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**Research Article**

# RAPID IDENTIFICATION OF SEMEN STAINS IN FORENSIC INVESTIGATIONS: STK SPERM TRACKER

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**Abstract**

Sexual offences are complex, necessitating expertise from various forensic disciplines for proper evidence handling. Detecting semen on clothing items in forensic laboratories is also challenging, as identifying the semen donor ultimately depends on DNA analysis. Therefore, employing a reliable and robust method for identifying semen stains is crucial for convicting offenders and exonerating the innocent. Several screening and confirmatory techniques have been developed over the years; however some are known to have limited in sensitivity, specificity and time consuming. The recent increase in forensic examinations performed on rape victims has led significant advancements in existing forensic techniques, improving the ability to identify and recovery semen. In 2016, the National Institute of Scientific Police (INPS/LPS69), France collaboration with AXO Sciences, launched STK® Sperm Tracker™ an advanced technology developed to detect semen on sexual assault evidence items. The technique is reported to be rapid, sensitive, environmental friendly, easy to use, highly specific and can use as a confirmatory test for semen. The two research studies discussed and compared its performance with traditional semen identification methods, assessed its impact on subsequent techniques like DNA analysis, evaluated its effectiveness in detecting semen from vasectomized individuals, and tested its sensitivity in identifying semen on fabric and degraded stains. It was revealed that STK is more suitable than the conventional tests on semen detection, compatible with downstream applications, and can detect semen from vasectomized donors. Additionally, it demonstrated higher sensitivity in detecting semen on various fabric types and degraded semen stain, relying significant advancement in the field of forensic science in future.

**Keywords:** Semen, Alternative Light Source, Acid Phosphatase, STK Sperm Tracker, DNA Analysis, Degraded semen.

## INTRODUCTION

Sexual offenses, including rape and sexual assault, pose a persistent challenge, with rising reported cases. The number of reported sexual offences has increased over the last few years compared to other offences. Identifying biological evidences like semen, blood and saliva is a fundamental requirement in sexual assault investigations. Detecting semen on clothing items received at the forensic laboratory is vital and several presumptive methods for semen detection have been investigated in forensic investigations to ensure accurate results[1,2]. Forensic scientists employ a combination of microscopic, biochemical and molecular techniques for semen identification. The presumptive indication of semen is based on the use of an ALS and AP activity on semen. Nevertheless, these tests are not specific only for semen and gives positive response for some other biological and physiological origin stains [3,4,5,6]. Beyond the generally low specificity, factors such as fabric type, fabric inherence and fabric absorbency also determine whether semen is detectable by ALS[7]. Microscopic examination for visualization of sperms and techniques like PSA and SVSA involving specifically target proteins found in semen are commonly used to confirm presence of semen. However, these methods also have their own limitations [8,9,10].
The STK® Sperm Tracker™ by AXO Sciences and French, National Institute of Scientific Police launched STK® Sperm Tracker™ offers an alternative solution, exhibiting specificity in detecting semen stains and overcoming challenges faced by other methods [11,12]. Two research studies concerned on optimization of STK Sperm Tracker technique, assessing its ability for applications in forensic laboratories and it revealed it can be used as a precise, efficient and reliable test for detecting semen.

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Furthermore, the study focused to evaluate the sensitivity level of STK Lab Sperm Tracker™ in detecting semen on fabric. The detection limit is explored by assessing sensitivity at different levels of semen dilutions. The products performance under different conditions is evaluated by investigating the effects of fabric substrate porosity and semen degradation on sensitivity. Understanding these nuances is crucial for optimizing forensic investigations, particularly in case involving different fabrics and delayed reporting of sexual assaults.

## METHODOLOGY

### Sample Preparation

In the study 1, Biological fluids of semen, blood, urine, saliva, vaginal secretions, semen from normal person (donor 1) and from vasectomy person (donor 2) were collected through voluntary donors. Solutions of biological samples from tea, detergent, broccoli and potato were obtained from commercially available sources. In the study 2, five different fabrics, namely 100% cotton, 100% acrylic, toweling, denim and 100% polyester were selected. 100% cotton was used as standard fabric for limit of detection and effect of time on semen degradation. In view of the previous evaluation of cotton fabric's ability to persist semen, it was used as the standard fabric[13]. The other four fabrics were selected due to their common encounter in forensic laboratories. For the study on the effect of substrate porosity and semen degradation, a dilution of 1:10 was used given in a recent study, it was recorded as the lowest dilution of semen the product could detect [12].

**Apparatus, Consumables and Reagents**

STK® Sperm Tracker™ paper was obtained from AXO Science. Crime-lite 42s (UV 350-380nm) and Crime-lite 2 (Blue 420-470nm) from Foster-Freeman were used to observe the results. Canon DSLR camera was used to take images. Image J software was used to quantify the intensity of the fluorescence of the reaction from the photograph's records in Study-2. In both study positive result is considered when a blue fluorescence is visible. In contrast, a negative result is considered when a bright blue fluorescence is not visible.

**Study-1**

A volume of 50µl of sample and quadruplicate were performed across the study. Section 3, 4, 5 6 and 7 were followed optimized parameters outlined in section 1 and 2. Fluorescence visualization was conducted with Crime-lite 42s (UV 350-380nm) in a dark room. Crime-lite 2 (Blue 420-470nm) was used only to obtain comparative results for sensitivity, specificity and semen from vasectomized person

**1. Detection of Fluorescence Intensity**

The grading system implemented to evaluate the intensity of fluorescence for semen, according to the results given for neat semen in different dilutions. The fluorescence intensity was categorized as Strong, Moderate and Weak.

**2. Optimizing Experimental Parameters**

The experimental parameters were optimized by considering supplier's recommendations [14]. The absorbent side of the STK paper was moistened with appropriate amount of water and placed in contact with the stained filter paper. The time of the STK paper contact with the stain, volume of water and weight applied were optimized for all possible combinations using implemented grading system. These optimized parameters were used for testing all subsequent analysis in Study 1.

**3. Sensitivity**

Serial dilution of 1:2, 1:5, 1:10, 1:20, 1:30, 1:40, 1:50, 1:100, 1:500 and 1:1000 of neat semen was prepared and tested for STK, Crime-lite 2 (Blue 420-470nm) and AP test.

**4. Specificity**

Specificity was assessed for vaginal secretion, blood, urine, saliva and household substances; detergent, organic extracts of tea, broccoli and potato. Generated stains were subjected to STK test. Independent examination was carried out for both AP and Crime-lite 2 (Blue 420-470nm) to ensure comparable results for specificity.

**5. Impact of STK on detecting semen from vasectomy donor**

Semen (50µl) from vasectomized person (donor 2) was deposited on filter papers and performed STK test. Comparative study was performed detecting from Crime-lite 2 (Blue 420-470nm) and AP test.

**6. Impact of STK on Phadebas Paper**

A mixture of semen and saliva (1:1) was tested on STK and same sample was immediately subjected to the Phadebas test. Colour change was monitored on the phadebas paper at intervals of 5 minutes starting from 5 minutes and continuing up to 40 minutes. The same procedure was followed for AP test instead of STK test; the intensities of Phadebas papers were compared.

**7. Impact of STK on DNA Analysis**

Ten samples were prepared from each individual (normal and vasectomized) and applied for STK test. STK treated and untreated were subjected for DNA Analysis. DNA extraction was carried out using QIAGEN® QIAcube® automatable robotic workstation following QIAGEN® QIAcube® manufacturer's guidelines. DNA quantification was performed with Qiagen Investigator Quantiplex kit on a Qiagen Rotor-Gene Q PCR thermo cycler according to manufacturer's protocol. DNA amplification was carried out using the Qiagen-Investigator ESSplex SE QS kit on SureCycler 8800 (Agilent Technologies) thermal cycler. Separation of amplified PCR products was carried out on Genetic Analyser 3130 (Applied Biosystems, Life Technologies, Foster City, CA, USA).

**Study-2**

A volume of 50µl and quadruplicates were performed across the study. The parameters were selected and observed in accordance with the manufacturers' manual[14]. The results were observed using Crime-lite 42s (UV 350-380nm) in a darkroom. The fluorescence intensity of semen on fabrics was quantified using Image J software.

**1. Detection of Fluorescence Intensity**

Image J software was utilized to quantify the fluorescence intensity from the photographs [15]. The mean fluorescence and area readings obtained from the software were used to calculate the integrated density of the fluorescence by multiplying these two values

Additionally, background readings were taken and subtracted from the integrated density to obtain the total corrected fluorescence.

**Integrated Density for Background** (RFU-Relative Fluorescence Units) = Area of selected background region x Mean fluorescence of the background region readings.

**Integrated Density for Sample** (RFU-Relative Fluorescence Units) = Area of selected sample region x Mean fluorescence of the sample region readings.

**Total Corrected Fluorescence = Integrated Density for sample - Integrated Density for Background**

The total corrected fluorescence results were used to classify the fluorescence intensity into three signals. Strong, Moderate and Weak giving an intensity of RFU $\geq$ 100,000 for Strong, RFU 50,001–100,000 for Moderate and RFU$\leq$50,000 for Weak signals.

**2. Limit of Detection on Fabric**

Dilutions of 1:10, 1:16, 1:32, 1:64, 1:100 and 1:1000 of neat semen were selected and deposited on 100% cotton in accordance with the literature review [12]. After 3 minutes, the

result was observed using Crime-lite 42s (UV 350-380nm) in a dark room. Due to anomalous results obtained from dilution 1:32, a repetition was performed for additional 10 and 15 minutes.

### 3. Effect of Substrate Porosity

In a recent study it was recorded as the lowest dilution of semen could be detected, was 1:10[12]. Hence, the impact of substrate porosity on sensitivity was explored by depositing 1:10 semen on different fabrics (100% acrylic, toweling, denim and 100% polyester). After recording the results an additional 10 minutes was added to study if any significant changes in the intensity of fluorescence will occur with an increasing time. The additional 10 minutes is the standard practice from the manufacture's manual[14].

### 4. Effect of semen degradation

1:10 diluted semen was applied on 100% cotton. Analysis was performed on both day 1 and day 25 days after the deposition dates in a similar manner.

## RESULTS

### Study 1

### 1. Detection of Fluorescence Intensity

The grading system used to assess the fluorescence intensity of semen is based on the results obtained from various dilutions of neat semen.

Strong - Strong fluorescence in all area
Medium - Moderate fluorescence in some area
Weak - Minimum fluorescence in some area

Figure 1 illustrates the implemented grading system.

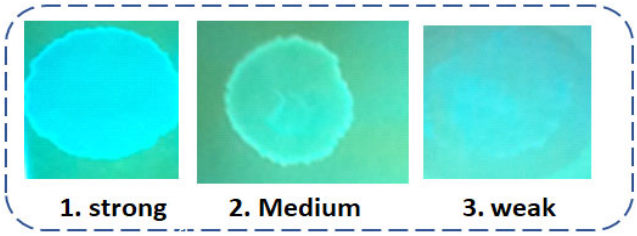


**Fig. 1. Grading system for fluorescence intensity**

### 2. Optimizing Experimental Parameters

Variable combinations of contact time, volume of water and pressure were used to identify potential favorable options. The combination observed the highest fluorescence, while utilizing minimum resources was confirmed as optimized set of parameters. The results revealed 100 to 200 µl/m$^2$ of water, pressure of 600kg/m$^2$ and duration of 5 minutes were recommended as validated parameters for the study.

### 3. Sensitivity

STK showed noticeably higher level of detection down to 1/20. However, Crime-lite 2 (Blue 420-470nm) and AP exhibited fluorescence only for 1/5 dilution with slightly more fluorescence for AP.

### 4. Specificity

For comparison studies the specificity was carried out for STK technique along with Crime-lite 2 (Blue 420-470nm) and AP test. STK displayed the highest specificity compared to other tests reacting only with detergents. Crime-lite 2 (Blue 420-470nm) exhibits weak fluorescence on saliva and detergent stains. Predominantly, semen gives strong purple reaction within 2 minutes for AP. In the case of vaginal secretions, tea, saliva, broccoli and potato gave color changes in between 2 to 10 minutes. Results revealed, AP reacted with many stains with different color variations and cut off times. Figure 2 illustrates reactivity of these stains on three tests.

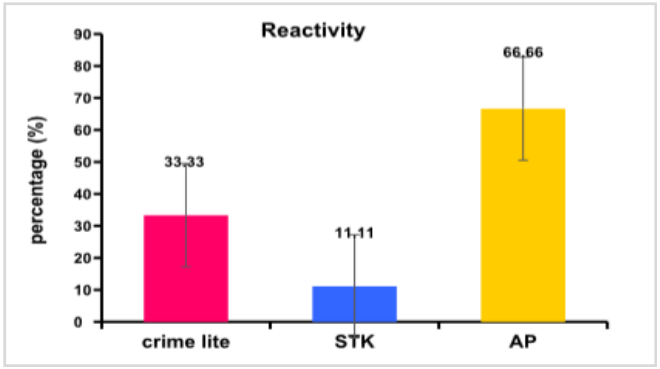


**Fig. 2 .Reactivity of Crime-lite 2 (Blue 420-470nm), STK and AP test for different type of stains**

STK was given the lowest reactivity, resulting in the highest specificity, whereas AP given lowest specificity.

### 5. Impact of STK on detecting semen from vasectomy donor

Semen from vasectomized person (donor 2), gave strong fluorescence for STK papers and Crime-lite 2 (Blue 420-470nm) respectively. The AP test also resulted in a strong purple within two minute time frame.

### 6. Impact of STK on Phadebas test

Strong fluorescence was observed for STK paper on mixed semen-saliva stain. Following the Phadebas test on the same spot observed initial light blue color after five minutes and gradually developed over 40 minutes to reach strong blue color. The similar procedure was employed for comparative studies, substituting STK analysis with AP test and observed consistent results.

### 7. Impact on DNA Analysis

Table 1 displays the average DNA quantity, along with corresponding standard deviation and standard error, both donors, before and after performing STK analysis.

**Table 1. Results for DNA analysis before and after performing STK test**

| Sample type | Average DNA quantity (ng/µl) | Standard Deviation | Standard Error |
|---|---|---|---|
| Extraction blank | 0 | - | - |
| Negative control | 0 | - | - |
| Donor-1-before STK | 6.98 | 2.80 | 1.3 |
| Donor-1-after STK | 7.93 | 4.46 | 2.0 |
| Donor-2-before STK | 1.99 | 0.75 | 0.34 |
| Donor-2-after STK | 2.96 | 1.82 | 0.81 |

Further details are illustrated in Figure 3

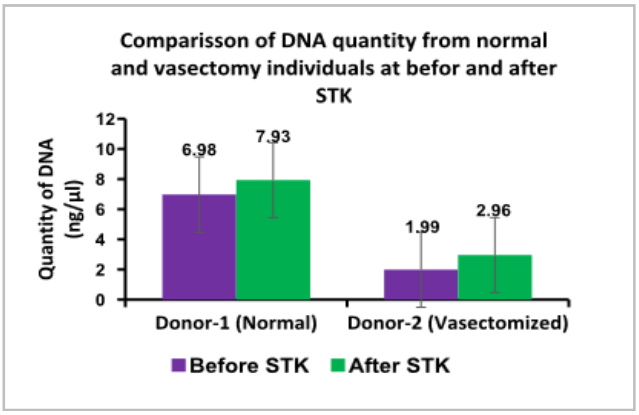


**Fig. 3. Impact of STK on DNA Analysis**

Quantity of DNA was slightly increased, following STK test on samples from both donors. Results were further confirmed by applying Two Sample-T Test given p values of 0.700 and 0.323 for before and after applying STK on both donor1 and 2 respectively. According to the p values ($p>0.05$) there was no statistically significant difference in DNA quantity between before and after performing STK on samples. The DNA analysis which resulted expected DNA profiles with corresponding alleles; further confirmed STK paper had no discernible impact on subsequent analysis.

## Study 2

### 1. Limit of Detection on Fabric

Various dilutions of semen deposited on 100% cotton fabric. When observing UV (350-380nm), it was found the detection threshold extended up to 1:32 dilution.

Table 2 illustrates results of different semen dilutions observed on 100% cotton.

**Table 2. An average qualitative results of different semen dilutions examined on 100%cotton fabric**

| Semen Dilution | Semen Stain (White light) | Stiffness | Fluorescence at UV 350-380nm |
|---|---|---|---|
| 1:10 | Visible | Detectable | Present |
| 1:16 | Visible | Detectable | Present |
| 1:32 | Visible | Detectable | Present |
| 1:64 | Not visible | Detectable | Absent |
| 1:100 | Not visible | Undetectable | Absent |
| 1:1000 | Not visible | Undetectable | Absent |
| 1:10000 | Not visible | Undetectable | Absent |

Figure 4 illustrate the images from image J software for the dilutions of 1:10, 1:16 and 1:32

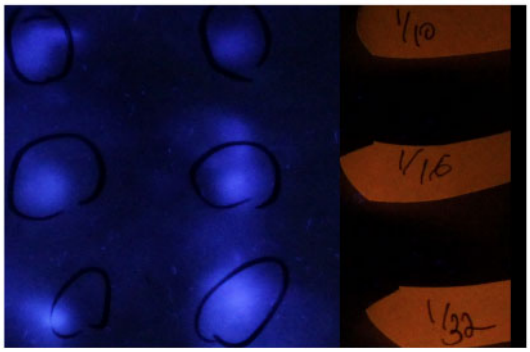


**Fig. 4. Images for diluted semen using image J software**

Figure 5 illustrates qualitative results of fluorescence intensity obtained using Image J software.

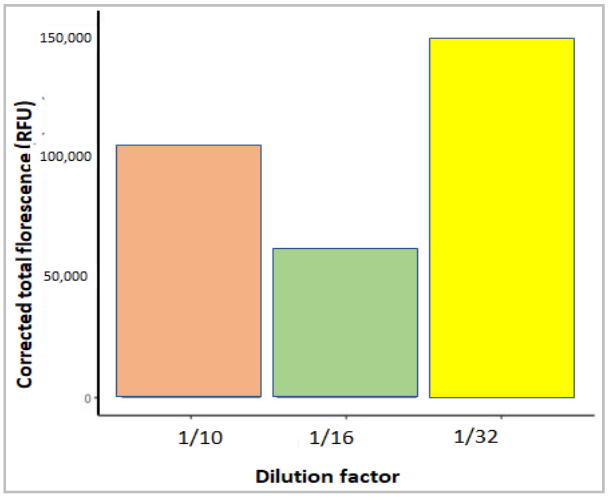


**Fig. 5. Quantitative results of the intensity of fluorescence using ImageJ software**

Semen dilution at 1:10 and 1:32 recorded strong intensity, 1:16 was moderate. To exclude the possibility of anomaly results, the study was repeated on the 1:32 dilution and recorded in Figure 6 and Figure 7

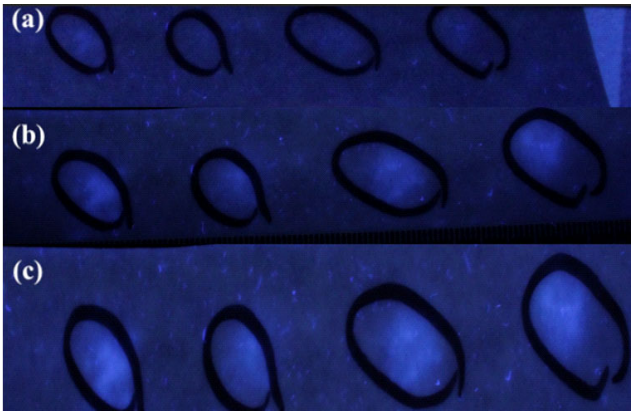


**Fig. 6- Detection of semen diluted at 1:32 for STK® Sperm Tracker™, using UV (a) 3 minutes, (b) 10 minutes and (c) 15 minutes on a 100% cotton fabric**

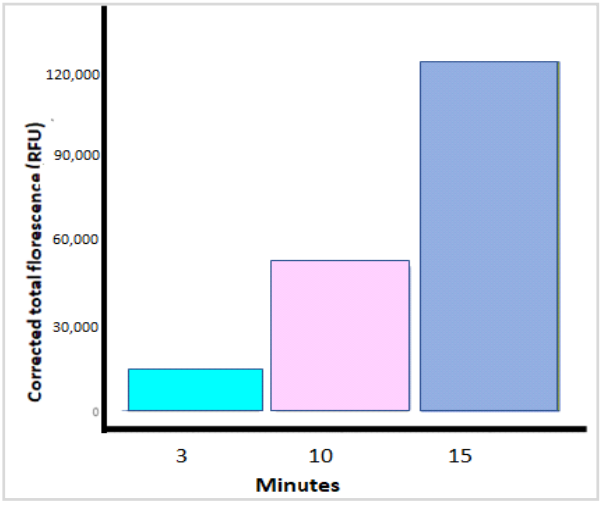


**Fig. 7. Quantitative results of the intensity of fluorescence for 1:32 dilution at different time intervals**

At the 3 minutes interval, the intensity of fluorescence was weak, but it increased to strong in the subsequent 15 minutes.

### 2. Effect of Substrate Porosity

When determining the effect of different fabrics, it was observed that three out of four, 100% polyester, blue denim and 100% acrylic given positive results within the first 3 minutes, whereas white towel fabric showed negative results. Figure 8 shows the intensity of fluorescence at different types of fabrics at 3 minute

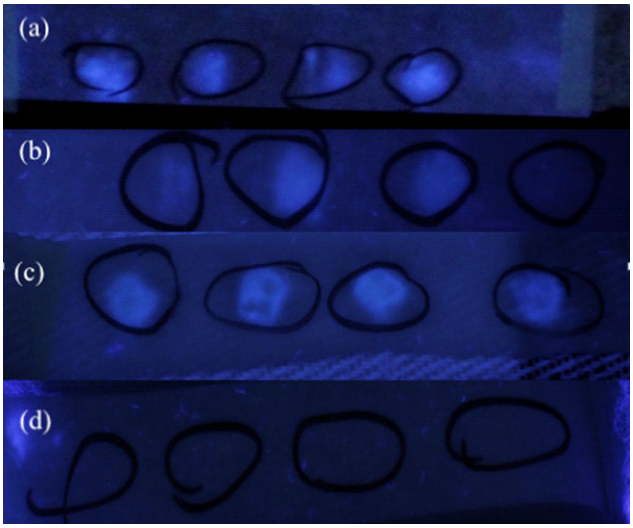


**Fig. 8. Effect of substrate porosity on STK® Sperm Tracker™. (a) 100% polyester, (b) Blue Denim Jeans, (c) 100% Acrylic and (d) White**

The fluorescent intensity displayed on the materials differs with 100% polyester and 100% acrylic producing a strong intensity, followed by the blue denim with moderate intensity. Figure 9 illustrates fluorescence intensities on different materials.

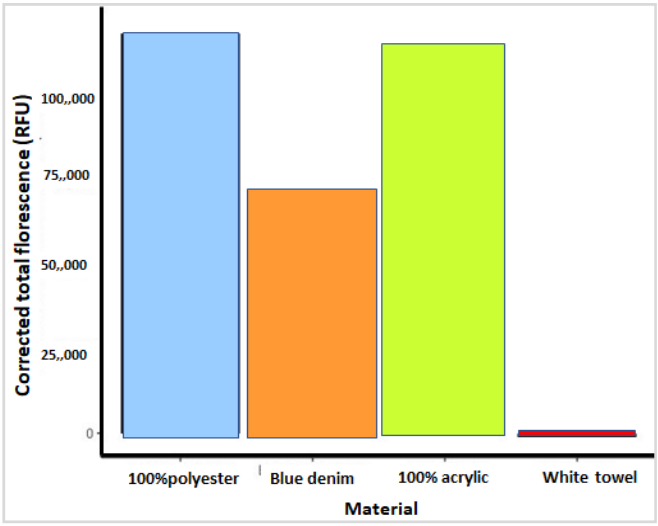


**Fig. 9 Fluorescent intensity of semen detected on 100% polyester, blue denim , 100% acrylic and white towel**

### 3. Effect of semen degradation

Semen stained exposed on fabric for 1 and 25 days both given positive results within 3 minutes time frame. Figure 10 illustrates the images for fluorescence on both days.

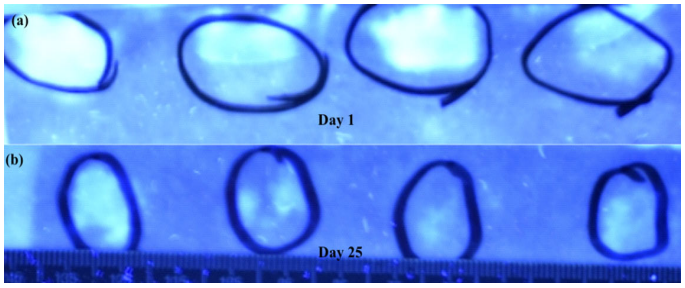


**Fig. 10. Fluorescence intensity for semen degradation on STK® Sperm Tracker™. (a) day 1, (b) day**

The intensity of fluorescence of the semen tested on day 1 was stronger compared to the intensity of day 25. Figure 11 illustrates the fluorescence intensities of semen tested on both days

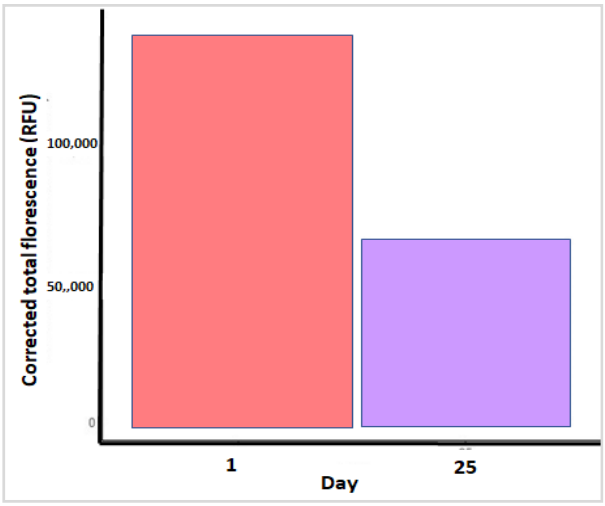


**Fig. 11 sensitivity study on the effect of semen degradation on STK® Sperm Tracker™. (a) day 1, (b) day 25**

## DISCUSSION

The optimization of parameters for STK technique conducted in study 1 revealed, that the achieved results for volume and pressure (100 -200 µl/m$^2$, 600kg/m$^2$) were compatible with supplier's recommendation values of 150 -200 µl/m$^2$ and 650kg/m$^2$. The time optimized (5 minutes) was not consistent with recommended time of 3 minutes [11,12]. The grading system used in study 1 was developed by monitoring fluorescence intensities and relies on examiners subjectivity. STK was given slight positive reaction only for detergent and given highest specificity when compared to two other tests. The STK Lab Sperm Tracker's sensitivity in detecting diluted semen found to have threshold of 1:20 .This is low compared to the AP test, where the threshold was is reported at a dilution of 1:400[16]. Since evidence received by forensic laboratories can arrive in various conditions, it is important to determine the dilution thresholds at which the product can reliably detect semen stains. The STK paper showed no impact on Phadebas test when a stain containing mixture of semen and saliva. After conducting the STK Test, a slight increase in DNA quantity observed for both normal and vasectomized individuals. However, the Two-Sample T-Test results indicate that this is not statically significant. In both instances, the quantity of DNA is found to be lower in vasectomy individual compared to normal person. This observation might be attributed to the absence of sperm cell production in vasectomized individual, with only other cells types contributing the overall output. In

the study 2, an initial test prior to STK showed the semen with dilution up to 1:32 under visible light and stiffness up to 1:64 dilutions. Nevertheless, the classification of a stain as semen cannot be confirmed by these parameters alone. The study has demonstrated that semen is detectable on cotton fabric by STK Lab sperm tracker up to a dilution on of 1:32, in contrast to previous reports of the limit of detection using at dilutions of 1:10 or 1:20[12,15]. The anomalous results obtained during the first trial and still detected during the second trial. However, the average intensity of fluorescence was weak (RFU<50,000). The study has demonstrated the advantage of increasing reaction time up to 10 minutes, due to the observed increase in the intensity of fluorescence. Increasing reaction time may reduce the likelihood of failing to detect the semen stain, particularly when the semen is highly diluted. Further research is needed to evaluate the tradeoffs between sensitivity and specificity when increasing the reaction time for the STK lab product. The evaluation of STK's sensitivity on various fabrics in study 2 revealed notable findings. The successful detection of semen on 100% polyester, blue denim and 100% acrylic aligns with its intended applications. The intensity of fluorescence different across fabric, with strong fluorescence found on 100% polyester and 100% acrylic fabrics and moderate fluorescence found on blue denim. The variation in fluorescence intensity may be explained by differences in fabric properties, including differences in absorbency. Whether the manufacturers given the enhanced results observed after extending the reaction time, the potential benefit of increasing the recommended reaction time from 3 to 10 minutes requires further investigation. It should be noted that the white towel exhibited auto fluorescence, which required substantial movement when disassembling the STK from the fabric to observe the reaction without interference from background fluorescence. This may result in negative outcomes for white towel samples. However, the observed challenges in detecting semen on white towel contradict the manufacture's claim of universal fabric suitability. Additional study is recommended to determine the causes and replicability of our findings of negative results on white towel fabrics. Finally, the extended detection capabilities of degraded semen up to 25 days post-deposition underscore the techniques potential in delayed forensic investigations. Further exploration of its suitability for towels and the establishment of standardized procedure for diverse fabric types could enhance the reliability and applicability of the STK sperm tracker in forensic settings.

## Conclusion

Validated parameters for the STK test in study 1 were 100 to 200µl/$m^2$ of water, 600kg/$m^2$ pressure and contacting time for 5 minutes. STK technique, against conventional acid phosphatase and ALS technique revealed many advantages. AP reagent is not recommended to direct applying to the stain as possible risk for subsequent tests and associated with chemical risk. The STK test is non-destructive and can directly apply for larger area without exposing to hazardous environment. Not involve any hazardous chemicals. Additionally, the STK Lab on subsequent saliva testing and DNA analysis. The incorporation of fabric density analysis enhances the comprehensiveness of the study, addressing the need for versatile semen detection techniques in forensic investigations. Overall, the STK Lab sperm tracker has more positive outcomes compared to classical AP and ALS techniques and can be used as confirmatory detection of semen on evidence items with higher reliability. I would like to extend my heartfelt gratitude to my current employer, the Government Analyst's Department, for the opportunities to pursue higher studies at the University of Strathclyde. Additionally, I'm deeply thankful to the University of Strathclyde for the resources and guidance that provided for the success of these two research projects.

**Conflicts of interest:** No potential conflicts of interest were reported by the authors.

**Ethical issues:** Ethical clearance for the research was granted by the University of Strathclyde.

**Author contribution:** RWRKR: Conceptualization, data acquisition, writing the manuscript. KOJ: Conceptualization, reviewing and approval of the final manuscript.

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