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Development of an Immunoassay Specific for the PSA-ACT Complex Without the Problem of High Background

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We have developed an assay specific for the PSA-ACT (PSA-α1-antichymotrypsin) complex that effectively diminishes the problem of high assay background commonly reported by other investigators. The assay follows a two-site ELISA format. Polyclonal anti-PSA antibodies were coated on the microplate to capture the PSA complex from the serum, whereas the biotinylated anti-ACT polyclonal antibodies and HRP-conjugated streptavidin were used for detection. The high background ordinarily associated with this assay was greatly reduced when milk casein was added in addition to albumin for blocking and when the Super Block™ was also included in the diluents for sample dilution and dilution of enzyme conjugated detecting antibodies. The assay has a sensitivity of 0.05 ng/mL. The within-run precision ranges from 4.2–7.2% and the between-run precision falls between 5.8–8.5%. Cross reactions with ACT and free PSA (fPSA) are 0.0001% and 0.02%, respectively. The highest concentration of PSA-ACT complex in the maternal sera was < 0.4 ng/mL by this assay, much less than reported in the literature. Using this improved assay, the sum of fPSA and PSA-ACT concentrations were less than that of their corresponding total PSA (tPSA) most of the time. We believe that this improved assay should be used to replace the current tPSA assay for screening, monitoring, and managing patients with prostate cancer. J. Clin. Lab. Anal. 12:14–19, 1998.

Key words: PAGE; prostate cancer; PSA complexes; free PSA; total PSA; protease inhibitor; antichymotrypsin; protease activity; anti-PSA antibody

INTRODUCTION: IMPROVING PROSTATE CANCER SCREENING

There are two major isoforms of prostate specific antigen (PSA) that are detectable immunologically in the serum: the free PSA (fPSA) and the serum PSA-α1-antichymotrypsin (PSA-ACT) complex. In patients with prostate cancer, the majority of the PSA found in the sera is in the form of the PSA-ACT complex. As much as 95% or more of the total PSA (tPSA) detected in the serum from patients with prostate cancer may be comprised of the PSA-ACT complex. However, the percentage of PSA-ACT complex is much lower in patients with benign prostate diseases, such as benign prostate hyperplasia (BPH) and prostatitis (1,2). It is therefore likely that serum levels of the PSA-ACT complex are more specific for prostate cancer.

The PSA test is in great demand. It was the first tumor marker used for screening (3). However, it was found only a few years ago that PSA values produced by different commercial kits were generally not compatible (4,5). The major discrepancies, as pointed out by several investigators (1,5,6), were due to the different calibrators used and different PSA values assigned to the calibrators by different kits. The presence of two immunologically detectable PSA isoforms in the serum also caused problems (1,8,9). Failure to select an anti-PSA-ACT antibody for their kits, which would react equally with both free and complexed PSA, also contributed to the different values produced by different commercial kits on the same specimens (6).

As has been demonstrated repeatedly (9–11), there are many advantages associated with an assay specific for the serum PSA-ACT complex. It simplifies the selection of anti-PSA antibodies and the preparation of calibrator for the assay, making it possible for all commercial kits to produce the same values. The assay also should be more specific for prostate cancer.

Assays so far developed specifically for the PSA-ACT complex invariably used a two-side, sandwich test format. For example, the anti-PSA was coated on the solid phase, such as the microplate, to trap the PSA complex. Anti-ACT

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antibodies were used to react with the trapped PSA-ACT complex for the purpose of detection (12). It is not possible to coat the microwells with anti-ACT antibody to capture the serum PSA-ACT complex for the assay, because much higher serum ACT concentrations than PSA-ACT concentrations are present in the serum. However, using anti-ACT antibody for detection results in a high assay background. Apparently, as pointed out by Lilja et al. (13), another protease—namely, cathepsin G—in the serum will form a complex with serum ACT and will bind to the microplate. The use of enzyme-conjugated anti-ACT for detection may raise the assay background because the anti-ACT antibody may also react with the cathepsin G-ACT complex bound to the microplate surface. This high assay background frequently associated with the serum PSA-ACT assay has been the principal obstacle to the widespread application of this test.

After repeated trial and error, we eventually found two ways to improve the assay that avoided the problem of high assay background. There are so many advantages to measuring serum PSA-ACT complex alone over measuring a mixture of free and complex PSA, as the current tPSA assay does. However, in the near future we need to reestablish the normal reference range as well as the age-dependent reference ranges for the serum PSA-ACT assay in order to improve the screening, monitoring, and management of patients with prostate cancer.

MATERIALS AND METHODS

We obtained Super Block from ScyTek Laboratories (Logan, UT). BSA (Bovine serum albumin, fraction V), Tween 20, casein, and thimerosal were all purchased from Sigma (St Louis, MO). Polyclonal anti-PSA antibodies came from Dako (Carpinteria, CA). Sheep antirabbit IgG HRP-conjugate solution was obtained from BioDesign International (Kennebunk, ME). TMB substrate kit came from Pierce (Rockford, IL). Both polyclonal anti-PSA and polyclonal rabbit antihuman ACT antibodies were from Dako. HRP-streptavidin was purchased from Zymed Laboratory (South San Francisco, CA). Microtiter Strips (immulon 4) were from Dynatech Laboratories (Chantilly, VA). NHS-LC-Biotin Kit and TMB Substrate were purchased from Pierce (Rockford, IL), and Hybritech Tandem E kits were purchased from Hybritech (San Diego, CA).

All serum specimens used for comparing the sum of serum concentrations of fPSA and PSA-ACT complex with the serum concentrations of tPSA were obtained from the Department of Urology at the University of Utah Health Science Complex, Salt Lake City. The diagnoses of these specimens were largely determined by biopsies. The maternal sera used for various experiments of this study were obtained from Special Chemistry Laboratory at ARUP, which were sent to ARUP for screening neural tube defect.

Biotinylation

The NHS-LC-Biotin kit from Pierce was used for the biotinylation of polyclonal anti-ACT antibodies following the instruction of the kit insert. The molar ratio of biotin to Anti-ACT antibodies was maintained at 11/1. The resulting reaction mixture after removal of unreacted biotin by centrifugation was washed once and then concentrated by Centricon-30.

Coating Microplate With Anti-PSA Antibodies

A total of 100 μL Dako polyclonal anti-PSA antibody (5 μg/mL) in 0.1 M carbonate buffer, pH 9.6 was added to each well and incubated overnight at 4°C. After the wells were emptied, 250 μL blocking solution (1% BSA + 0.05% casein + 0.05% Tween 20 in PBS) was added, and the microplate was incubated overnight at 4°C. The microplate was allowed to dry after the blocking solution was aspirated and discarded. Plates should be stored in a sealed bag at 4°C with desiccant.

PSA-ACT Assay (in-house)

1. Pipette 50 μL of Super Block (1:4 diluted with buffer) and then 50 μL of sample or control into each microwell.
2. Incubate for 1 hr at rt. with constant shaking.
3. Wash the wells three times, each with 250 μL of Wash solution (0.05% Tween in PBS).
4. Just prior to use, mix equal volumes of biotinylated anti-ACT antibody (1:2,000) with Streptavidin-HRP in Super Block (1:2,000 diluted with 1:4 diluted Super Block). Add 100 μL of the mixture to each well.
5. Incubate for 1 hr at rt. with constant shaking. Repeat washing (same as described in Step 3).
6. Color development (10 min.) with the addition of 100 μL TMB solution to each well.

RESULTS

Effect of Super Block

Traditionally, 1% BSA has been used for blocking after coating the antibody on the solid phase. For the majority of immunoassays, the use of 1% BSA has been effective in blocking any remaining reactive sites on the solid phase. The use of anti-ACT antibodies as detecting antibody and the likely binding of cathepsin G-ACT complex to the microplate frequently gave high assay backgrounds for this assay. Blocking with traditional 1% BSA did not give us the low assay background desired. The addition of casein with 1% BSA in the blocking solution resulted in considerable improvement (12). Following the additional use of a 20 μL serum sample, we did not experience as many difficulties as described by other investigators with this assay (13), even before the Super Block became available.

Super Block is a new product that recently became commercially available and has been found to be an effective

blocking agent in many solid phase immunoassays and for Western blots. However, when we compared the Super Block with a traditional blocking agent, such as 1% BSA, we found that it did not lower the assay background as effectively as when it was used as a diluent. As shown in Figure 1, the use of Super Block only reduced the background to 0.3 ng/mL, not as low as when Super Block was included in the diluents (close to 0.1 ng/mL). Female sera have been used to check the assay background, since it was assumed that they did not contain any PSA but all other serum proteins, such as ACT and cathepsin G.

We found that it was most effective to reduce the assay background if we not only diluted the serum specimen with Super Block but also included the Super Block in the diluent for anti-ACT antibodies (Fig. 2). In fact, the results shown in Figure 2 indicate that including Super Block in the diluent for the dilution of anti-ACT antibodies (BSA & S) is more effective than including Super Block only during sample dilution (S & BSA).

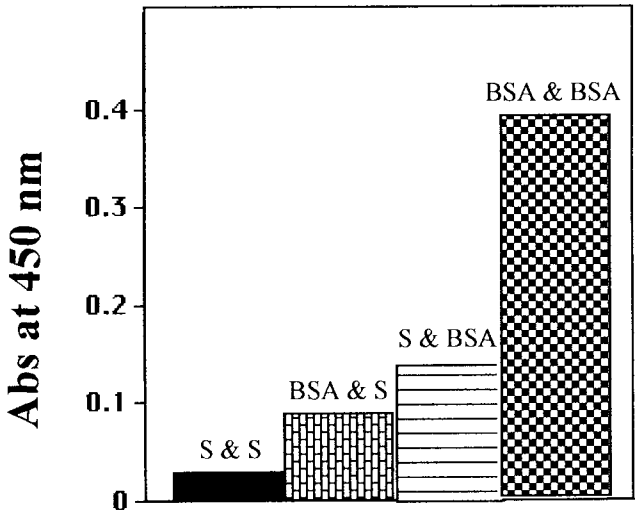

Fig. 2. Effect of Super Block as diluent for only one or two steps during the assay; 50 µL female serum containing undetectable PSA was used as sample in the PSA-ACT assay. Super Block was included in either the diluent for sample dilution (1:1 dilution) and/or in the diluent for anti-ACT antibodies.

Effect of Sample Volume

Apparently the volume of serum specimen used for the assay can have an impact on the assay background. As shown in Figure 3, when Super Block was not included in the diluents, a very high assay background was observed with female serum. However, high assay background could be greatly reduced if a smaller amount of serum was used for the assay (such as 5 µL). The success we had with our earlier PSA-ACT assay had to do not only with the use of casein as a blocking agent, but also because we used only 20 µL serum for the assay (12). However, when Super Block was included in the diluent, up to 80 µL of serum could be accommodated without raising the background. Using a larger aliquot of serum has the advantage of increasing the assay sensitivity and also improving the precision at the low sample concentration range. We concluded that a sample size of 50 µL when mixed with 50 µL of Super Block provides a sufficient level of sensitivity for our needs.

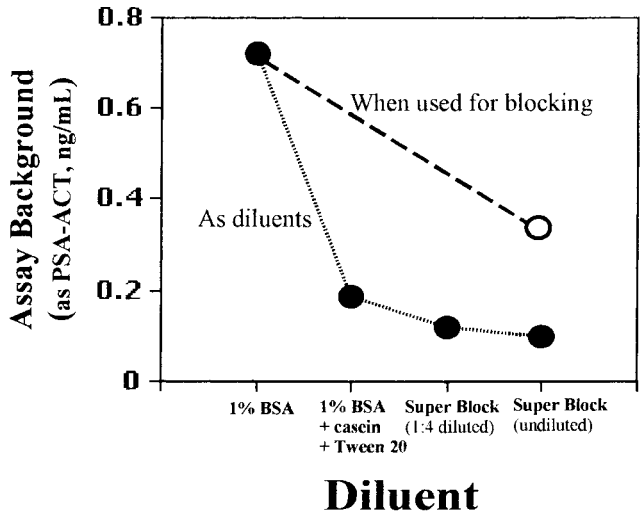

Fig. 1. Effect of Super Block on the assay background when used either for blocking or as diluents. The effects (expressed as ng/mL of PSA-ACT) were determined when assayed on a female specimen known to give high assay background. Undiluted Super Block was used either for blocking or used as diluents for the sample dilution (50 µL + 50 µL) and for the dilution of anti-ACT antibodies. Listed on the x-axis are compositions of various diluents used for purpose of comparison.

Total PSA vs. Sum of fPSA Plus PSA-ACT

Although there are only two major PSA isoforms detectable immunologically in the serum, other PSA isoforms could

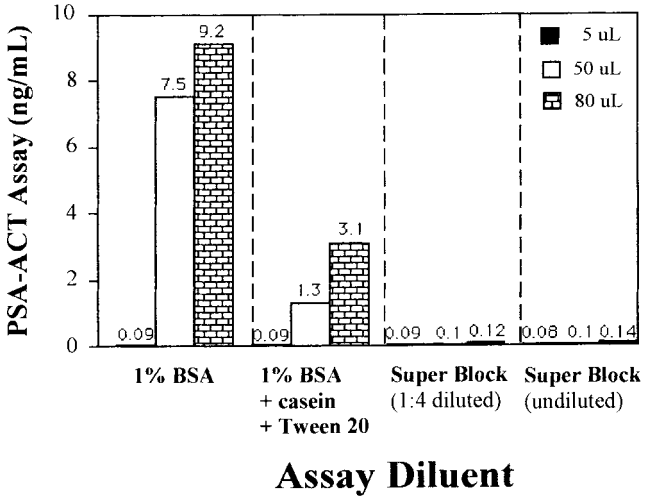

Fig. 3. Effect of the composition of assay diluent and sample size on the assay background. Values listed on top of the bars are assay background expressed as concentration of PSA-ACT. See legends of Figures 1 and 2 for other details.

also contribute to the value of total PSA determined by the current tPSA assays. In fact, we found low immunoreactivity with PSA-A2M (α2 macroglobulin) in a tPSA assay (14). PSA-AT (antitrypsin) complex could also contribute to the total PSA concentration even though the serum concentration of PSA-AT complex could be very low. The detecting anti-PSA antibody used in the tPSA assay can react with PSA molecules in any form to a varying degree. Consequently, the value of total PSA determined by the tPSA assay may include contributions from all PSA isoforms in addition to fPSA and PSA-ACT complex. In other words, there should be no surprise if the serum values of total PSA are greater than the sum of serum concentrations of fPSA and PSA-ACT complex. However, due to the problems of high assay background, we frequently found the reverse was also often true. Before the Super Block was available, we found that the concentrations of both free and complex PSA were greater than that of tPSA in approximately one-fourth of the specimens we assayed, even when casein was used as a blocking agent and only 20 μL serum specimen was used (Fig. 4). However, after including Super Block in the diluents, as the result of reduced assay backgrounds, almost all tPSA values became greater than the combined values of free PSA and PSA-ACT assays (Fig. 4). Among a total of 70 specimens, there were only two specimens where the concentrations of fPSA and PSA-ACT complex was slightly above that of tPSA.

PSA-ACT Concentrations in Female Sera

We also determined the PSA-ACT concentrations in a group of female maternal sera sent to us for screening neural tube defect and down syndrome. The average level determined by the improved PSA-ACT assay was much lower than that reported by Lilja et al. (13). This is additional evidence proving that the problem of high assay background had been eliminated or greatly reduced by the current PSA-ACT assay (Fig. 5).

Characteristics of the Improved PSA-ACT Assay

The overall assay characteristics of the improved PSA-ACT test are listed in Table 1. Because we biotinylated our anti-ACT antibodies and used the HRP-streptavidin for quantification, the high affinity between streptavidin and biotin allowed us to premix the biotinylated anti-ACT antibodies

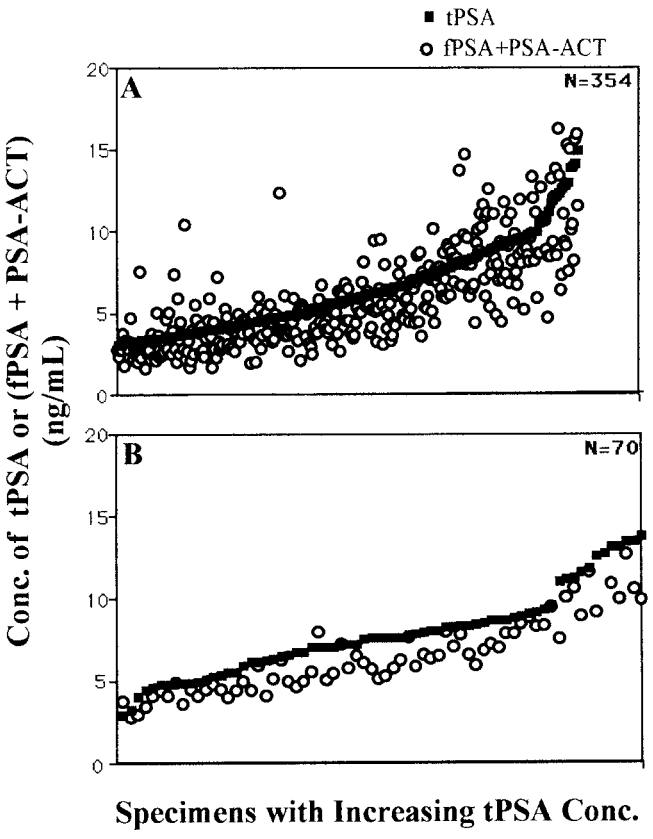

Fig. 4. Comparison of tPSA concentration with combined concentrations of fPSA and PSA-ACT complex on the same specimens. Values of total PSA were assayed by Hybritech Tandem E kits whereas the fPSA and PSA-ACT concentrations were determined separately by two in-house kits.

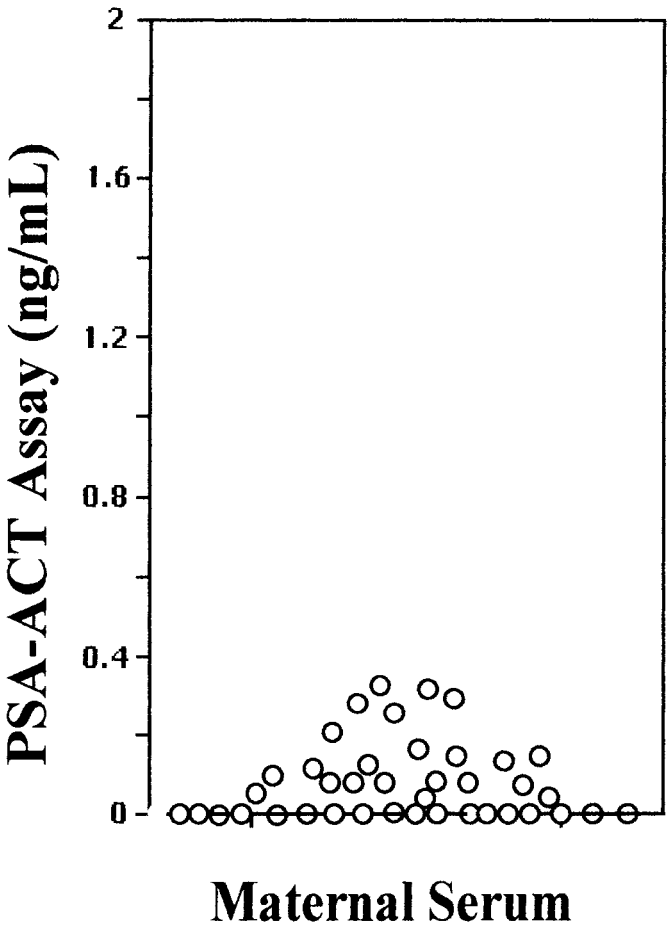

Fig. 5. PSA-ACT concentrations measured in the maternal sera.

TABLE 1. Characteristics of Improved PSA-ACT Assay

Assay format (add figure)	Microplate two-site ELISA
Sample volume recommended	50 µL
Sensitivity[a]	0.05 ng/mL
Calibrators	Pooled sera from cancer patients (0 to 10 ng/mL)
Assay precision	
Within day	CV = 6.3% (6.4 ± 0.4 ng/mL, N=12)
	CV = 6.8% (4.6 ± 0.31 ng/mL, N=12)
	CV = 4.2% (2.4 ± 0.1 ng/mL, N=14)
	CV = 7.2% (1.1 ± 0.08 ng/mL, N=14)
Day-to-day	CV = 8.5% (4.7 ± 0.4 ng/mL, N=11)
	CV = 5.8% (2.4 ± 0.14 ng/mL, N=14)
	CV = 7.1% (1.4 ± 0.1 ng/mL, N=12)
Crossreactivity with fPSA[b]	<0.02%
Crossreactivity with ACT[c]	<0.0001%

[a]Results of 12 determinations (mean + 2SD) using 1% BSA (zero calibrator) containing no PSA.
[b]Value provided by PSA-ACT assay on specimen containing only fPSA isoform.
[c]Value provided by the PSA-ACT assay on specimen containing pure ACT.

with the HRP-streptavidin and used in a single addition. We also changed our sample volume from 20 µL to 50 µL. As reported earlier (12), our PSA-ACT assay did not cross-react with either fPSA or ACT to any significant extent. In Figure 6, we also demonstrated that there was no effect on the PSA-ACT determination in the presence of as much as 50% fPSA in the serum sample, a situation frequently encountered in sera from benign prostate diseases.

The dose response curves shown in Figure 7 indicate that our current assay, using 50 µL of sample, has limited linearity in concentration. Therefore, computer programs, such as four parameters, available on the microplate reader (Molecular Device, Menlo Park, CA) should be used to construct the calibration curve. Failure to use a computer program for curve fitting could result in serious errors (15). It should be noted that the PSA-ACT calibrator when stored lyophilized at –20°C is stable for at least seven months (Fig. 8).

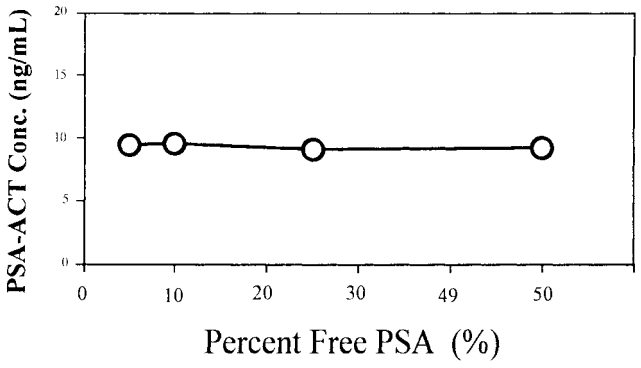

Fig. 6. Effect of % fPSA on the determination of PSA-ACT concentration by the PSA-ACT assay. Free PSA concentration, up to 50%, had no effect on the PSA-ACT assay.

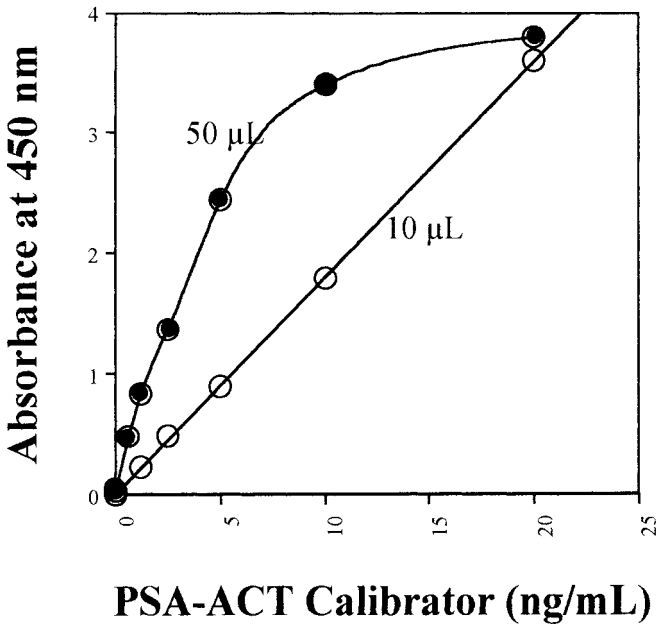

Fig. 7. Effect of sample size on the shape of the calibration curve. The calibration curve will not be linear when using 50 µL sample size. Computer curve fitting program should be used to construct the calibration curve.

DISCUSSION

The importance of measuring specifically the serum PSA-ACT complex is not only because the serum concentration of PSA-ACT is closely associated with the progression and regression of prostate cancer, but also due to the fact that many technical problems found with the tPSA assay will be avoided. For example, the problem of selecting antibodies for the assay will be greatly simplified. The problem of calibrator preparation also will be much easier to manage because we no longer have to deal with two PSA isoforms whose serum ratio constantly varies with the total PSA concentration. Since the PSA-

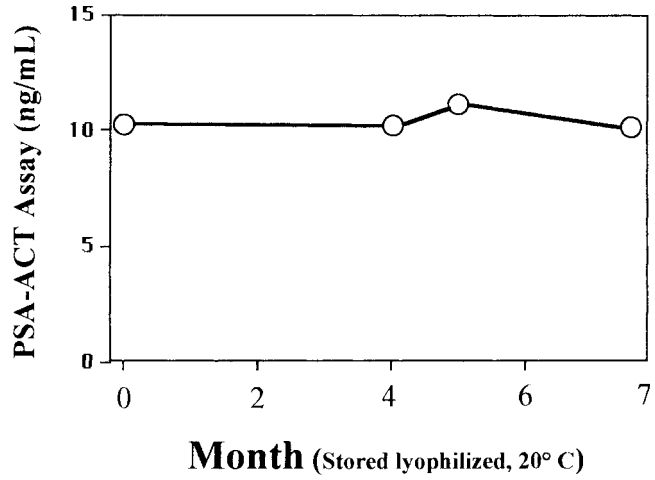

Fig. 8. Stability of lyophilized PSA-ACT calibrators stored at –20°C.

ACT assay measures only a single molecular species, we do not have to be concerned about the troublesome subject of "equal molarity" as we would for the tPSA assay. We found sometimes that the increase of the tPSA value was due to an increase of fPSA and not PSA-ACT complex. Since the increase of fPSA and the increase of PSA-ACT concentrations carry different clinical meanings, conceivably the increase of tPSA may not always associated with progression of prostate cancer. Contributions from many other serum PSA isoforms to the tPSA value can further distort the clinical implication of tPSA. Aside from serum fPSA and the PSA-ACT complex, we know very little about the associated clinical significance of all the other PSA isoforms. The measurement of the PSA-ACT complex also can help to distinguish prostate cancer from benign prostate diseases. Because of the higher % fPSA associated with benign prostate diseases, serum from BPH patients, for example, contains lower PSA-ACT concentration, whereas the PSA-ACT concentration associated with prostate cancer remains high due to low % fPSA in the serum.

The mechanism in which Super Block works to reduce the assay background is unclear. Without knowing the chemical composition of Super Block, it is practically impossible to know exactly why it works. The effectiveness of Super Block apparently has to do with the prevention of nonspecific binding of anti-ACT antibodies. As shown in Figure 2, Super Block was effective in reducing the assay background when included in the diluent for anti-ACT antibody, more effective than when used during sample dilution. Since Super Block did not appear to interfere with the assay in any fashion, we decided to include it in the diluents for both sample dilution and dilution of the detecting anti-ACT antibodies in order to achieve a maximum effect.

Conceivably, all commercial kits in the future could produce identical values for the same specimen if they all adopted the PSA-ACT assay. It also should be noted that, in our experience, some abnormal tPSA values, between 4–10 ng/mL, were falsely elevated due to the high percent of fPSA in the sample. Specimens with a high percentage of fPSA are supposedly associated with good prognosis. The current tPSA assay may provide misleading information regarding disease prognoses. Replacing tPSA by PSA-ACT test would diminish these types of problems.

We are now in the process of establishing normal reference level for serum PSA-ACT complex and levels of PSA-ACT in prostate cancer patients and patients with benign prostate diseases. We are also making an attempt to find out what are the clinical benefits of replacing tPSA with the PSA-ACT assay during monitoring the disease for patients with prostate cancer. We noticed that during monitoring treatment of prostate cancer patients, the changes of tPSA might sometimes be due to changes of fPSA and not PSA-ACT, which could lead to misinterpretation of the test result. We are also looking into the possibility of expression fPSA as ratio of fPSA/PSA-ACT to avoid the contributions from various minor PSA complexes to the tPSA value that we know very little about. We believe that the availability of the PSA-ACT assay will provide more precise and accurate screening and improved management of patients with prostate cancer.

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