Forensic Detection of Semen II. Comparison of the Abacus Diagnostics *OneStep ABAcard p30 Test* and the Seratec *PSA Semiquant* Kit for the Determination of the Presence of Semen in Forensic Cases

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Introduction

Prostatic specific antigen (PSA) or p30, was first described in 1971 by Hara, et.al. (1) which they called γ-seminoprotein. Li and Beling (2) described what most likely was the same protein that they called E1 in 1973. A thorough biochemical analysis of a protein isolated from semen utilizing electrophoretic methods was made by Sensabaugh (3) in 1978. He termed the protein p30 as its molecular weight was approximately 30 kdaltons. Graves, et.al. (4) studied the protein extensively and the work was published as partial fulfillment of his Ph.D. thesis in 1985. Antisera to the protein quickly became utilized in the forensic field for the detection of semen.

Initially believed to be a prostate specific protein (3,4) it is now known to be found in many different fluids and tissues including breast tissue and tumors(5,6), periurethral glands (7,8,9), breast milk (10), amniotic fluid (11), female urine (12) and endometrium (13).

Methodologies utilized for detection of the protein included Ouchterlony diffusion, crossover electrophoresis, Laurel rocket electrophoresis and ELISA. Recently, membrane based detection methods have been utilized and commercial kits have been manufactured for forensic use. The sensitivity of these commercial kits has been listed as low as 2 ng PSA/mL.

Several laboratories have validated these commercial kits, primarily the one manufactured by Abacus Diagnostics (14,15,16,17,18). Validation has examined sensitivity and specificity issues and the kits have been used extensively throughout the United States. To our knowledge, no one has studied the issue of PSA degradation as it relates to these kits, to which this research is aimed.

Both the Abacus Diagnostics *OneStep ABAcard p30 Test* and the Seratec *PSA Semiquant* Kit are immunochromatographic one-step tests for the detection of p30. If p30 is present in a sample, the p30 reacts with a mobile monoclonal antihuman PSA antibody in the

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strip forming a mobile antigen-antibody complex. This antigen-antibody complex then migrates through the absorbent device toward the test area. A monoclonal antihuman PSA antibody is attached to the membrane in the test area. This immobilized antibody captures the above complex resulting in an antibody-antigen-antibody complex. When the p30 concentration is greater than 2 - 4 ng/mL, the dye particles will form a pink colored band in the test area indicating a positive result (Figure 1). Both tests also have an internal control to ensure that the test is properly working and that proper procedures have been followed. This internal control contains a polyclonal anti-mouse-antibody that captures the monoclonal antibody and forms a complex.

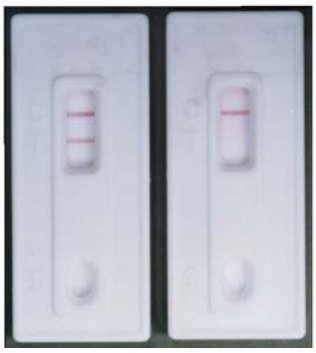

Figure 1. ABAcard Results (positive and negative results)

The Seratec *PSA Semiquant* Kit was developed as a screening test for the detection of human prostate cancer. Therefore it was designed as semiquantitative test. In this regard, it contains a 4ng PSA/mL internal standard (Figure 2). The company literature states that for the detection of seminal fluid, this test is used as a qualitative test and the internal standard is insignificant. However, in this analysts study, this internal standard can be quite helpful in estimating the concentration of PSA present in an unknown.

Figure 2. A Positive Reaction for PSA with the Seratec Kit (From Seratec's website, www.seratec.com.)

As proteins degrade, they lose their three dimensional conformation. It is possible that the monoclonal antibodies used in the two kits differ in their ability to bind to partially degraded psa. This experiment was designed to compare these two kits in detecting degraded psa.

Methods

Sexual assault samples were simulated by applying psa to vaginal, anal and oral samples and incubating them at 37°C.

Eleven swabs were collected from vaginal, anal and oral regions from 3 volunteers who had no sexual contact for 5 days (a total of 33 swabs per person) using sterile cotton-tipped swabs (Pur-Wraps Hardwood Products Company). 40 µL of 500 ng/mL PSA standard (Stanford PSA standard, Catalog number L-F 500) was immediately added to each swab (**20 ng PSA added to each swab)**. Swabs were placed in 2.0 mL microfuge tubes (Costar, Catalog number 3213), capped, and incubated at 37 °C in dry bath incubators (Fisher Scientific) and collected at 0, 2, 4, 8, 12, 16, 24, 32, 48 and 72 hours. Swabs were extracted in 1 mL HEPES (0.24 %, pH 7.2) for 2 hours. The tubes were gently shaken, opened and the swabs were removed and placed in spin baskets (Costar, Catalog number 9301), recapped and centrifuged at 13 K rpm for 3 minutes. 200 µL of supernatant was added to each test chamber and results were recorded after 10 minutes. Any visible line or band (sometimes a dot) was recorded as a positive result.

Dilution Study

A human PSA standard (Stanford, #060 5/96) was prepared using deionized water resulting in 2 mL of a 500 ng/mL solution. Serial dilutions were prepared resulting in concentrations of 500, 100, 50, 25, 12.5, 6.25, 3.125, 1.56 and 0.78 ng/mL. 200 µL samples were added to each test chamber and the results were recorded after 10 minutes.

Concordance Study

One of us (AJT) conducted tests using both kits on 70 case samples consisting of a variety of swab types and stains. Stacy Shipman (personal communication) conducted tests using both kits on 31 case samples. 200 µL samples from a single extract were added to each test chamber and results were recorded after 10 minutes.

Results

The results in this study are summarized in Table 1. The initial results for the ABAcard using Lot # 23221024 (expires 5/2004) were negative and it was decided to use a different lot to check the results (Lot # 23220621, expires 12/2003). This different lot of cards gave positive results.

Very little difference was noted between the two cards over the time period of the experiment. The Seratec cards were positive to the end of the experiment (96 hours) while the ABA cards were positive to 72 hours.

Subject 1																						
Hours	0		2		4		8		12		16		24		32		48		72		96	
Kit	A	S	A	S	A	S	A	S	A	S	A	S	A	S	A	S	A	S	A	S	A	S
Vaginal	-	+	+	+	+/+	+/+	-/+	+/+	-/+	+/+	-/+	+/+	-	+	+	+	+	+	+	+	-	+
Anal	+	+	-	+	+/+	+/+	-/+	+/+	-/+	+/+	-/+	+/+	-	+	+	+	+	+	+	+	-	+
Oral	+	+	+	+	+/+	+/+	+/+	+/+	-/+	+/+	-/-	+/+	-	+	+	+	+	+	+	+	-	+
Subject 2																						
Hours	0		2		4		8		12		16		24		32		48		72		96	
Kit	A	S	A	S	A	S	A	S	A	S	A	S	A	S	A	S	A	S	A	S	A	S
Vaginal	+	+	-	+	-/+	+/+	-/+	+/+	-/+	+/+	-/-	+/+	-	-	-	+	-	-	nt	nt	nt	nt
Anal	-	+	-	+	+/+	+/+	-/+	+/+	-/+	+/+	-/-	+/+	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt
Oral	+	+	-	+	-/+	+/+	-/-	+/+	-/-	+/+	-/-	+/+	-	+	-	+	-	+	nt	+	nt	-
Subject 3																						
Hours	0		2		4		8		12		16		24		32		48		72		96	
Kit	A	S	A	S	A	S	A	S	A	S	A	S	A	S	A	S	A	S	A	S	A	S
Vaginal	-	+	-	+	-/+	+/+	-/-	+/+	-/-	+/+	-/-	+/+	-	-	-	-	+	+	-	+	-	+
Anal	+	+	-	+	-/+	+/+	-/+	+/+	-/+	+/+	-/+	+/+	-	+	+	+	+	+	+	+	+	+
Oral	+	+	-	+	-/+	+/+	-/-	+/+	-/-	+/+	-/-	+/+	-	+	-	+	+	+	+	+	-	+

Table 1. Results of tests on swabs at designated times. Shaded areas are Lot #23220621 and unshaded areas are Lot#23221024 for ABAcard. Where two symbols appear, the top symbol is Lot # 23221024 and lower symbol is Lot#23220621 for ABAcard and Lot#64803 upper and Lot#60636 lower for Seratec. A = ABAcard S = Seratec
nt = not tested

Results of the PSA dilution study are shown in Table 2. Negative results were obtained with the ABAcards at a concentration of 6.25 ng/mL. Seratec kits were positive down below 1 ng/mL PSA.

The bands in the Seratec kits were tight, well defined and dark. Faint bands appeared at low levels of PSA, below 4 ng/mL. The internal 4 ng standard in the Seratec kit was consistent in intensity between tests and correctly approximated the concentration of PSA in the sample. It is concluded that this internal standard can be used to estimate the concentration of PSA in a sample to some degree, certainly over and under 4 ng/mL. This may aid in determining the size of sample to extract for DNA analysis.

Weak positive reactions were obtained with the ABAcard at levels below 100 ng/mL PSA. A spot occurred in one test and faint lines were observed in other tests. These were recorded as positive results but would have to be repeated for case work. Inconsistencies were observed between lots and within a lot. ABAcard Lot 23220621was positive to a dilution of 6.25 ng psa/mL while Lot 23221024 was negative at this dilution. One batch from this lot gave weak results at 50, 25 and 12.5 ng psa/mL.

PSA	Seratec[1]	Seratec[2]	Seratec[3]	ABA[4]	ABA[5]	ABA[5]
500	+	+	+	+	+	+
100	+	+	+	+	+	+
50	+	+	+	+	Weak +	+
25	+	+	+	+	Spot +	+
12.5	+	+	+	+	Faint +	+
6.25	+	+	+	Weak +	-	-
2.13	+	+	+	-	-	-
1.56	+	+	+	-	-	-
0.78	-	+	+	-	-	-

Table 2. Results of the PSA dilution study. The values along the y-axis are ng PSA/mL. PSA Lot #060 5/96. The lot numbers of the kits are: [1]50742, [2]60636, [3]64803, [4]23220621, and [5]23221024. The last two columns are tests removed from different boxes from the same lot number of ABAcards. The shaded areas showed either very weak, spotty or negative results.

The ABAcard is not designed to be quantitative. The Technical Information Sheet supplied with the ABAcard states “the intensity of either the control band or the test band should not be compared between tests or to each other for ABAcard p30 test and no quantitative interpretation should be made based upon differences in the intensity”.

Substrate	Seratec	Abacus	n
Stain on clothing	-	-	7
	+	+	2
	+	-	4
Vaginal swab	-	-	14
	+	+	12
	+	-	3
Rectal swab	-	-	17
	+	+	3
	+	-	6
Oral swab	-	-	18
	+	+	0
	+	-	0
Vaginal wash	-	-	3
	+	+	3
	+	-	1
Labia swab	-	-	2
	+	+	1
	+	-	0
Penile swab	-	-	1
	+	+	0
	+	-	0
Dried stain	-	-	1
	+	+	0
	+	-	0
Feminine pad	-	-	1
	+	+	0
	+	-	0
Condom	-	-	1
	+	+	0
	+	-	1

Table 3. Results of the concordance study. No positive ABAcard negative Seratec results were obtained.

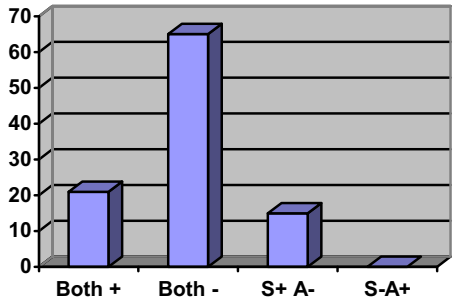

Figure 3. Diagram illustrating the results of the concordance study. (S) = Seratec (A) = Abacus; Values along the y-axis are number of cases

Results of the concordance study are shown in Table 3 and in Figure 3. For (85%), the kits were in agreement, i.e., both were positive or both were negative. In 15% of the cases, a difference was noted between the kit results. In all of these cases, a positive result was obtained with the Seratec kit and a negative result was obtained with the ABAcard. There were no occurrences of a positive ABAcard and a negative Seratec.

A large percentage of the differences between kits occurred in extracts prepared from stains and from rectal swabs. Seratec's greater sensitivity may play a role in obtaining more positive reactions from rectal swabs where degradation most likely occurs.

Discussion

PSA is now known to occur in a variety of fluids and tissues from both men and women. The term prostatic specific antigen is universally used in the clinical and forensic fields but a more appropriate name may be p30, referring to its molecular weight.

The results in this paper indicate that the Abacus Diagnostics *OneStep ABAcard p30 Test* and Seratec *PSA Semiquant* Kit are quite sensitive in detecting p30. Issues regarding these sensitivity levels and the presence of p30 in other body fluids have been discussed[20] and will be discussed in another paper [21].

The time study, dilution study and concordance study demonstrate that the Seratec *PSA Semiquant* Kit is more sensitive and consistent in detecting p30 from samples that may be encountered by forensic biologists than the Abacus Diagnostics *OneStep ABAcard p30 Test*.

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Forensic Detection of Semen III. Detection of PSA Using Membrane Based Tests: Sensitivity Issues with Regards to the Presence of PSA in Other Body Fluids

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Introduction

Prostatic specific antigen (PSA) or p30, was discovered in the 1970's independently by three groups [1-3]. After antisera to the protein was developed, detection of p30 in forensic samples quickly became the method of choice in determining the presence of semen in the absence of sperm.

Initially believed to be a prostate specific protein, it is now known to be found in many different fluids and tissues including breast tissue and tumors [4, 5], periurethral glands [6-8], breast milk [9], amniotic fluid [10], and female urine [11].

Membrane based detection methods have been utilized and commercial kits have been validated for forensic use [15-18]. The sensitivity of these commercial kits has been listed as low as 2 ng PSA/mL. Issues regarding sensitivity versus specificity and PSA detection have been raised [19]. The question arises that if PSA is detected, e.g., in a stain in a pair of panties, in extremely small amounts, can one state with certainty that semen is present?

This paper examines the detection of PSA using membrane based tests and the potential for detecting PSA from fluids other than semen.

Methods

Filtered water was added to sterile cotton-tipped swabs in varying amounts to saturation. Two brands of swabs were tested; both were cotton-tipped with wooden shafts. The brands were Puritan, Ref 806-WC and Pur-Wraps, 25-806 1WC, both manufactured by Hardwood Products Company, Guilford, Maine.

A green dye was added in varying amounts to a pair of cotton underwear and Whatman #3 filter paper and allowed to dry. Photographs of the stains were taken and the diameter of each stain was measured and recorded.

Neat breast milk and urine samples were collected from five nursing mothers (post-partum from 1 week to 8 months). Neat urine and whole blood samples were collected from three females. Breast milk samples were centrifuged and the resulting extracts added directly to Seratec PSA *Semiquant* Kits. Neat blood samples were centrifuged and

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100 µL of serum was mixed with 100 µL of HEPES (0.24 %, pH 7.2) to facilitate absorption, and added to the membrane. Blood samples from four nursing mothers were collected and dried on DNA cards. The bloodstains were extracted in 1 mL HEPES for two hours. The stains were centrifuged in Spin-Ease and 200 µL of extract was added to Seratec PSA *Semiquant* Kits.

Results of the Seratec PSA *Semiquant* Kits were read after ten minutes

Results and Discussion

It is now quite clear that the term prostatic specific antigen (PSA) is a misnomer. Although present in great amounts in seminal plasma, its presence has been detected in a variety of other body fluids (Table 1). The greatest concentrations of PSA outside of semen have been in breast milk and amniotic fluid. Generally, the forensic biologist does not encounter these fluids, however, one unusual case of the detection of PSA in a diaper originating from the colostrum in breast milk from a nursing child has been reported [20].

Fluid	Concentration PSA (ng/mL)	Reference
Semen	200,000 to 5.5 million	Sensabaugh [3]
Semen	820,000 (mean)	Lovgren, et.al [21]
Amniotic fluid	0.60 (avg.) 8.98 in one case	Lovgren, et.al [21]
Breast milk	1 (avg.) 2100 in one case	Lovgren, et.al [21]
Breast milk	Majority < 1.0; > 100 in one case	Filella, et.al.[22]
Breast milk	0.47 (median)	Yu and Diamandis [9]
Saliva	None	Lovgren, et.al [21]
Female urine	3.72 (mean)	Breul, et.al.[11]
Female urine	1.73 (mean)	Breul, et.al.[12]
Female urine	0.12 – 1.06; 0.29 mean	Schmidt, et.al.[13]
Female serum	0.53 (mean)	Breul, et.al.[11]
Female serum	Majority < 0.01	Yu and Diamandis [14]
Female serum	Majority < 0.1	Diamandis and Yu [23]

Table 1. Concentration of PSA in various body fluids (liquid).

Substantial levels of PSA have been found in amniotic fluid and breast milk. Cases involving lactating or pregnant women should be treated with due caution.

Of particular concern to this analyst is the detection of PSA in female urine and female serum. The finding of urine on a pair of underwear from a rape survivor would not be uncommon. In addition, if trauma is present or the survivor is menstruating, blood may be present on vaginal swabs or on stains in underwear. When an extract is prepared from a stain on the underwear and PSA is detected, how sure can the analyst be that the result is from semen? In other words, what is the likelihood that the stain is from female urine or serum?

The Abacus Diagnostics *OneStep ABAcard p30 Test* is used quite extensively throughout forensic laboratories in the United States. It has a listed sensitivity of 4 ng PSA/mL.

The Seratec *PSA Semiquant* Kit was developed as a screening test for the detection of human prostate cancer. It was designed as a semiquantitative test and contains a 4ng PSA/mL internal standard. Company literature states the sensitivity of the kit as 2 ng/mL of PSA.

This author has found the Seratec kit to be the more sensitive of the two PSA kits with positive reactions obtained at 0.78 ng/mL PSA [24]. At this level of detection the test is certainly in the range of known concentrations of PSA in female urine and near the limit detected in female serum.

Swabs

Filtered water was added to 10 swabs of each brand until saturation (at the point water began to pool at the swab-stick interface). The Puritan swabs could hold 150 µL of water and the Pur-Wraps 120 µL.

Stains

Results of the stain experiment are shown in Table 2 and Figure 1.

	Diameter, mm	
Stain volume, µL	**Cotton fabric**	**3M Whatman**
100	40	32
50	30	23
10	15	12

Table 2. Size of stains made from corresponding volumes of stain.

The size of the stains generated on cloth and filter paper can be seen in Table 2. The amount of material cut out from a stain depends on, among other things, the size of the stain present and it's intensity. If the clothing item is examined by an alternate light source, the areas giving off fluorescence are generally circled or marked in some manner.

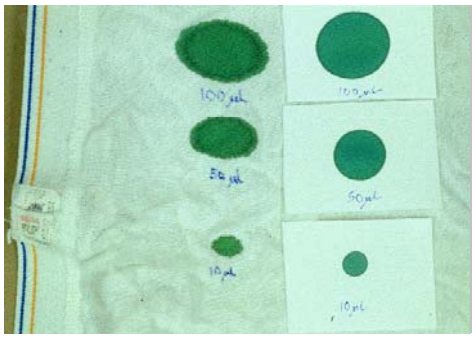

Figure 1. Green dye applied to cotton fabric and Whatman #3 filter paper at the listed volumes. Volumes from top to bottom: 100µL, 50 µL and 10 µL.

Generally, the smallest amount of material is cut out for extraction. Routinely, this analyst takes 0.25 cm^2 cuttings. I could not imagine one cutting out and extracting a stain larger than 1.0 cm^2.

		Urine		Serum		Semen	
Sample	**Volume µL**	**Concentration in fluid, ng/mL[11]**	**Extract Concentration ng/mL**	**Concentration in fluid, ng/mL[11]**	**Extract Concentration ng/mL**	**Concentration in fluid, ng/mL[21]**	**Extract Concentration ng/mL**
1 swab	150	3.72	0.558	0.53	0.08	820,000	123,000
½ swab	75	3.72	0.279	0.53	0.04	820,000	61,500
¼ swab	38	3.72	0.141	0.53	0.02	820,000	30,750
1 cm^2 stain	10	3.72	0.037	0.53	0.005	820,000	8,200
0.25 cm^2 stain	5	3.72	0.019	0.53	0.003	820,000	4,100

Table 3. Concentration of PSA in urine, serum and semen

The amount of PSA expected to be found in female urine, female serum and semen, based on published findings, is found in Table 3. The amounts of PSA expected vary according to the source of the material and the amount extracted. The table shows the amounts of PSA expected from the extraction of an entire swab, one-half of a swab, one-quarter of a swab, a 1cm by 1cm stain and a 0.5 cm by 0.5 cm stain. The values used for the concentration of PSA were the maximum amounts observed in urine and serum and the mean value of PSA in semen.

Even extracting an entire vaginal swab in 1 mL of HEPES, one would not expect to find PSA from urine or serum. Female urine and serum collected on a cotton-tipped swab, air dried, and extracted in 1 mL HEPES would not yield enough PSA to be detected by the Seratec kit. A 1 cm^2 stain from a pair of panties with female urine and blood extracted in 1 mL of HEPES will not be expected to yield enough PSA to be detected by the Seratec test chamber.

The dilution factor for a cotton-tipped swab (150 µL volume) is 0.15 and for a 1 cm^2 stain is 0.01. This means that the minimum concentration to obtain a positive reaction for a fluid dried on a cotton-tipped swab is 6.7 ng/mL, assuming extraction of the entire swab in 1 mL HEPES. For a 1-cm2 stain, a concentration of 100 ng PSA/mL would be required to obtain a positive reaction.

Recently, a study was conducted by Gartside, et.al. [25] in which they attempted to determine the efficiency of extracting psa from forensic samples. In their study, they obtained an extraction efficiency of 0.11% for swabs and 0.34% efficiency from stains using water and 1.03% efficiency from stains using HEPES.

I added known amounts of psa (Stanford) to cotton-tipped swabs and let them air dry. The entire swab was extracted in 1 mL HEPES using Sin-Ease baskets and 200 µL of extract was added to the Seratec membranes. Results were read at ten minutes.

Figure 2. Results of the extraction of 10 and 5 ng PSA/mL samples from cotton-tipped swabs. A weak band is visible in the 10 ng PSA/mL sample.

Figure 3. Results of the extraction of 15, 25 and 100 ng PSA/mL samples from cotton-tipped swabs. A band equal in intensity to the 4 ng/mL internal standard is visible in the 25 ng/mL sample.

As seen in Figure 2, a weak band can be seen in the 10 ng/mL sample. In Figure 3, bands are visible in the 15, 25 and 100 ng/mL samples. The band in the 25 ng/mL sample equals the 4 ng internal standard on the Seratec card. This equates to a 16% recovery rate. This corresponds well with the 100 ng sample that has a band significantly darker than the 4 ng standard (~16 ng/mL).

When considering such a low extraction efficiency, one does not have to be concerned with obtaining a positive result using the Seratec *PSA Semiquant* Kit on any sample other than semen.

A word of caution in analysis of a liquid urine sample from a sexual assault survivor. Addition of 200 µL of urine directly to a Seratec test chamber may result in a positive result from the urine, without the presence of any semen. Such analysis is not recommended. In fact, the addition of neat liquid samples from any source is not recommended. However, no psa was detected in neat breast milk, urine, or serum samples in this study.

It is also apparent that the instructions supplied with the test must be followed precisely. The swab or stain must be extracted in a minimum volume of 1 mL HEPES (or suitable buffer), only 200 µL of the extract must be added to the test and the results must be read within 10 minutes. Failure to follow these instructions may lead one to an inaccurate conclusion.

Conclusion

The Seratec *PSA Semiquant* Kit has been validated for use in the forensic identification of semen stains [15, 23]. PSA is now known not to be specific to the prostate and can be found in small amounts in fluids and tissues from women. The results of this study indicate that the forensic biologist can extract material from vaginal swabs and stains on clothing and be confident that a positive result is due to the presence of semen.

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