Advanced International Journal for Research (AIJFR)
E-ISSN: 3048-7641 ● Website: www.aijfr.com ● Email: editor@aijfr.com

# Evaluation of Seminal Stains: A Comparative Analysis of Acid Phosphatase and Prostate-Specific Antigen Tests

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**Abstract**

In the forensic examination of sexual assault cases, the Acid Phosphatase (AP) test is commonly used as a rapid presumptive screening method, while the PSA immunoassay is applied for confirmatory purposes due to its superior sensitivity. Immunochromatographic PSA (p30) tests are capable of detecting minute quantities of seminal material and often produce weak or trace positive results at dilutions where the AP test has already become negative. Because AP relies on an enzyme-based color reaction, its visible response diminishes at higher dilutions, limiting its practical detection threshold. PSA, a glycoprotein secreted by the epithelial cells of the prostate and present in high concentrations in seminal plasma, serves as a dependable biomarker for semen identification, particularly in cases where spermatozoa are absent due to azoospermia, vasectomy, or sample degradation. Comparative findings from both tests indicate that the PSA assay exhibits markedly higher sensitivity, detecting seminal fluid at greater dilutions (up to 1:2048 or more), whereas AP activity typically declines and becomes undetectable around 1:512–1:1024.Overall, while both AP and PSA tests are useful for identifying seminal stains, the PSA test offers a more sensitive and reliable means of detection at extreme dilutions where AP reactions become weak or inconclusive. Consequently, in rape investigations, PSA testing plays a critical role by enabling the sensitive detection of seminal fluid, reinforcing biological evidence, corroborating victim statements, facilitating DNA analysis, and providing robust scientific support within the criminal justice system and courts of law.

**Keywords:** Sexual assault investigation, POCSO Act, semen detection, forensic evidence, acid phosphatase (AP) test, prostate-specific antigen (PSA) and immunological assays.

## 1. Introduction

The frequency of sexual assault cases, particularly under the POCSO Act, continues to rise. Identifying semen plays a vital role in the investigation of rape and other sexual assault cases. The confirmation of semen in forensic samples not only helps to establish sexual contact but also strengthens the evidence in linking the suspect to the crime scene. Early and reliable detection methods are essential, as they assist investigators in corroborating victim statements and reconstructing the sequence of events. The ability to detect semen in aged or degraded stains significantly improves the chances of solving cases where

evidence may not be fresh. Since spermatozoa may sometimes be absent (due to azoospermia, vasectomy, or degraded samples), the use of biochemical markers such as acid phosphatase (AP) and prostate-specific antigen (PSA) becomes crucial. Acid phosphatase is an enzyme secreted by the prostate gland and is found in abundance in seminal fluid. The resilience of the enzyme, even in dried biological stains, enables forensic experts to identify seminal residues long after they have been deposited (Sensabaugh, G.F.1978). The acid phosphatase (AP) test serves primarily as a screening tool rather than a confirmatory method in forensic analysis. While it is useful for the preliminary detection of semen, it is not fully reliable because acid phosphatase is also found in other biological substances that do not contain semen, such as vaginal secretions, fecal matter, and certain food residues. This cross-reactivity may lead to false-positive results**,** limiting its specificity (Keating, S.M., & Peters, A. 1999). The Prostate-Specific Antigen (PSA) test is widely utilized in forensic science for the detection and confirmation of seminal fluid, particularly in cases of sexual assault. PSA is a glycoprotein secreted by the epithelial cells of the prostate and is found in high concentrations in seminal plasma, making it a reliable biomarker for identifying the presence of semen, especially when spermatozoa are absent due to conditions like azoospermia or vasectomy. (Sensabaugh, 1978). The forensic application of the PSA test offers several advantages, including high sensitivity and the ability to detect minute quantities of seminal fluid on various substrates (Hochmeister et al., 1999). However, concerns regarding cross-reactivity with other body fluids such as urine and female vaginal secretions, as well as the potential degradation of PSA in aged or exposed samples, pose limitations to its specificity and reliability (Stowell & Gulliksen, 1997). PSA is a one of the most important biomarker present in precum a protein secreted by the prostate and widely used in forensic science as a sensitive indicator of seminal fluid. Studies have shown that PSA can be detected in pre-ejaculate(precum) using immunological assays, making it a useful tool in confirming sexual contact even when no visible signs of semen are found (Stowell & Gulliksen, 1997; Hochmeister et al., 1999). This is especially relevant in cases involving vasectomized or azoospermic individuals, or when ejaculation is incomplete or intentionally avoided. Hara, M., et al. (1971) foundational work explaining PSA abundance in semen and its suitability for high-dilution detection. Numerous studies have demonstrated that PSA testing has largely superseded acid phosphatase analysis because of its higher sensitivity and more consistent performance at greater dilution levels.

## 2. Material and Method

This study, conducted at the Biology Section, Forensic Science Laboratory, Bihar, Patna. In this study, 25 human seminal fluid samples were analysed. Serial dilution of seminal fluid following a geometric dilution pattern (two-fold series) were performed by using distilled water.Acid phosphatase test and Prostate Specific Antigen Test were done (DFS Manual , 2019).

## 3. Result

**Table 1.1- Acid Phosphatase Test Results at Different Dilutions**

| Dilution | Colour Development | Interpretation |
|---|---|---|
| 1:2 → 1:32 | Strong, fast purple development (clear positive) | Positive |
| 1:64 → 1:256 | Moderate purple; slower but usually clear | Positive (weaker) |
| 1:512 → 1:1024 | Faint or patchy purple; | Weak positive / borderline |
| | Very patchy purple*; | unreliable |
| 1:2048 | No visible purple | Negative |

**Table 1.2- The optimal level of seminal fluid dilution by using Acid –Phosphates Test**

| Sl.No. | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 | 1:256 | 1:512 | 1:1024 | 1:2048 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| A/1 | +++ | +++ | +++ | +++ | +++ | ++ | ++ | + | +(w) | - | - |
| A/2 | +++ | +++ | +++ | +++ | +++ | ++ | ++ | + | +(w) | - | - |
| A/3 | +++ | +++ | +++ | +++ | +++ | ++ | ++ | + | +(w) | -* | - |
| A/4 | +++ | +++ | +++ | +++ | +++ | ++ | ++ | +(w) | - | - | - |
| A/5 | +++ | +++ | +++ | +++ | +++ | ++ | +(w) | - | - | - | - |
| A/6 | +++ | +++ | +++ | +++ | +++ | ++ | ++ | + | +(w) | - | - |
| A/7 | +++ | +++ | +++ | +++ | +++ | ++ | ++ | + | +(w) | - | - |
| A/8 | +++ | +++ | +++ | +++ | +++ | ++ | ++ | + | +(w) | - | - |
| A/9 | +++ | +++ | +++ | +++ | +++ | ++ | ++ | + | +(w) | -* | - |
| A/10 | +++ | +++ | +++ | +++ | +++ | ++ | ++ | + | +(w) | - | - |
| A/11 | +++ | +++ | +++ | +++ | +++ | ++ | ++ | +(w) | - | - | - |
| A/12 | +++ | +++ | +++ | +++ | +++ | ++ | ++ | + | +(w) | - | - |
| A/13 | +++ | +++ | +++ | ++ | +(w) | -* | - | - | - | - | - |
| A/14 | +++ | +++ | +++ | +++ | +++ | ++ | ++ | + | +(w) | - | - |
| A/15 | +++ | ++ | ++ | ++ | + | + | +(w) | - | - | - | - |
| A/16 | +++ | +++ | +++ | +++ | +++ | ++ | ++ | + | +(w) | - | - |
| A/17 | +++ | +++ | ++ | ++ | + | +(w) | - | - | - | - | - |
| A/18 | +++ | +++ | +++ | +++ | +++ | ++ | ++ | + | +(w) | - | - |
| A/19 | +++ | +++ | +++ | +++ | +++ | ++ | ++ | + | +(w) | - | - |
| A/20 | +++ | ++ | + | + | + | +(w) | - | - | - | - | - |
| A/21 | +++ | +++ | +++ | +++ | +++ | ++ | ++ | + | +(w) | - | - |
| A/22 | +++ | ++ | ++ | + | + | +(w) | - | - | - | - | - |
| A/23 | +++ | +++ | +++ | +++ | +++ | ++ | ++ | + | +(w) | - | - |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **A/24** | +++ | +++ | +++ | +++ | +++ | ++ | ++ | + | **+(w)** | - | - |
| **A/25** | +++ | +++ | +++ | +++ | +++ | ++ | ++ | + | **+(w)** | - | - |

**Table 2.1: Prostate Specific Antigen (PSA) Test Results at Different Dilutions**

| **Dilution** | **Test line (T)** | **Interpretation** |
|---|---|---|
| 1:2 – 1:16 | Strong, dark test line (clearly visible) | Strong positive — high PSA |
| 1:32 – 1:64 | Clear test line (slightly less intense) | Positive —PSA readily detectable |
| 1:128 – 1:256 | Visible but lighter test line (may appear slower) | Moderate positive |
| 1:512 – 1:1024 | faint or borderline test line; some samples negative | Weak/ Borderline positive |
| 1:2048 – 1:4096 | Very faint or trace line (inconsistently seen) | Trace Positive or near detection limit |
| | No visible test line in most samples | Negative (below visual detection) |
| 1:8192 | No test line | Negative |

**Table 2.2- The optimal level of seminal fluid dilution by using Prostate Specific Antigen Test**

| **Sl.No.** | **1:2-1:16** | **1:32-1:64** | **1:128-1:256** | **1:512** | **1:1024** | **1:2048** | **1:4096** | **1:8192** |
|---|---|---|---|---|---|---|---|---|
| **A/1** | +++ | +++ | ++ | + | + | **+(w)** | - | - |
| **A/2** | +++ | +++ | ++ | + | + | **+(w)** | - | - |
| **A/3** | +++ | +++ | ++ | + | + | **+(w)** | - | - |
| **A/4** | +++ | +++ | ++ | + | **+(w)** | - | - | - |
| **A/5** | +++ | ++ | + | **+(w)** | - | - | - | - |
| **A/6** | +++ | +++ | ++ | + | + | **+(w)** | - | - |
| **A/7** | +++ | +++ | ++ | + | + | + | **+(w)** | - |
| **A/8** | +++ | +++ | ++ | + | + | **+(w)** | - | - |
| **A/9** | +++ | +++ | ++ | + | + | **+(w)** | - | - |
| **A/10** | +++ | +++ | ++ | + | + | **+(w)** | - | - |
| **A/11** | +++ | +++ | ++ | + | **+(w)** | - | - | - |
| **A/12** | +++ | +++ | ++ | + | + | **+(w)** | - | - |
| **A/13** | +++ | ++ | + | - | - | - | - | - |
| **A/14** | +++ | +++ | ++ | + | + | **+(w)** | - | - |
| **A/15** | +++ | +++ | ++ | **+(w)** | - | - | - | - |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **A/16** | +++ | +++ | ++ | + | + | +(w) | - | - |
| **A/17** | +++ | +++ | +(w) | - | - | - | - | - |
| **A/18** | +++ | +++ | ++ | + | + | +(w) | - | - |
| **A/19** | +++ | +++ | ++ | + | + | + | +(w) | - |
| **A/20** | +++ | ++ | + | - | - | - | - | - |
| **A/21** | +++ | +++ | ++ | + | +(w) | - | - | - |
| **A/22** | +++ | ++ | + | - | - | - | - | - |
| **A/23** | +++ | +++ | ++ | + | + | + | +(w) | - |
| **A/24** | +++ | +++ | ++ | + | + | +(w) | - | - |
| **A/25** | +++ | +++ | ++ | + | + | +(w) | - | - |

## 4. Disscussion

The study shown that purple colour formed during reaction in Acid Phosphatase test get progressively weaker as dilution increases. (Table-1.1,1.2). At lower dilutions (1:2 to 1:32) when the enzyme activity was highly concentrated, gave an intense and rapid development of purple colour .At moderate dilutions (1:64 to 1:256) enzyme concentration reduced, indicated slower but still reliable positive reactions. Faint purple colour produced around Higher dilutions 1:512 and very patchy purple at 1:1024 which is almost unreliable due to enzyme activity falls near the detection threshold, resulting very faint colour , and no visible purple at very high dilutions (1:2048) because enzyme activity is usually too low to produce a visible purple colour. The present study findings were consistent with those reported by Singh et al. 2015. Maximum samples tested for acid phosphatase activity by serial dilutions gives strong positive results at lower dilutions (1:2 to 1:32) (FBS05 2018). Sample no.17, Sample no.20 and Sample no.22 gave weak positive result even at dilution 1:64 while sample no .13 gave weak positive at such a lower dilutions at 1:32 and produce very patchy purple at dilution 1:64 gave inconclusive result. The findings indicates when diluting, some samples already have lower starting enzyme activity, making them more susceptible to being undetectable at higher dilutions which may be due to acid phosphatase levels vary among individuals. The findings additionally suggest that, a dilution level that still produces a detectable result in one sample may fall below the detection threshold in another due to donor-to-donor sample variation Keel(2006) . Out of only 25 samples , 2 samples were gave very patchy purple up to 1:1024 dilution which is almost unreliable. The results further showed that on applying a fixed dilution factor, those samples have lower AP may drop below limit of detection while others with higher AP may still be detectable. Lewis *et al* 2012 reported that allowing AP test papers to develop for a longer period increases the chances of detecting semen at higher dilutions. Since the volume of ejaculate in casework is rarely known and AP levels differ widely between individuals, extended development time can significantly improve the likelihood of identifying seminal material in sexual-assault examinations. The Table 2.1, 2.2 demonstrates Prostate-Specific Antigen (PSA) detection changed as seminal samples were serially diluted. At lower dilutions (1:2 – 1:16)**,** PSA concentration was high, produced a strong and distinct test line**,** which confirmed a strong positive result. As dilution increases **(**1:32 – 1:64**),** clear test line appeared which was slightly less intense, shown positive reaction. At dilutions (1:128 to 1:256) visible but lighter test line indicated moderate positive result. Around 1:512 – 1:1024**,** faint test line detected gave weak positive

result while some samples were found negative. As dilution increases **(**1:2048 – 1:4096**)** the test reached its detection threshold some samples were still gave a very faint or trace line, while others become negative. This findings were consistent with those reported by Lewis, J., et al. (2012) that PSA detection at low dilutions and faint/borderline positives near the assay detection limit (around 1:1024–1:4096). Beyond 1:8192**,** the test line disappears completely**,** which indicates PSA concentration has fallen below the device's detection limit, though the control line remains visible (confirming test validity).Old, J., et al .(2009) also **c**onfirms immunochromatographic PSA/p30-based tests remain detectable at high dilutions but become inconsistent at extreme dilution levels. A comparison of the findings indicated that Acid Phosphatase (AP) test yielded weak positive reactions for Samples 17, 20, and 22 at a dilution of 1:64 and gives negative result on further dilution however, when examined using the PSA test, these samples yielded positive results even at higher dilutions ranging from 1:128 to 1:256. In contrast, Sample 13 exhibited only a weak positive reaction at a lower dilution of 1:32 and showed a very patchy purple coloration at 1:64, rendering the AP result inconclusive. Nevertheless, PSA testing of Sample 13 produced a positive result at a dilution of 1:128. These findings also indicated that PSA concentrations detected in seminal fluid show heterogeneous responses among donors and across different detection methods. Its levels and detectability can vary between individuals and under different testing conditions, which can influence forensic interpretation of seminal evidence (Shubham et al 2023), (Hochmeister, M. N., et al. 1999). Kamijo, T., et al. (1992) also established the high sensitivity of PSA assays compared to acid phosphatase, especially at low seminal concentrations. Acid Phosphatase test detects the enzymatic activity of acid phosphatase, which is present in relatively high concentrations in seminal plasma. The Prostate-Specific Antigen (PSA) test detects PSA i.e a protein highly concentrated in semen and is used to confirm the presence of seminal fluid, especially in forensic analysis (e.g., sexual assault investigations). AP (a color reaction enzyme test) tends to lose a visible signal at lower dilutions (i.e. when semen is more diluted) because the enzyme reaction has a higher practical detection threshold and the color readout becomes too faint to call reliably (Peonim et al 2013). PSA rapid kits are antibody-based immunoassays designed to detect the PSA protein at low ng/mL concentrations. Antibody capture and visual line amplification make them intrinsically more sensitive than a simple colorimetric enzyme substrate reaction. This lets PSA strips produce a faint test line at very high dilutions 1:2,048–1:4,096 range (Hobbs etal2009).PSA (p30) is extremely abundant in seminal fluid; depending on the assay and extraction, tested semen dilutions can still carry detectable PSA far beyond the point where AP activity produces visible colour. Studies and validation reports show PSA membranes giving positives at dilutions that produce no visible AP colour(Gartside et al 2003).Immunoassay lines are read visually but result from antigen–antibody complexes accumulated at the test line; even a tiny amount of antigen can produce a visible trace line. AP depends on enzyme concentration and rate of substrate conversion at low enzyme concentration the colour may be too weak or too slow to be read within the prescribed time. The PSA test shown slightly higher sensitivity at extreme dilutions (1:2048–1:4096), detecting some samples as trace positives that AP might miss while AP test loses visible signal earlier (around 1:1024). Because the AP test is enzymatic, diluting the seminal fluid reduces the concentration of acid phosphatase in the reaction volume. That lowers the reaction velocity and prolongs the time required to reach a detectable colour signal. When dilutions are sufficiently high, the enzyme activity may be too low to generate a visible colour change within the stipulated cut off period (e.g. 2 minutes), thus reducing the sensitivity of the assay. Several studies have observed that at high dilutions (e.g. 1:256, 1:512) the AP test fails to give a positive colour within the standard time, and extending the allowed reaction time can recover detection in more dilute

samples (Redhead & Brown, 2013; Siemieniuk et al.2013). Hochmeister, M. N., et al. (1999) also demonstrated that PSA rapid tests detect semen at high dilutions, with weak or trace reactions at extreme dilutions depending on sample variability. Thus, the present study suggests that Immunochromatographic PSA tests (the p30/PSA rapid kits) are generally more sensitive than the classic AP test for detecting very low amounts of seminal material. Overall, the PSA test shows superior sensitivity compared to the acid phosphatase test, remaining positive at higher dilutions where the AP reaction becomes weak or negative.

## 5. Conclusion

Thus, both AP and PSA are useful for seminal fluid detection, but PSA has a slight edge in sensitivity**,** especially at higher dilutions. AP is simpler and faster for high-concentration samples, but PSA is more reliable for detecting very diluted seminal stains. Thus, in rape case investigations, the PSA test plays a crucial role by enabling the sensitive detection of seminal fluid even when sperm cells are absent, strengthening biological evidence, supporting victim accounts, assisting DNA analysis, and providing reliable scientific support to the courts within the criminal justice system.

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