

# Assay of prostate-specific antigen from whole blood spotted on filter paper and application to prostate cancer screening

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We report the measure of prostate-specific antigen (PSA) from extracts of blood dried on filter paper. Five 3-mm (diameter) paper discs containing ~25  $\mu\text{L}$  of dried whole blood were punched from the filter paper and extracted with 500  $\mu\text{L}$  of buffer. Recovery of PSA was >92%. Imprecision of the filter paper procedure was <10% when corresponding whole-blood concentrations were >0.35  $\mu\text{g/L}$ . PSA recovery was unaffected whether blood was applied to the filter paper as one 85- $\mu\text{L}$  aliquot, two 43- $\mu\text{L}$  aliquots, or three 28- $\mu\text{L}$  aliquots. PSA is contained in the plasma fraction. Variation in hematocrit from 0.61 to 0.31 caused  $<\pm 10\%$  change in filter paper PSA. Regression analysis showed: filter paper PSA = 0.86 whole-blood PSA – 0.02;  $S_{yx} = 0.44$ . Men (153) without prostate cancer gave a 95th percentile of 4.8  $\mu\text{g/L}$ . PSA in filter paper dried blood was stable for >1 month at –20 to 37 °C and showed no loss of recovery after being mailed to a hot climate. We conclude that the filter paper procedure can reliably distinguish normal from increased concentrations of PSA and that it could facilitate screening to detect occult prostate cancer in large-scale mail-in programs to centralized laboratories.

INDEXING TERMS: immunoassay • spot immunodetection

Prostate-specific antigen (PSA) is currently the most sensitive serum marker of adenocarcinoma of the prostate [1, 2].<sup>3</sup> Although its role in monitoring the treatment of known prostate cancer is well established, its utility, along with that of other tests, in screening aging men for prostate cancer remains

uncertain [3, 4]. This is largely due to the lack of convincing data showing reduction in disease-specific mortality by earlier identification of the cancer in an asymptomatic population [5]. There is currently disagreement on whether screening should [6] or should not [7, 8] be recommended. Nevertheless, PSA has performed well in a number of recent screening studies of large cohorts of asymptomatic men [9–11]. PSA has been found to be more sensitive than other markers in identifying gland-contained and presumably curable cancer [10] and in detecting a preponderance of clinically significant tumors as opposed to latent, indolent microcarcinomas [12, 13]. PSA would become an even more attractive screening test marker if the confounding effects of benign prostatic hypertrophy on cancer detection could be attenuated [14]. Several means to achieve this, including the use of age-specific reference ranges [15], rate of PSA concentration change [16], correction for prostate volume [17], and ratio of circulating molecular forms [18, 19], have been proposed and are currently being investigated. It is likely that PSA will increasingly be used to screen asymptomatic older men for occult prostate cancer. In certain settings, it may suffice to screen with PSA alone and to dispense with a visit to a physician and digital rectal examination (DRE). For example, Labrie et al. [11] showed in their study of 7350 unselected, unscreened men that 97% of the cancers detected at annual follow-up by DRE plus PSA testing were PSA positive. Thus, a minimal benefit would accrue from including DRE in the annual work-up. Particularly when the screening procedure requires only blood-work, any means that simplifies the collection and testing of the blood would greatly facilitate the screening for occult prostate cancer.

Blood collected by capillary puncture from the heel, finger, or earlobe and dried on filter paper has been used to advantage in large-scale infant screening programs throughout the world to detect inborn errors of metabolism and congenital defects [20, 21] and in the testing of populations for infectious disease [22, 23] and nutritional deficiency [24]. In particular, filter paper dried blood provides a suitable delivery system where transport delays, safety concerns, and high temperatures preclude the shipment of liquid whole blood. Moreover, capillary puncture is less invasive and more convenient than venipuncture, and only

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<sup>3</sup> Nonstandard abbreviations: PSA, prostate-specific antigen; DRE, digital rectal examination; BSA, bovine serum albumin; RBC, red blood cell; and S & S, Schleicher and Schuell.

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minimal volumes of dried blood need be collected when sensitive analytical methods such as immunoassay to measure protein or polymerase chain reaction to test DNA are available. The only caveats are that the analyte to be measured must be stable to drying and must be released from the paper upon elution.

The measurement of PSA from one or several drops of dried blood collected on filter paper has recently become much more feasible with the advent of increasingly sensitive immunoassays [25, 26] that are capable of reliably measuring the very low concentrations that would be present in the eluate. This development, along with the increasing prominence of serum PSA as a screen for occult prostate cancer, has made it timely to consider if filter paper dried blood is a suitable specimen from which to quantify PSA. We were also interested in examining whether this type of specimen could support a simplified and convenient home-centered screening program in which one or two drops of blood collected from the finger and applied to filter paper in the home would be subsequently mailed to a central laboratory for PSA analysis. We show here that PSA can be reliably measured from dried filter paper blood, although variation in hematocrit is a potential complicating factor. We found that the five-disc sampling protocol from the blood spot is minimally affected by variations in the way blood drops are applied to the filter paper and that no PSA activity is lost from dried blood spots after 3 weeks in the mail system halfway around the world to a hot climate and back.

## Materials and Methods

### MATERIALS

Highly purified seminal plasma PSA was provided by Tom Stamey, Stanford University, Palo Alto, CA. Mouse serum was purchased from OEM Concepts, Toms River, NJ. All other chemicals were purchased from Sigma Chemical Co., St. Louis, MO.

### GENERAL PROCEDURES

*Whole-blood specimen.* Unless otherwise specified, whole blood precollected by venipuncture into heparin-containing evacuated glass tubes (Vacutainer Tubes; Becton Dickinson, Franklin Lakes, NJ) was used throughout this study. Blood was gently but thoroughly mixed immediately before being applied to filter paper.

*Spotting of blood onto filter paper.* Blood collection on filter paper was carried out in accordance with the standards issued in the National Committee for Clinical Laboratory Standards (NCCLS) publication LA4-A2 [27]. After thoroughly mixing the previously collected heparinized blood, 85  $\mu\text{L}$  was aspirated by air-displacement pipette and applied in one continuous application without pressing to Schleicher and Schuell (S & S) #903 filter paper to form a discrete circular spot ( $\sim 15$  mm in diameter) and allowed to air dry at room temperature for at least 3 h. Unless otherwise indicated, blood dried on filter paper was stored at room temperature. S & S #903 filter paper conforms to NCCLS specifications, is specially manufactured and quality tested for specimen collection, and has been widely used in neonatal thyroid/phenylketonuria dried blood screening pro-

grams [28, 29]. Screening programs have available specialized forms incorporating this filter paper. For this study, we used the preassembled form issued by the Ontario Government.

*Extraction of PSA from dried blood spots.* Five 3-mm-diameter, blood-impregnated paper discs scattered over the breadth of the dried blood spot were punched from the filter paper with a commercially available hand-held single-hole paper puncher. These five discs corresponded to  $\sim 30\%$  of the surface area of the entire 85- $\mu\text{L}$  dried blood spot. The paper punch was cleaned with tissue paper before being used on the next blood spot to minimize carryover from one dried specimen to the other. The discs were placed in a round-bottomed test tube containing 500  $\mu\text{L}$  of extraction buffer [0.1 mol/L Tris, pH 7.4, containing per liter 60 g of bovine serum albumin (BSA), 0.5 mol of potassium chloride, 5 mL of Tween 20, and 10 mL of mouse serum] and shaken for 1 h at room temperature, during which time dried specimen leached from the filter paper and dissolved in the liquid phase. PSA was measured in the liquid phase.

*PSA analysis.* The concentration of PSA in whole blood and in filter paper extracts was determined by a sensitive assay ("third-generation PSA") on the automated Immulite chemiluminescent immunoassay analyzer. Both the assay kit and analyzer are available commercially from Diagnostic Products Corp. (Los Angeles, CA). The assay is equimolar in that it is equally reactive to  $\alpha_1$ -antichymotrypsin-bound and free forms of PSA [30]. Assay sensitivity is 0.002  $\mu\text{g/L}$ , with a dynamic range to 20  $\mu\text{g/L}$ . For a single assay, 150  $\mu\text{L}$  of specimen is used, 50  $\mu\text{L}$  for the analysis itself and 100  $\mu\text{L}$  to accommodate the minimum volume requirements of the automated aspiration probe. Simultaneous replicate analyses can be done at an incremental volume of 50  $\mu\text{L}$  per replicate. Several analyses could be carried out if desired on the 450  $\mu\text{L}$  routinely recovered from the 500- $\mu\text{L}$  extraction procedure.

*PSA assay calibration.* Two sets of calibrators were used to calibrate the Immulite PSA assay:

- 1) The manufacturer's calibrators designed for use in measuring serum/plasma PSA. The calibration curve was routinely stable for 2 weeks. A two-point adjust procedure was carried out at 2-week intervals.

- 2) Locally prepared whole-blood calibrators with mid-normal hematocrit dried onto filter paper and freshly extracted for each assay run on the Immulite. This approach was instituted to correct for potential differences in run-to-run extraction efficiency and to incorporate an average hematocrit space-filling effect into the standardization. Heparinized whole blood freshly collected from women with PSA concentration  $< 0.002$   $\mu\text{g/L}$  and a hematocrit of  $\sim 0.45$  was supplemented with various amounts of purified seminal plasma PSA and then stored overnight to permit equilibration of free and protein-bound forms. Each supplemented whole-blood specimen was assayed six times on the Immulite analyzer against the manufacturer's calibrators and assigned a PSA value corresponding to the average of the six replicate analyses. Whole-blood specimens were mixed just before aspiration by the Immulite to fully

resuspend red blood cells (RBCs); otherwise, the procedure was identical to serum analysis. Multiple 85- $\mu\text{L}$  aliquots of each whole-blood calibrator were applied to filter paper, dried, and stored at room temperature until needed. For each run of the assay, the entire series of filter paper whole-blood calibrators (i.e., 0, 0.12, 0.23, 0.74, 1.23, 5.0, 14.1, and 76  $\mu\text{g/L}$ ) was extracted alongside filter paper dried whole-blood specimens of unknown PSA content. A run-specific, spline-fitted calibration curve was constructed from the logarithm of the Immulite chemiluminescence signal (arbitrary counts) vs the logarithm of the assigned whole-blood calibrator value.

#### HEMATOCRIT STUDIES

*Plasma vs RBC distribution of PSA.* Heparinized whole blood of normal hematocrit but containing about four times the normal limit of PSA was freshly collected from prostate cancer patients. Aliquots of this pool were centrifuged and the resulting plasma was either added to or taken away from the RBC fraction to create a series of pools derived from the original but differing in hematocrit. Hematocrit and PSA concentration were experimentally determined on each of these pools. Hematocrit ranged from 0 (plasma) to 0.61. The plasma PSA concentration was compared with that in each of the other pools and a whole-blood-to-plasma ratio derived in each case. Ratios were compared with the fractional plasma volume calculated as the proportion of the whole-blood volume not occupied by RBCs (i.e.,  $1 - \text{hematocrit fraction}$ ). Comparable values were taken to indicate that the PSA was present solely within the plasma fraction in whole blood.

*Change of measured PSA concentration with hematocrit in extracted dried blood and in whole blood.* A series of related whole-blood pools of various hematocrits ranging from 0 to 0.61 was prepared from freshly drawn whole blood (hematocrit mid-normal at 0.45) as described above. Aliquots of each pool were spotted onto filter paper, dried for 2 h, and extracted. PSA was measured in the extract and in the whole blood of each pool. The percent change in whole-blood value from that obtained on the originally drawn blood indicated the effect of hematocrit on PSA measurement in whole blood. The same was calculated for the extract PSA values, using the dried blood extract from the originally drawn blood as the basis for comparison. The magnitude of the changes observed with each specimen type (i.e., whole blood, dried blood extract) was compared.

#### DRIED BLOOD STUDIES

*Optimal extraction conditions of filter paper dried blood.* Extraction efficiency was investigated with a variety of extraction volumes, durations of extraction, and liquid media (phosphate-buffered saline, 60 g/L BSA, and 60 g/L BSA containing, per liter, 0.5 mol of potassium chloride, 5 mL of Tween 20, and 10 mL of mouse serum).

*Recovery of PSA from dried blood.* Whole-blood PSA concentrations were compared with those obtained from extracts of the entire 85- $\mu\text{L}$  dried blood spot to determine the recovery of PSA from filter paper. Because the entire blood spot contained about

three times the amount of dried blood present in the five 3-mm discs used routinely, extraction volumes of 1000  $\mu\text{L}$  and 1500  $\mu\text{L}$  were included in these studies along with the usual amount of 500  $\mu\text{L}$  in the event that the latter proved inadequate to completely extract the increased load.

The PSA concentrations in whole blood and corresponding dried blood filter paper extracts were compared to assess the effect of sample dilution from the five-disc extraction procedure on filter paper assay sensitivity.

Whole blood was applied to filter paper as one continuous 85- $\mu\text{L}$  aliquot, two closely superimposed 42.5- $\mu\text{L}$  aliquots, and three somewhat overlapping 28- $\mu\text{L}$  aliquots to simulate the range of applications that could realistically be expected from individuals using finger or ear lobe capillary puncture to draw the required volume of blood to fill the designated circle marked on the filter paper. Repeat aliquots to the application circle were applied either 30 s or 2 min apart. Extraction was carried out as usual with five 3-mm discs scattered over the breadth of the blood spot. Recovery of PSA activity in the extract was compared with all the conditions to determine the robustness of the five-disc extraction procedure.

#### *Reproducibility of PSA determination from blood dried on filter paper.*

Four heparinized pools of whole blood containing, respectively, low-normal, mid-normal, four times normal, and 20 times normal concentrations of PSA were prepared and multiple aliquots of each pool were spotted onto filter paper, dried, and stored at room temperature until needed. The Immulite assay was standardized as described under *General Procedures* both with manufacturer's calibrators and with the dried whole-blood calibrators, and precision was determined for each type of calibration over eight separate runs spanning 3 weeks. Eight replicates were included per run, encompassing two separate blood spots, two five-disc extracts per blood spot, and two duplicate analyses per extract.

#### *Stability of dried blood PSA.*

Stability was determined as follows:  
1) Filter paper dried blood stored at different temperatures. Three pools of heparinized whole blood containing normal and increased concentrations ( $4 \times$  normal limit,  $20 \times$  normal limit) of PSA were prepared. Multiple aliquots of each pool were applied to filter paper and dried. Dried filter paper blood was stored in manila envelopes at ambient humidity at either  $-20^\circ\text{C}$ ,  $4^\circ\text{C}$ , room temperature, or  $37^\circ\text{C}$ . Specimens were assayed at 0, 8, 17, 24, and 109 days of storage to determine the PSA activity still remaining.

2) Filter paper dried blood sent through the mail. Pairs of 85- $\mu\text{L}$  filter paper dried blood spots were prepared from freshly collected heparinized whole blood from 33 men. One of each blood-spot pair was sent in the mail for a 2.5-week excursion to Southern Europe and back. In accord with NCCLS standards (document H5-A3) pertaining to the shipment of blood dried onto filter paper [31], these specimens were shipped in an enclosed, sealed, high-quality bond envelope separated from one another with paper overlay. No special precautions were undertaken to control humidity, once again in accord with NCCLS guidelines. The other dried blood spot of each pair was stored in

the laboratory at room temperature over this time period. Upon receipt of the mailed specimens, paired blood spots were assayed to determine and compare the PSA that remained.

*Comparison of PSA in whole blood and corresponding dried blood extract.* Heparinized whole blood was collected from 158 men presenting to a hospital phlebotomy service. The freshly collected whole-blood specimens were assayed for PSA. An 85- $\mu$ L aliquot of each whole-blood specimen was spotted onto filter paper, dried, extracted, and the extract assayed for PSA. PSA concentrations in paired whole-blood and dried blood extracts were compared by regression analysis.

#### PRELIMINARY REFERENCE RANGE

PSA values from five individuals with a documented diagnosis of prostate cancer were removed from the 158-man database referred to above. The distribution of filter paper PSA from the remaining 153 men was analyzed to determine median, mean, and 95th percentile values. In addition, the data were grouped by the age of the donor, and the distribution of PSA concentrations was analyzed to determine age-specific median and 95th percentile values.

#### STATISTICS

Regression relations were determined by linear regression analysis, with scatter about the regression line expressed as the standard error  $S_{y|x}$ .

### Results

Measured PSA concentrations in whole blood and the corresponding plasma fraction were compared to provide insight into the distribution of PSA among whole-blood fractions. Whole-blood-to-plasma PSA ratios over a wide range of hematocrit closely approximated the plasma volume fraction. For example, at plasma fractional volumes of 0.39, 0.46, 0.55, 0.61, 0.69, and 0.76, the ratio was, respectively, 0.35, 0.45, 0.53, 0.63, 0.71, and 0.78. This indicated that PSA was present predominantly, if not solely, in the plasma fraction of circulating blood and that PSA was essentially excluded from RBCs. RBCs significantly affected PSA concentration by diluting the plasma fraction. The effect was variable, depending on the packed cell volume and the corresponding degree of dilution.

Applying whole blood to filter paper caused RBCs to lyse, leading to a cell-free extract upon rehydration. Despite this, the hematocrit of the applied whole blood did influence PSA recovery from filter paper dried blood extracts with the five-disc procedure. PSA concentration increased as hematocrit decreased and vice versa because the proportion of the PSA-containing plasma applied to the filter paper varied with the packed cell fractional volume. If this were the only factor, then hematocrit-induced variation in measured PSA should have been of similar magnitude for whole blood and filter paper dried blood. However, changes in hematocrit had much less effect on PSA concentration per unit volume in dried blood extracts than in whole blood (Table 1). In the latter, measured PSA concentration changed  $\sim 15\%$  for every 0.06 to 0.07 change in packed cell fractional volume. In dried blood extracts, on the other

**Table 1. Hematocrit effect on measured PSA concentration in whole blood and extracted dried blood.**

Hematocrit	Percent change from 0.45 hematocrit specimen	
	Whole blood	Extracted dried blood
0.61	-35	-10
0.54	-14	-6
0.45	0	0
0.39	+18	-0.5
0.31	+34	+8
0.24	+47	+18
0 (plasma)	+89	-3

hand, the corresponding change in measured PSA was less than one-third that observed in whole blood. The hematocrit effect was blunted in dried blood extracts because an additional factor came into play when applying the blood to the filter paper. Blood formed a larger circle when the packed RBC volume was decreased and a smaller circle when the packed RBC volume was increased. This meant that not only did hematocrit influence the volume fraction of plasma applied to the filter paper spot but it also influenced the amount of dried specimen per unit area on the filter paper because of its effect on diffusion. Because these two influences partially counteracted one another, the net overall effect was reduced, with the upshot that the hematocrit now affected PSA analysis in dried blood extracts much less than it did in whole blood. Specifically, PSA measured in extracts was changed  $<6\%$  by hematocrit spanning the reference range (0.37 to 0.52) and  $\leq \pm 10\%$  over a wider range of hematocrit from 0.31 to 0.61. This made it feasible to use dried whole-blood calibrators with a mid-normal hematocrit to standardize the filter paper assay, since only modest systematic errors would be introduced over the limited range of hematocrit likely to be encountered in the ambulatory male population for whom this procedure was designed to serve.

Several aspects of the extraction procedure were investigated to optimize performance. PSA was readily solubilized from the dried blood spot and it made little difference whether BSA or detergent or salt was included in the extraction liquid. All three solutions tested performed equally (data not shown). Extraction was  $\sim 80\%$  complete after 5 min of shaking, and fully complete after 1 h. Consistent differences were not observed when 1-, 2-, and 4-h extractions were compared. Concentration of extracted PSA varied linearly with the inverse of the extract volume over a range from 250  $\mu$ L (one-half usual volume) to 2000  $\mu$ L (four times usual volume). A linear relation was observed between number of 3-mm discs extracted and PSA concentration in the 500- $\mu$ L extract over a range of 2 to 10 discs.

The recovery of PSA from the entire 85- $\mu$ L aliquot of whole blood applied to the filter paper was determined by using two preparations of whole blood, one containing a mid-normal concentration of PSA (0.8  $\mu$ g/L) and another containing an eightfold increased concentration (8  $\mu$ g/L). Different extract volumes (500  $\mu$ L, 1000  $\mu$ L, and 1500  $\mu$ L) gave similar results. Recovery averaged 100% for the mid-normal specimen and 92% for the specimen with the increased concentration of PSA. The high recovery suggested that PSA was stable to drying and

that it did not form an adherent complex with the filter paper fibers.

PSA in the dried blood extract was considerably diluted, compared with that present in the original whole-blood specimen. Under routine conditions, with extraction of somewhat less than a third of the applied blood (five 3-mm discs) and a 500- $\mu\text{L}$  to 85- $\mu\text{L}$  sixfold increase in final volume, PSA concentration was reduced about 28-fold. This limited and hampered the sensitivity of the filter paper procedure, and restricted its application to whole-blood specimens containing  $>\sim 0.35 \mu\text{g/L}$  PSA. Below this, concentrations readily detected in whole blood were diluted to either the sensitivity limit of the assay or to concentrations at which analytical imprecision became unacceptable.

Recovery of PSA activity via the routine procedure was fairly consistent whether whole blood was applied to the filter paper as one continuous 85- $\mu\text{L}$  aliquot, two closely superimposed 42- $\mu\text{L}$  aliquots separated by 30 s, two superimposed 42- $\mu\text{L}$  aliquots separated by 5 min, or three overlapping 28- $\mu\text{L}$  aliquots separated by 30 s each. Recovery ranged from 91% to 105% relative to the single 85- $\mu\text{L}$  aliquot. The use of five discs punched from different areas of the blood spot successfully averaged out local inhomogeneities in specimen density to give an overall representative estimation of PSA concentration.

The precision of the dried blood procedure for low-normal, mid-normal, four times normal, and 20 times normal concentrations of PSA is shown in Table 2. Precision was calculated from eight separate runs involving 16 different blood spots and a total of 64 replicate analyses. The calibration procedure had some bearing on the precision obtained, but the strategy of extracting in parallel a set of dried blood calibrators with each run to correct for inconsistent extraction did not dramatically improve precision. In fact, the opposite occurred with PSA in the normal range. Precision was somewhat poorer than when manufacturer's calibrators were used to standardize the assay at 2-week intervals. However, at increased concentrations of PSA, precision was marginally better. With manufacturer's calibrators and a 2-week standardization interval, precision was  $<10\%$  in the mid-normal and increased range, and  $\sim 15\%$  in the low-normal PSA range.

The stability of PSA in dried blood spots stored at various temperatures is shown in Fig. 1. Recovery in general exceeded 80% under all conditions at 3 weeks and, except at 37°C, recovery exceeded 80% at 3.5 months.

PSA in dried blood was equally stable when sent in the mail to a hot climate as it was to storage in the laboratory at room temperature (Fig. 2). Mailed and room-temperature-stored paired specimens gave virtually identical PSA values.

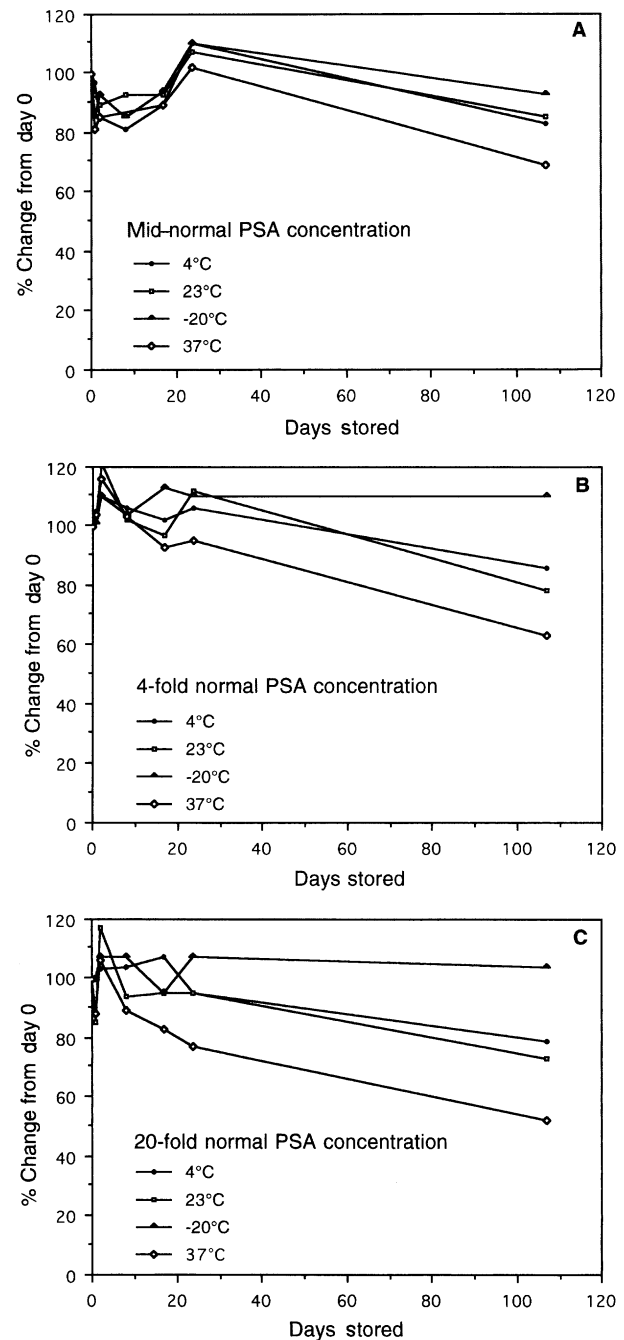


Fig. 1. Stability of PSA in dried filter paper blood stored at various temperatures for up to 109 days.

Five-disc extracts of the filter paper dried blood were assayed for PSA at the times indicated.

Table 2. Precision of filter paper dried blood PSA assay.<sup>a</sup>

Calibrators	Calibration frequency	Mean PSA conc ( $\mu\text{g/L}$ ) in spotted blood and corresponding precision (CV, %)			
		Low-normal	Mid-normal	4 $\times$ normal	20 $\times$ normal
Immulite	Every 2 weeks	0.014 (16)	0.026 (9)	0.29 (9.5)	1.5 (9)
Dried whole blood	Each run	0.32 (19)	0.63 (12)	8.5 (8.5)	37 (7.4)

<sup>a</sup> Precision was calculated from eight separate runs involving a total of 16 blood spots and 64 replicate analyses over 3 weeks.

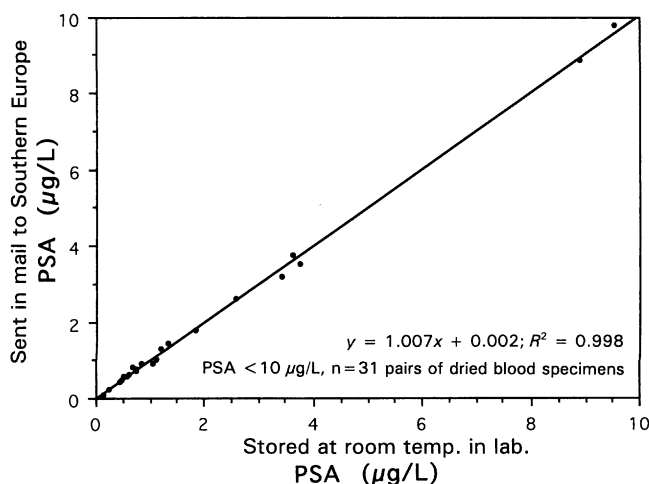


Fig. 2. Stability of PSA compared between paired filter paper dried blood specimens stored in the laboratory and sent in the mail for 3 weeks to a hot climate in Southern Europe and back.

Paired specimens gave virtually identical recoveries of PSA. Regression analysis showed one-to-one correspondence.

Extracted dried blood and corresponding whole-blood concentrations of PSA were compared in specimens received from 158 men presenting to a hospital phlebotomy service (Fig. 3). The filter paper procedure was standardized with freshly extracted dried blood calibrators. A linear and reasonably consistent relation was observed over the low-normal, mid-normal, and increased concentrations of PSA encountered. The overall slope of the regression line was 0.86, with  $S_{y|x} = 0.44$  for data <13  $\mu\text{g/L}$ .

The distribution of PSA values from the above 158 men (ages

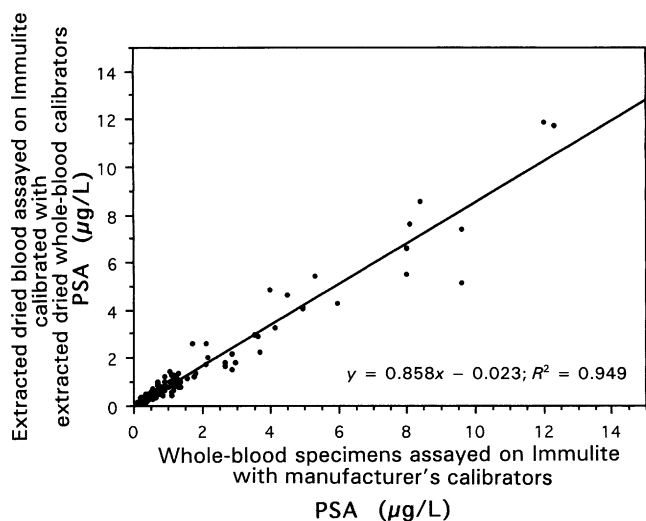


Fig. 3. Comparison of PSA in paired whole blood and filter paper dried blood specimens from 158 men presenting to a hospital phlebotomy service.

Heparinized whole blood was obtained by venipuncture and 85- $\mu\text{L}$  aliquots spotted onto filter paper within 2 h of collection. Whole-blood PSA was measured the same day of collection and extracts of filter paper dried blood specimens were assayed within 24 h. PSA assay of each specimen type was calibrated as indicated on the axes of the graph.

20–95 years) was analyzed after eliminating data from individuals with documented diagnosis of prostate cancer to determine a tentative reference interval for the filter paper procedure (Fig. 4). The median value was 0.4  $\mu\text{g/L}$  with the 95th percentile set at 4.8  $\mu\text{g/L}$ . Age-specific grouping of the data (Fig. 5) showed the expected rise in the 95th percentile with advancing age.

**Discussion**

The data show that PSA can be reliably measured from filter paper dried blood. PSA activity is not lost when the blood is dried, and recovery, with the five-disc sampling protocol, is the same whether blood is applied as one, two, or three drops, with variable delays, onto the application area of the filter paper. The S & S #903 filter paper used in this work is relatively thick and

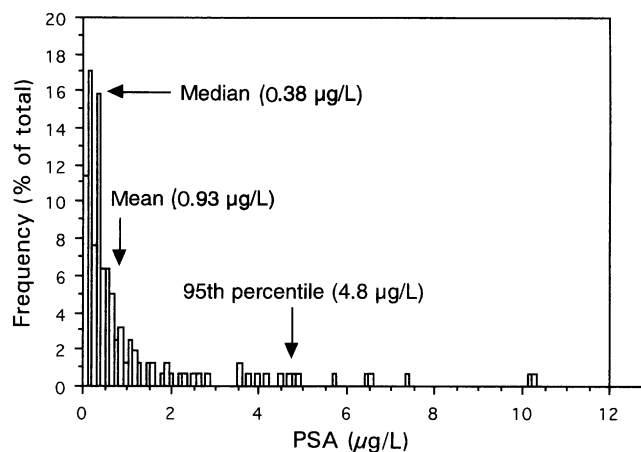


Fig. 4. Distribution of PSA in extracts of filter paper dried blood collected from 153 unselected men without documented diagnosis of prostate cancer who presented to a hospital phlebotomy service.

Ages ranged from 20 to 95 years. The PSA assay was calibrated with locally prepared extracted dried whole blood calibrators.

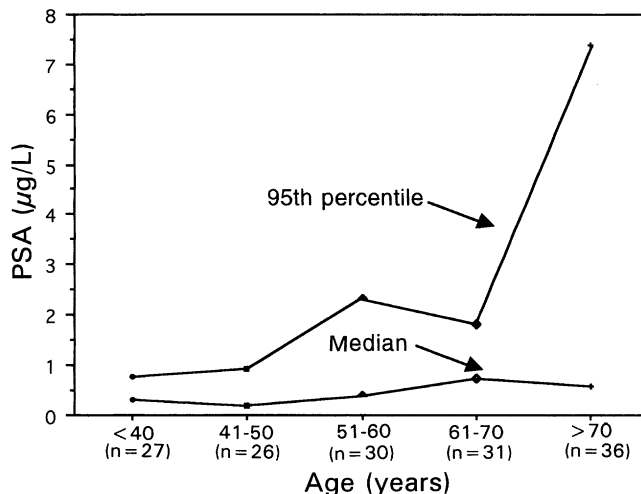


Fig. 5. Age-specific distribution of PSA in dried blood extracts obtained from unselected men without documented diagnosis of prostate cancer who presented to a hospital phlebotomy service.

Age of men vs PSA concentration (median and 95th percentile).

very absorbent, and applied drops of blood readily diffuse radially outward to form overlapping circles. This filter paper has been used extensively to collect blood in screening programs since Guthrie and Susi pioneered its use in 1963 [32] to detect phenylketonuria in newborns. It is now the recognized standard for collecting dried blood and the absorbency of each manufactured batch is kept within tight limits as certified by quality-control testing carried out by the Centers for Disease Control and Prevention. With this filter paper, a suitable format for the home collection of blood for PSA screening could readily be prepared.

We have shown that PSA in the circulation is present in the plasma fraction. When whole blood is used instead of plasma for the measure of PSA, as is the case here, the packed cell volume influences the final concentration and complicates interpretation inasmuch as the hematocrit can vary from individual to individual. The hematocrit space-filling effect is sufficiently pronounced with liquid whole blood to invalidate its use as a specimen from which to directly measure PSA without careful correction for packed cell fractional volume. Fortunately, this is not routinely necessary when filter paper dried blood is used as the specimen, thereby making filter paper collection attractive in screening programs to detect prostate cancer. Hematocrit effects are greatly reduced with the filter paper protocol and at most would be responsible for a  $\pm 10\%$  perturbation in the final measured PSA concentration, given the fractional packed cell volumes likely to be encountered in the reasonably healthy, asymptomatic aging male population for whom this test is intended. It is really only in the anemic range, when packed cell fractions drop to 0.25, that the perturbation becomes sufficiently great and exceeds acceptable limits for a serial screening program.

We anticipated that including dried whole-blood calibrators within each assay run would correct for systematic variation in run-to-run extraction efficiency and lead to an improvement in run-to-run precision. Our precision data show that the expected improvement did not materialize. At best, the dried whole-blood calibrators permit a mid-normal 0.45 hematocrit space-filling effect to be explicitly built into the standardization of the assay, but this in itself is not of critical importance, since it is variation in hematocrit per se within the population that confounds the issue, and the calibrators with their constant fractional packed cell volume do nothing to alleviate this variable. Perhaps the only benefit accruing from the use of dried blood calibrators is that they directly transform PSA concentrations in the filter paper extract to those present in the original whole-blood specimen; some might find this convenient in that they consider the latter to be more recognizable and prefer to report the data in this form. In summary, the use of dried whole-blood calibrators in the assay protocol can be considered optional.

PSA in the mid-normal and increased range is reliably measured by the filter paper dried blood procedure. Overall precision is  $<10\%$ . Thus, the procedure can distinguish normal from increased concentrations of PSA and is sufficiently sensitive analytically to screen asymptomatic populations for prostate cancer. However, analytical sensitivity becomes rate limiting at  $<0.4 \mu\text{g/L}$  with precision rising to  $>10\%$ . This precludes the

use of the filter paper protocol as currently constituted to serially monitor patients after prostatectomy for the recrudescence of prostate cancer where small changes in PSA concentrations in the range of  $0.4 \mu\text{g/L}$  [33],  $0.1 \mu\text{g/L}$  [34], and even lower [35] have clinical significance and must be reliably detected. This limitation is unfortunate in that home monitoring in this clinical setting would have been an ideal application for the filter paper technique.

The analytical sensitivity of the filter paper dried blood procedure is  $\sim 50$ -fold that obtained with plasma. Dilution of the plasma fraction by the RBCs contributes somewhat to this and accounts for about a twofold reduction. Much of the remaining 25-fold reduction in sensitivity stems from the dilution of applied PSA activity in the filter paper eluate. The most sensitive, commercially available immunoassays can reliably measure plasma PSA concentrations down to  $0.005\text{--}0.01 \mu\text{g/L}$ , which is more than adequate to meet the requirements as currently contemplated for postprostatectomy monitoring of prostate cancer relapse. However, when applied to the dried blood filter paper eluates, these lower limits are transposed 50-fold higher to the  $0.25\text{--}0.5 \mu\text{g/L}$  range, which no longer gives adequate sensitivity for monitoring postprostatectomy patients. The filter paper protocol could be made somewhat more sensitive by extracting more of the applied blood and (or) using a smaller extraction volume. Both approaches appear to be feasible and could easily lead to a doubling of the sensitivity. We have shown that the threefold greater amount of blood in the entire blood spot can be readily extracted into just twice the usual extraction volume. In addition, extraction from the five discs is equally efficient when the extraction volume is halved to  $250 \mu\text{L}$  instead of  $500 \mu\text{L}$ . It is important to emphasize that PSA is readily extracted from the filter paper and that persistent adherence does not in any way contribute to the loss of sensitivity encountered with the filter paper method.

The filter paper method correlated well with the analysis of PSA in liquid whole blood. The 158 paired specimens of blood obtained from men using a hospital phlebotomy service gave a reasonably consistent relation despite the wide array of packed cell fractional volumes in this population. This is encouraging inasmuch as hematocrit would likely be somewhat less variable in the asymptomatic ambulatory population for whom the filter paper screening test is intended. Somewhat surprisingly, given the limitation in sensitivity of the filter paper procedure, good correlation was observed even at PSA concentrations  $<0.35 \mu\text{g/L}$ , down to  $0.1 \mu\text{g/L}$ .

The age-specific reference ranges presented here are merely preliminary estimates, since only 27–38 individuals comprised each age group, and medical chart review, which was not carried out in depth, may have missed some individuals with prostate cancer. Nevertheless, the data show that the upper limit of the reference interval rises with age, and this pattern is consistent with the findings of others [36, 37] who have noted a similar age dependence of serum PSA concentration. Oesterling et al. [15] have recently investigated the clinical impact of using age-specific reference ranges and suggested that they will improve the specificity of the PSA test with minimal loss in the ability to detect clinically significant prostate cancers. Reference ranges

determined with whole blood will be lower than those determined with serum because of the dilutional effect of the RBC fraction.

PSA is remarkably stable when spotted as dried blood onto filter paper. Less than 20% activity was lost from dried blood after 21 days of storage in the laboratory at various temperatures. The most relevant test of stability in terms of large-scale screening, however, was the excellent recovery of activity from dried blood spots after 3 weeks of circulating in the mail to a far-off hot climate and back, exposed to ambient humidity. The latter point is particularly relevant in that humidity and not temperature is frequently the primary culprit in causing instability of analytes in dried blood specimens. There can be no doubt that PSA is robust to the rigors of the postal system and that stability of PSA would not be a rate-limiting factor in a screening program designed to receive large numbers of specimens through the mail at centralized testing laboratories.

Screening for prostate cancer by using PSA can be simplified and made more convenient by encouraging the home collection of the blood specimen. On-site testing of the specimen is one alternative, and a 20-min fluorophore-conjugated two-site immunometric point-of-care test involving 25  $\mu$ L of heparinized blood has recently been described [38]. Alternatively, specimens could be mailed to central testing laboratories. In this paper, we have shown that this is feasible with aliquots of blood collected by venipuncture, but there is nothing to suggest that two to three drops of blood obtained from capillary skin puncture and directly applied to filter paper would not also suffice. We have established that PSA can be reliably measured from such blood spots, that the sensitivity of the analysis is adequate to distinguish normal from abnormally increased concentrations, and that PSA in dried blood does not deteriorate when sent through the mail. For these reasons, the filter paper method may be of value in screening programs to detect prostate cancer.

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