0.72% with buffer A, 1.82% with buffer G2.

**Discussion**:

The findings of this comparative study support the use of appropriate buffer and incubation timings in the differential extraction method for improved recovery of male perpetrator DNA from mixed biological samples. Maximum recovery of male DNA with least quantity of female DNA carryover in the spermic fraction is referred to as pre-eminent male DNA recovery(16). Spermic fraction data indicates that the male DNA recovery increased gradually from 15 min to 45 minof lysis time in buffer A. While in buffer C and buffer G2, there was a significant enhancement in male DNA recovery from 15 min to 30 min of lysis time which declined a little in 45 min of incubation time. Besides, the female DNA carryover reduced with increasing the lysis time. 15 min of incubation time indicates the maximum female DNA carryover with all buffers, which decreased gradually with 30 min and 45 min in the spermic fraction. The rupture of epithelial cells was gradually increased in the initial lysis as a result of increased lysis duration, resulting in the least amount of female DNA carryover in the spermic fraction(17). Subsequently, buffer C showed the minimum carryover of female DNA, followed by buffer G2 and buffer A at 30 and 45 min of incubation time, respectively. Non-spermic fraction revealed that the recovery of female DNA along with male DNA loss were maximum at 45 min incubation, followed by 30 and 15 min in all buffers. The minimum loss of male DNA was found in the 15 min lysis time. Thus, more lysis time may lead to an increase in loss of male DNA in all buffers(18). However, at 30 min incubation time, the recovery was maximum with buffer C and the male DNA loss was minimum as compared to 45 min. Buffer C indicated the maximum recovery as compared to buffers G2 and A. Buffer C data showed that a lysis time of 15 min indicated the minimum quantity of male DNA recovery as compared to an enhanced lysis time. Simultaneously, female DNA carryover in the spermic fraction was found higher due to the carryover of female epithelial cell in spermic fraction(19). Lysis time enhanced to 45 min showed that recovery of male DNA was reduced as compared to a 30 min lysis time. Hence, male DNA recovery is inversely proportional to incubation time, as lysis of sperm cells increases with more incubation time. The male recovery was minimum with a 15 min lysis time and the female reduction was maximum in the spermic fraction. Similarly, the recovery of female DNA and male DNA loss were also minimal with a 15 min lysis time in the non-spermic fraction. This study showed that buffer C is an efficient lysis buffer at 30 min incubation time This preliminary study using differential recovery of DNA demonstrated that 30 min is the optimal conditions for DNA extraction, including suitable pH and ionic strength in buffer C. These factors contributed to efficient cell lysis and DNA recovery, particularly from male cells, thereby enhancing the identification of male perpetrators(13). The use of most efficient buffer and selection of incubation timings further makes the procedure more useful for maximizing yield of pure male DNA in mixed samples. Further validation studies are going on with real sexual assault case exhibits(20).

**Conclusion**

The differential extraction method is an effective technique for improving the recovery of male perpetrator DNA from mixed biological samples. However, the optimization of buffers and incubation timings in the differential extraction process can further enhance the efficiency of DNA recovery. This comparative study evaluated the impact of different buffers and incubation times on the recovery of male DNA using the differential extraction method in mixed biological mock samples. Buffer C is found to be the most compatible and productive buffer as it gave the highest male recovery value at 30 minutes of incubation time with least female DNA carryover in spermic fraction. There was the tiniest quantity of male DNA loss in the non-spermic fraction, wash and re-digested cotton swab at an incubation time of 30 min.It is worth noting that the optimization of buffers and incubation times should consider the specific characteristics of the mixed biological samples and the target DNA of interest. Buffer C is useful for fast and efficient differential lysis in sexual assault cases for male DNA recovery. In the future, it may be useful for DNA analysis of the huge pendency of sexual assault cases across the country

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