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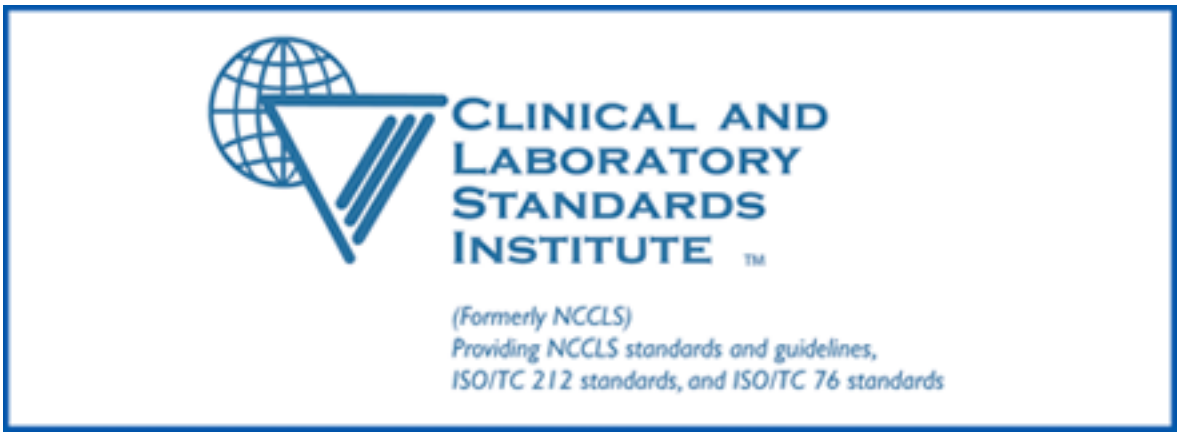
Primary Reference Preparations Used to Standardize Calibration of Immunochemical Assays for Serum Prostate Specific Antigen (PSA); Approved Guideline



This document provides procedures for purifying PSA from seminal fluid and ACT from human plasma. These are for use in preparing and biochemically defining primary standards for free PSA, PSA-ACT, and a 90:10 blend (PSA-ACT: free PSA) of the two.

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Primary Reference Preparations Used to Standardize Calibration of Immunochemical Assays for Serum Prostate Specific Antigen (PSA); Approved Guideline

Abstract

Primary Reference Preparations Used to Standardize Calibration of Immunochemical Assays for Serum Prostate Specific Antigen (PSA); Approved Guideline (NCCLS document I/LA19-A) is written for developers and manufacturers of prostate-specific antigen products for in vitro diagnostic use. This guideline describes procedures for the purification, characterization, and concentration value assignment of primary standards for PSA and PSA-ACT, and a blend (90:10 molar ratio) of PSA-ACT:PSA. These standards are intended to ensure consistency in determined concentration values between assay formats, equipment, operators, and laboratories.

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Primary Reference Preparations Used to Standardize Calibration of Immunochemical Assays for Serum Prostate Specific Antigen (PSA); Approved Guideline

Volume 17 Number 6

Robert M. Nakamura, M.D.
Barry I. Bluestein, Ph.D., M.T.(ASCP)
Barry L. Dowell, Ph.D.
Howard C.B. Graves, M.D., Ph.D.
Roger L. Sokoloff, M.B.A., Ph.D.
James T. Wu, Ph.D.

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Area Committee on Immunology and Ligand Assay

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Linda Ivor **Vice Chairholder**	**Del Mar, California**

Subcommittee on PSA Reference Material Specifications

Robert M. Nakamura, M.D. **Chairholder**	**Scripps Clinic & Research Foundation** **La Jolla, California**
Barry I. Bluestein, Ph.D., M.T.(ASCP)	Chiron Diagnostics Corporation East Walpole, Massachusetts
Barry L. Dowell, Ph.D.	Abbott Laboratories Abbott Park, Illinois
Howard C.B. Graves, M.D., Ph.D.	San Francisco General Hospital San Francisco, California
Joan H. Howanitz, M.D.	West Los Angeles VA Medical Center Los Angeles, California
Alan J. McNally, Ph.D.	Roche Diagnostic Systems Branchburg, New Jersey
Roger L. Sokoloff, M.B.A., Ph.D.	Hybritech Incorporated San Diego, California
S.K. Vadlamudi, D.V.M., Ph.D.	FDA Center for Devices/Radiological Health Rockville, Maryland
James T. Wu, Ph.D.	Associated Regional and University Pathologists Salt Lake City, Utah
Norman S.T. Yang, Ph.D.	Yang Laboratories Bellevue, Washington

Advisors

Dennis Brogan, M.P.H.	Bridgeport Hospital Bridgeport, Connecticut
Sanda Clejan, Ph.D.	Tulane University Medical Center Hospital New Orleans, Louisiana
Majula K. Gupta, Ph.D.	The Cleveland Clinic Foundation Cleveland, Ohio

Masood Haider, Ph.D.	Roche Biomedical Laboratories Raritan, New Jersey
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Aaron Malkin, M.D.	FML Consultants Don Mills, Ontario, Canada
Peter Maxim, Ph.D.	FDA Center for Devices/Radiological Health Rockville, Maryland
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Contents

Foreword

I/LA19-A is written for developers and manufacturers of prostate-specific antigen (PSA) products for in vitro diagnostic use. In general, primary standards provide a means for increased consistency in determined concentration values across time, regardless of the assay format, equipment, operator, and the laboratory. This document describes procedures for the purification, characterization, and concentration value assignment of free PSA (PSA), of the complex of PSA with alpha-1-antichymotrypsin (PSA-ACT), and of the 90:10 mixture (PSA-ACT:PSA).

In 1986, the FDA approved the first commercial immunoassay for quantifying serum PSA to aid in the prognosis and management of patients with prostate cancer. Since then, additional applications for PSA diagnostic tests have been explored, including (1) prostate cancer screening,* (2) detecting residual tumors in patients after a prostatectomy, and (3) differential diagnoses (e.g., to distinguish prostate cancer from benign prostatic hypertrophy). Recent clinical studies support the utility of serum PSA as an adjunct or alternative to digital rectal examination for screening men at risk for prostate cancer. In response to the conclusions of these studies, the American Cancer Society recently recommended that men 50 years and older, as well as younger men in high-risk groups, be screened annually for prostate cancer using a digital rectal examination and serum PSA.[1] This practice of routine screening of asymptomatic men has been questioned and further studies are being conducted to determine its appropriateness.[2,3]

Because of this large market, the number of commercial PSA assays has proliferated. Many more PSA assays are expected to be marketed within the next few years. The potential widespread use of PSA as a screening test makes it imperative that the different commercial PSA tests be standardized to give consistent values in patient serum samples. Only in this way can critical boundary values for PSA be maintained to ensure the accurate clinical interpretation of test results.

Prostate-specific antigen is a member of the kallikrein enzyme family[4] with a molecular weight of approximately 30 kiloDaltons (kDa).[5-7] It demonstrates primarily chymotrypsin-like enzymatic activity.[8-10] Recently, it has been recognized that PSA appears in serum in several forms. [11-14] It is possible that the low molecular weight form (approximately 30 kDa) represents a pro-enzyme of PSA or an enzymatically inactive form. Because of its enzymatic activity, PSA also complexes extensively with the serum serine protease inhibitors, alpha-1-antichymotrypsin (ACT) and alpha-2-macroglobulin.[12] Current immunoassays for PSA detect primarily two forms of PSA: a low molecular weight form and the PSA-ACT complex.[11,15] Studies of PSA in human serum have demonstrated the predominant form in most patients to be a high relative molecular mass (M_r) species of approximately 90 to 100 kDa, which is equivalent to the added masses of PSA and ACT.[11,15] However, the ratio of the two forms varies in different patients, particularly in the range of PSA values that is important for the early detection of prostate cancer.[15]

Some of the current FDA-approved immunoassays for PSA detect these two forms of PSA differently, which results in significant differences in the molar response ratios of the assays of the two forms.[13,15] Future assays that detect either only free PSA or only PSA-ACT are anticipated, and they could have different clinical utilities than the current generation of tests.

Because current immunoassays detect both free and complexed forms of PSA, and immunoassays for PSA are being developed to recognize these forms specifically,[13,15,16] the NCCLS Subcommittee on PSA Reference Material Specifications recommends that three standards be developed: one for PSA (PSA primary standard); one for PSA-ACT (PSA-ACT primary standard); and a mixture of the two, which will contain molar percentages of 10:90 (mixed PSA/PSA-ACT primary standard).

*In 1994, the FDA cleared a commercial immunoassay for use in conjunction with digital rectal examinations for the detection of prostate cancer in men 50 years and older.

Note that the purpose of this guideline is to define methods for preparing primary standards. It is not intended to prescribe to manufacturers how PSA and PSA-ACT should be prepared for use in their commercial immunoassays. The manufacturers of PSA immunoassays retain the flexibility to optimize recommended procedures for production purposes.

Primary Reference Preparations Used to Standardize Calibration of Immunochemical Assays for Serum Prostate Specific Antigen (PSA); Approved Guideline

1 Introduction

It has been recently recognized that PSA appears in serum in several forms.[11-14] The possibility exists that the low molecular weight form (approximately 30 kDa), referred to as free PSA, represents a pro-enzyme of PSA or an enzymatically inactive form. PSA also complexes extensively with the serum serine protease inhibitors, alpha-1-antichymotrypsin and alpha-2-macroglobulin. Only free PSA (PSA) and the PSA-alpha-1-antichymotrypsin complex (PSA-ACT) appear to be recognized by current PSA immunoassays. Because current immunoassays detect two forms of PSA, and immunoassays for PSA can recognize these forms of PSA in differing molar responses,[13,15,17] the NCCLS Subcommittee on PSA Reference Material Specifications recommends that three standards be developed: one for PSA (PSA primary standard); one for PSA-ACT (PSA-ACT primary standard); and a mixture of 90% PSA and 10% PSA-ACT (mixed PSA/PSA-ACT primary standard).

I/LA19-A describes the production and definition of primary reference materials (PRMs) for PSA and PSA-ACT. The document represents an initial step toward the ultimate goal of standardization of PSA immunoassays.

2 Scope

This guideline describes procedures for preparing and biochemically defining PSA and PSA-ACT primary standards and a mixture of 90% PSA-ACT and 10% PSA. Preparation of the materials should be in accord with good manufacturing practices, as described in the *Code of Federal Regulations* (Section 21, Part 820 [1994]).

3 Definitions

In science and technology the English word, "standard," has two different meanings[18]: (1) A widely adopted written technical standard, specification, technical recommendation, or similar document, and (2) A measurement standard (standard material).

In the context of this guideline, the term “standard” is defined in the following ways:

Standard, *n* - A material measure, measuring instrument, reference material, or measuring system intended to define, realize, conserve, or reproduce a unit or one or more values of a quantity to serve as a reference.

Primary standard, *n* - A standard that is designated or widely acknowledged as having the highest metrological qualities and whose value is accepted without reference to other standards of the same quantity.

Secondary standard, *n* - A standard with a value assigned by comparison with a primary standard of the same quantity.

For use at the local level, the terms "reference standard" (a standard, generally having the highest metrological quality available at a given location or in a given organization, from which measurements made there are derived) and "working standard" (a standard that is used routinely to calibrate or check material measures, measuring instruments, or reference materials) are available.

Additional definitions that are relevant within this guideline include the following:

Equimolar-response immunoassay, *n* - For PSA, quantitates free PSA and PSA-ACT equivalently, i.e., reports *the same* value for solutions containing equal molar concentrations of free PSA or PSA-ACT. **Note**: Reported values for total PSA therefore depend only upon the sum of the molar concentrations of free PSA and PSA-ACT present in the samples.[13,19,20]

Skewed-response assay (nonequimolar), *n* - For PSA, detects free PSA and PSA-ACT differently, i.e., reports *different* values for solutions containing equal molar concentrations of free PSA or PSA-ACT.[13,19,20] **Note**: Values reported for total PSA in a sample by this assay are affected by multiple variables, including total molar concentration of PSA, relative proportion of the

two PSA-forms, molar response ratio of the assay to the two PSA-forms, and the PSA-form used to calibrate the assay.[19-21] The approximate degree of skewing can be estimated from these variables by a mathematically derived skewing diagram.[21]

4 Primary Standard of PSA: Preparation, Characterization, and Value Assignment

4.1 Description

A primary standard of PSA is derived by purification from human seminal fluid. Diluted solutions of the PSA primary standard can be used for calibration of equimolar response assays for total PSA and for assays for free PSA. They may also be used to calibrate reference equimolar response assays to assign values to serum-based secondary PSA standards (see Figure 1). Dilutions of the PSA primary standard and the PSA-ACT primary standard (Section 5) may be used to define the molar response ratio (to the two forms of PSA) of assays for total PSA.[13] Finally the PSA primary standard may be used in conjunction with the purified PSA-ACT primary standard in the preparation of a defined mixure composed of a proportionality of both PSA and PSA-ACT (see Section 6.2)

Methods for PSA purification that are described in the literature can produce PSAs of comparable quality.[5,22-24] The following procedure does not require monoclonal antibodies for purification.[5,23] This procedure is recommended by the PSA Workgroup of Standardization of PSA, which was sponsored by the American Cancer Society, the College of American Pathologists,[25] and the first Stanford Conference on the International Standardization of PSA (December 1992).

4.2 Preparation of a Seminal Fluid Pool from Semen

PSA that is suitable for a primary standard may be prepared from seminal fluid using the following procedure. Collect semen from donors who test negative for the human immunodeficiency virus (HIV), hepatitis B surface antigen (HbsAg), and the hepatitis C virus (HCV); allow it to liquify at room temperature for approximately 1 hour. Flash freeze this material in liquid nitrogen (or isopropanol and dry ice) and store it at -70 °C until further processing is required. After a cumulative target volume sufficient for a purification cycle is achieved (i.e., between 50 and 100 mL), thaw the samples in a water bath (approximately 20 °C), pool and centrifuge at approximately 10,000 x *g* for 20 minutes in a refrigerated centrifuge at 4 °C. Decant the supernatants (cheesecloth may be used) and remove the required number of aliquots from the pool for subsequent testing. Pools prepared by this method typically contain 0.5 to 1.0 mg of PSA/mL.

4.3 Purification of PSA from the Seminal Fluid Pool

PSA for the primary standard may be obtained from the seminal fluid pool using the modified purification protocol described below, which is based on previously published procedures.[5,23] Perform all operations at 2 to 6 °C. Exchange a pool of 50 to 100 mL of seminal fluid, processed as previously described, with Buffer A [10 mmol/L potassium phosphate (dibasic), adjusted to pH 6.9 with concentrated HCl] using conventional dialysis or with the aid of a commercially available rapid dialysis/concentrating device. Recover the retentate and introduce it onto a fresh CM-Sephadex C-50 (Pharmacia) column (2.5 x 40 cm) that was equilibrated previously with Buffer A. A fresh CM-Sephadex column should be poured for each purification cycle. Load the seminal plasma onto the column until the absorbance (at 280 nm) of the eluate falls below 0.1. Set up a 1-L gradient mixer containing 500 mL of Buffer A and 500 mL of Buffer B [0.1 mol/L potassium phosphate (dibasic), 0.5 mol/L NaCl, with pH adjusted to 6.9 using concentrated HCl] and start a linear gradient at a flow rate of approximately 1 mL/minute (fraction size 10 mL). PSA elutes as the first peak of a biphasic peak.[5] The fractions from the proximal to the distal inflection points of the PSA-containing peak should be pooled, typically a volume of 110 to 130 mL. Fractions containing PSA may be contained by subjecting aliquots to an immunoassay for PSA and to sodium dodecyl sulfate-polyacrylamide gel electrophoresis SDS-PAGE) to confirm that only a single band consistent with the molecular weight of PSA is present.

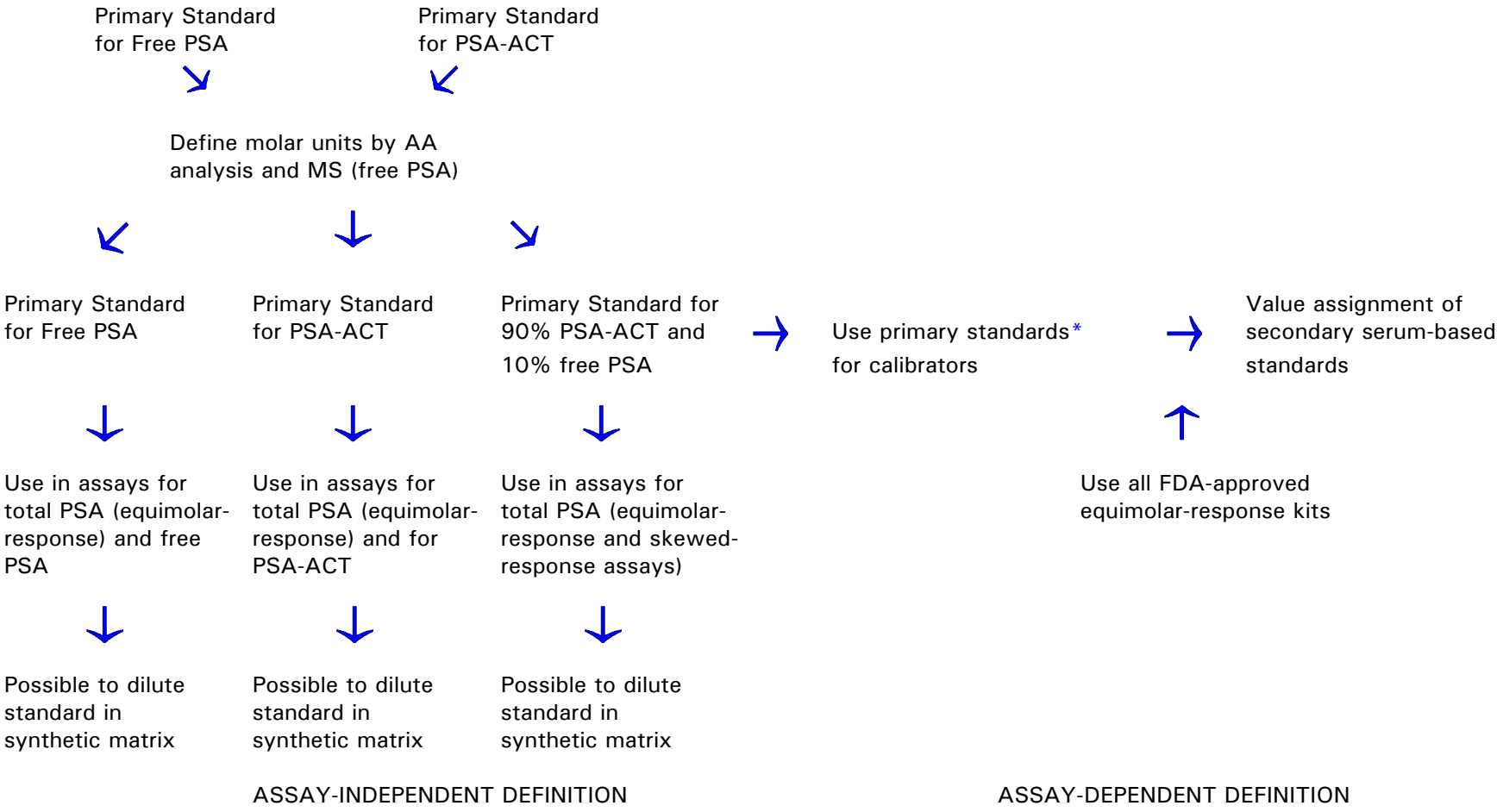

Figure 1. Options for use of primary standards in the production of secondary standards and calibration materials.

*Possible to use PSA primary standard, PSA-ACT primary standard, or PSA-ACT/PSA primary standard.

AA = amino acid.
ACT = alpha-1-antichymotrypsin.
MS = mass spectrometry.
PSA = prostate-specific antigen.

Reduce the volume of the pooled CM-Sephadex peak to approximately 10 mL either (1) using a rapid dialysis/concentration device, or (2) by precipitation with ammonium sulfate (90% saturation = 0.6 g/mL) overnight at 4 °C, followed by centrifugation and resuspension of the pellet in Buffer B. This crude PSA preparation can be stored as an ammonium sulfate precipitate at 4 °C, or it can be fractioned immediately on a molecular sieving column. The second column is a Sephacryl S-200 column (2.5 x 100 cm) (Pharmacia). The column should be tested for symmetry of flow and separation efficiency [height equivalent to a theoretical plate (HETP)] as recommended by the manufacturer. The column should then be equilibrated with Buffer B. PSA is eluted with Buffer B at a flow rate of approximately 0.5 mL/minute. Typically, PSA elutes from the Sephacryl S-200 column as a large peak (eluting molecular weight approximately 28 to 29 kDa) preceded by two small peaks.[5,23] Fractions containing PSA are pooled and the pooled volume (typically approximately 60 mL) is reduced as above to roughly 10 mL. As described previously, this pool can be stored as an ammonium sulfate precipitate. In the final step, the PSA pool is rechromatographed on the Sephacryl S-200 column as before and it elutes as a single large peak.

The PSA concentration in the pool can be estimated based on the absorbance at 280 nm ($A^{280}_{mg/mL,1\ cm}$ = 1.84).[6] Subject the pool to a buffer exchanged with 10mmole/L phosphate buffered saline, pH 7.4 (10 mM PBS, pH 7.4) [10 mmol/L sodium phosphate (dibasic), 0.15 mol/L NaCl, adjusted to pH 7.4 using concentrated HCl] and concentrate it to a value greater than 1 mg/mL (based on absorbance at 280 nm). Then flash freeze the pool and store it at –70 °C.

Repeat this previously described purification procedure using additional seminal plasma pools until the targeted amount of purified PSA is accumulated. Before pooling each preparation, evaluate the lot-to-lot variability of the immunological activity by comparing the concentration (defined by absorbance at 280 nm or quantitative amino acid analysis) with the results of each Food and Drug Administration (FDA)-approved assay for PSA. This last step is included to ensure that the immunoreactivity of various epitopes is reproducible from lot-to-lot during each performance of the PSA purification procedure.

Note: Since the subcommittee first convened, knowledge of another prostate-specific protein—human glandular kallikrein (hGK-1, hK2)—has greatly increased. hK2 is now known to be highly homologous to PSA, and is a potential contaminant of PSA preparations. However, application of an immunoassay that is specific for hK2, has indicated that the level of hK2 contamination in PSA preparations obtained by the reference purification procedure is ≤1%.[26] More recent work has indicated that the hK2 content of these preparations is ≤0.2% (unpublished observations).

4.4 Pooling

Once sufficient PSA has been obtained, thaw the pools in a water bath at 20 °C and consolidate the pools into a grand stock pool. Centrifuge this stock pool (10,000 x *g* for 15 minutes); decant the supernatant and filter it through a 0.22-μm filter under sterile conditions. Estimate the PSA concentration of the pool based on the absorbance at 280 nm. Flash freeze the stock pool in desiccation-resistant storage vessels and maintain them at –70 °C.[27]

4.5 Ensuring Sterility

The PSA stock material should be cultured on media that are appropriate for ensuring sterility.

4.6 Biochemical Evaluation

The PSA stock material should be biochemically evaluated and it should meet certain criteria for acceptance as a primary standard. Following are descriptions of the tests and criteria used.

4.6.1 SDS-PAGE

Subject a sample to SDS-PAGE under both reducing and nonreducing conditions, stain it with Coomassie Blue, destain it, and scan at a wavelength of 540 nm. The nonreduced gel should display a band that corresponds to PSA (apparent molecular weight on SDS-PAGE of approximately 33,000). Moreover, this band should account for >95% of the total absorbance.

NOTE: SDS-PAGE of PSA under reducing conditions generally results in multiple bands, which occur from internal cleavages in the polypeptide chain. A portion of the molecule is clipped, and the fragments are held together by internal disulfide bonds.

4.6.2 High-Pressure Liquid Chromatography (HPLC)

Subject a sample to size-exclusion HPLC analysis under nonreducing conditions. It should exhibit a single major chromatographic peak, as detected by total ion current or by absorbance at 280 nm.

4.6.3 HPLC Mass Spectrometry

Using an appropriate protein standard for calibration, subject a sample to HPLC mass spectrometry under nonreducing conditions. Several peaks will be observed, presumably due to variations in glycosylation of the PSA molecule. Studies indicate that the major PSA peak should occur at a molecular weight of approximately 28,430.[7]

4.6.4 Amino Acid Analysis

Subject a sample to quantitative amino acid analysis using vapor-phase acid hydrolysis of PSA at 24, 48, and 72 hours (using reference purified amino acids for calibration). The theoretical amino acid sequence of 237 amino acids (deduced by Lundwall and Lilja from cDNA[4] and Schaller, et al., from direct protein sequencing[28]) should be used for comparison. The molar ratios of the PSA samples should closely correspond to the molar ratios of the amino acids in the theoretical sequence.

4.6.5 N-Terminal Sequence Analysis

Subject a sample to N-terminal sequence analysis. The following N-terminal sequence should be present:

I-V-G-G-W-E-C-E-K-H-S-Q-P-W-Q-V-L-V-A-S.[4,23,28]

Another sequence, due to cleavage at an internal lysine-lysine bond, can also be detected:

K-L-Q-C-V-Q-L-H-V-I-S-N-D-V-C-A-Q-V-H-P.[4,23,28]

4.6.6 Enzyme Activity

Based on sequence homology, PSA belongs to the kallikrein family of serine proteases. Its major enzymatic activity is chymotrypsin-like,[8,9,12] but it also possesses a minor amount of trypsin-like or kallikrein-like activity.[10] Therefore, it is appropriate to subject the candidate PSA stock material to studies of enzymatic activity using a synthetic chymo-trypsin-like peptide substrate, such as MeO-Suc-Arg-Pro-Tyr-pNA (MeO-Suc = methoxy-succinyl; Suc = succinyl; Arg = Arginine; Pro = Proline; Tyr = Tyrosine; pNA = paranitroanilide) (Kabi Pharmacia Hepar, Inc.).[12] Also, an alternative chymotryptic-like substrate, Suc-Phe-Leu-Phe-SBzl (Phe = phenylanine; Leu = leucine; SBzl = thiobenzyl ester) is described for PSA, and it can be used.[10] The candidate PSA material should be analyzed for its trypsin-like activity using a synthetic, trypsin-like peptide substrate, such as H-D-Pro-Phe-Arg-pNA[29] or Z-Trp-Arg-SBzl (Z = benzyloxycar-bonyl; Trp = tryptophan).[10] The enzymatic ac-tivity may be expressed as micromoles of substrate cleaved per minute multiplied by micromoles of PSA at 37 °C.

The percentage of enzymatically active PSA can be determined by size exclusion chromatography [liquid chromatography (LC) or HPLC)] of an aliquot subjected to complexation with ACT (see Section 5.3). The area (absorbance 280) of the peak that corresponds to free PSA is compared to the area of a matched control (ACT absent) that was identically chromatographed. The difference in areas is attributed to the PSA-ACT complex formation.

4.7 Assignment of the Reference PSA Concentration and Absorption Coefficient

Once the stock PSA material has met the previously described biochemical criteria and is accepted as a primary standard, assign a reference concentration based on quantitative amino acid analysis.[6] Standard reference solutions of purified amino acids of known concentration are used for calibration of the amino acid analyzer. The reference con-centration of the primary standard should be based on replicate runs (minimum of three) at several sites (minimum of three), and each run should include at least one pertinent control. Vapor-phase acid hydrolysis is a commonly

recommended method that can be used for this analysis.

Calculate the molar concentration of each residue in the undiluted primary standard from the averages of the amino acid analysis data. Based on the concentration of each residue, calculate a corresponding PSA molar concentration (mol/L) by dividing the determined residue concentration by its molar ratio from the theoretical sequence.[4,28] The average value, based on those amino acid residues with molar ratios within 10% of the theoretical composition, is the reference value that should be assigned to the primary standard. To obtain a primary standard con-centration in milligrams per milliliter (mg/mL), rather than in amount of substance (mol/L), the reference value should be corrected by the ratio of the average weight of glycosylated PSA determined by mass spectrometry (28,430)[7] compared to the calculated weight of the theoretical sequence without the carbohydrate moiety (26,079).[28]

The absorbance at 280 nm of the primary standard should be determined by at least three different reference laboratories. The primary standard should be diluted in the same manner by each laboratory into the linear range of the spectrophotometer (e.g., between approximately 0.1 and 0.7 absorbance units) before determination. Appropriate light scatter corrections and corrections for diluent (e.g., dual beam spectrophotometers) should be used.[30] The average absorbance of the primary standard and the average concentration of the primary standard can be used to calculate an absorption coefficient at 280 nm for the primary standard. This value can be applicable to quantify the concentration of future preparations of PSA after demonstration of equivalent purity.

4.8 Aliquoting and Storage of PSA Primary Standards

Following the establishment of an absorptivity at 280 nm for PSA, thaw the stock material (in a water bath at 20 °C) and dilute it in sterile, dibasic-buffered saline (0.01 mol/L phosphate, 0.15 mol/L NaCl, pH 7.4) to a final concentration of 35.2 μmol/L (1 mg/mL PSA mass equivalent). Aliquot it into cryostorage vials that are impervious to desiccation; the contents should be flash frozen and stored frozen at –70 °C. The concentration assigned to the final PSA primary standard should be confirmed by spectrophotometric measurement by at least three different laboratories, as previously noted.

Recent stability studies indicate that the free form of PSA purified from seminal plasma has excellent stability both at –20 °C and 4 °C. In these studies, the immunoreactivity of free PSA was maintained in equimolar-response types of assays for total PSA as well as assays for free PSA.[31]

The preferred mode of storage of the PSA primary standard is as lyophilized powder. The PSA stock material (1 mg/mL) should be thawed in a water bath at 20 °C, then cooled to 4 °C and pooled. It is important to save aliquots of this PSA stock material for production of the 90:10 primary standard described in Section 6. The PSA stock should then be diluted in a reagent previously sterilized by ultrafiltration through a 0.22 μm membrane; the reagent consists of 1% bovine serum albumin (BSA) in 20 mmoles/L phosphate buffered saline, pH 7.4 (20 mM PBS, pH 7.4) [20 mmol/L sodium phosphate (dibasic), 0.15 mol/L NaCl, adjusted to pH 7.4 using concentrated HCl.] The PSA stock is diluted to a final PSA concentration of 500 ng/mL. The mixture containing the diluted PSA is refiltered through a 0.22 μm filter. Sections 6.3 and 6.3.1 describe general procedures for the aliquoting, lyophilization, and reconstitution of this material.

5 Primary Standard of PSA-ACT: Preparation, Characterization, and Value Assignment

Human ACT is a member of the superfamily of proteins referred to as serpins (serine protease inhibitors). ACT is synthesized in the liver and is one of the acute phase reactants. Serum levels rise five-fold or more in response to a wide variety of injuries. Methods of purifying this molecule to homogeneity from human plasma are well described.[32,33] The native molecule is a glycoprotein with a molecular weight between 58,000 and 66,000 with a peptide backbone of 398 amino acids and an M_r of 45,031.[34] Approximately 22 to 30% of the molecule by weight is carbohydrate. The gene for human ACT has been cloned and sequenced.[35]

5.1 Description

The PSA-ACT primary standard is a purified reference material with a biochemically determined concentration. The PSA-ACT complex is formed by incubating the PSA from the same batch of well-characterized bulk PSA used to make the PSA primary standard (Section 4.6) with purified ACT. Diluted solutions of the PSA-ACT primary standard can be used as calibrators for assays for PSA-ACT and for total PSA (equimolar-response assays). Moreover, they may be used as calibrators to assign values to serum-based secondary materials by use of equimolar-response reference assays (see Figure 1). Both the PSA and PSA-ACT primary standards can also be used directly as secondary standards when diluted in a defined synthetic matrix (see Figure 1).

5.2 Preparation and Characterization of Purified Human ACT

ACT is prepared by modifying the procedure described by Siddiqui et al.[33] Commercially available ACT has demonstrated purity of greater than 95% by SDS-PAGE, and it can be reacted with PSA primary standard to form a PSA-ACT complex. In the event that a different purification method for human ACT is adopted by the World Health Organization (WHO) for creation of a primary ACT reference material, this section may be revised in accordance with the new WHO guidelines. Revision of the procedure described here should not be adopted until the ACT prepared by WHO procedures is verified by enzymatic and immunologic methods to be fully equivalent to the purified ACT described in this section.

The Protein Standards Committee of the International Federation of Clinical Chemistry (IFCC) has recently assigned a concentration value for ACT to RPPHS/CRM-470. In the process, different purification methods were evaluated, especially as related to purity and native state of the ACT produced. The report of the committee, prepared by Prof. J. Whiches, is available from the Bureau Communitaire de Réference (Brussels, Belgium).

5.2.1 Isolation of ACT from Human Plasma

A modified method for large-scale purification of purified ACT from human plasma has been developed.[33] Outdated human plasma shown to be negative for HIV, HBsAg, and HCV by FDA-required tests is stored at 4 °C until use. All operations are performed at 4 °C. A pool of 10 L of plasma from approximately 50 donors, regardless of their blood type or gender, is processed according to the procedure described below.

(1) Ammonium Sulfate Precipitation

Fractionate the pooled plasma by ammonium sulfate precipitation with the addition of 0.209 g of solid ammonium sulfate per milliliter of plasma; the final saturation should be 35%. Stir this suspension for 60 minutes at 4 °C and centrifuge it at 12,300 x *g* for 20 minutes. Make the supernatant 75% saturated by the addition of 0.275 g of solid ammonium sulfate per milliliter; stir and centrifuge as before. Dissolve the resulting 35 to 75% ammonium sulfate-precipitated fraction in Buffer C [20 mmol/L sodium phosphate buffer (dibasic), adjusted to pH 6.8 using HCl] and dialyze it against the same buffer using a Pellicon cassette filter membrane system (Millipore) equipped with a PTGC 10,000 MW-cutoff membrane or similar system.

(2) Ion-Exchange Chromatography

Filter the dialyzed sample; adjust the final volume to that of the original plasma pool. Apply it at 4 L per hour to an Amicon P180 x 500 acrylic column packed with 8 L of 20m Silica PAE-1000. Wash the unbound proteins with Buffer C. Elute ACT with a 40-L gradient of 0 to 0.35 mol NaCl/L in Buffer C. Collect 500-mL fractions. Then, analyze the fractions for ACT using an immunoassay for human ACT. Pool the fractions containing ACT and dialyze the pool against Buffer C.

(3) Affinity Chromatography

Apply the dialyzed sample from the previous step at 100 mL per hour to a 1-L bed volume of DNA-Sepharose CL 4B in an XK 50 column (Pharmacia). This DNA affinity matrix can be prepared according to the method described by Arndt-Jovin.[36] Wash the column with Buffer C. Elute ACT with Buffer C containing 0.35 mol NaCl/L. Collect 25-mL fractions and assay them for ACT activity by determining inhibition of cathepsin G enzymatic activity. Pool ACT-enriched fractions and concentrate them using a

PM10 (Amicon) membrane in a stirred cell concentrator.

(4) Gel Filtration

Divide the concentrated sample mentioned in step 3 into three equal aliquots and apply them to an XK50 column packed with 2 L of Sephacryl S300 HR and attached to the Gradifrac system (Pharmacia). Equilibrate the column with 20 mmol/L (hydroxymethyl) aminomethane, pH 7.8 (Tris, pH 7.8) [Trizma ®base in water, adjusted to pH 7.8 with HCl, concentration adjusted to 20 mmol/L with additional water and sodium azide added to 1g/mL]. (Tris) buffer (adjusted to pH 7.8 using concentrated HCl), and 0.1% sodium azide and operate at 100 mL/hour. Collect 10mL fractions.

The major peak from this column contains ACT; pool it, divide it into 60-mg batches, and store it at –70 °C until the next step is carried out.

(5) FPLC, Ion-Exchange Chromatography

Remove each frozen aliquot (from the previous step) from storage at –70 °C as needed and thaw at 4 °C (in an ice bath). Apply the sample to a MonoQ 10/10 column (Pharmacia) equilibrated with Tris, pH 7.8 using an FPLC system. Apply the sample with an auto-injector at 2 mL/minute and elute with a 0 to 40% NaCl gradient in Tris, pH 7.8 for 30 minutes. Use the major symmetrical peak containing pure ACT immediately or store it at –20 °C until needed.

5.2.2 Biochemical Evaluation of Purified ACT

The following procedure details how to perform a biochemical evaluation of purified ACT:

(1) Concentration Determination

Estimate the concentration of the purified ACT based on the absorbance:[37,38]

$$A^{280}_{\text{mg/mL,1 cm}} = 0.6$$

(2) Gel Electrophoresis

Recover a separate 5- to 10-μg aliquot of ACT that has been stored under similar conditions as the other aliquots; subject it to 12% SDS-PAGE under reducing conditions, using the method of Laemmli et al.[39] Stain the gel using Coomassie Blue R 250. A visual examination of the gel should account for greater than 95% purity, based on the stained ACT band with the approximate molecular weight of 64 kDa.

(3) Immunoelectrophoresis

Apply an aliquot of ACT (5 to 10 μg) in 4 μL of buffer to a 1%-agarose gel with multiple wells and electrophorese it.[40] Apply the antiserum to whole human serum and the antiserum to human ACT to separate troughs. Incubate the plate at room temperature for 48 hours before staining it with Coomassie Blue R250; follow this by subsequent destaining. Upon visual examination of the plate, only a single arc is precipitated with each of these antisera and both arcs are aligned in the same position along the troughs on the plate. Also, the ACT preparation should be tested and shown to be nonreactive with antisera specific for other plasma protease inhibitors, including alpha-1-antitrypsin, alpha-2-macroglobulin, alpha-2-antiplasmin, and antithrombin III.

(4) Absence of Inhibitors to Other Enzymes

The inhibitory activity of ACT is determined with respect to cathepsin G from human neutrophils and human pancreatic chymotrypsin (Athens Research and Technology). Active site titration of chymotrypsin is determined using the method of Schonbaum et al;[41] cathepsin G activity is determined using titrated alpha-1-antitrypsin.[42] One suggested assay utilizing an equal molar ratio of enzyme and inhibitor with Suc-Ala-Ala-Pro-Phe-pNA as substrate, at pH 8.0, can be used for both enzymes. Add an increasing amount of inhibitor to the assay system until 100% inhibition is achieved. Then, calculate the percent of inhibitory activity based on the ratio of enzyme to ACT.

Using this assay system, the activity of the purified ACT is greater than 90%. Also, the ACT preparation is assayed to ensure the absence of inhibitors to trypsin, thrombin, and plasmin, using their specific substrates to rule out possible contamination with alpha-1-antitrypsin, alpha-2-macroglobulin, antithrombin III, and alpha-2-antiplasmin, respectively. Pure preparations of ACT do not inhibit these three enzymes.

5.3 Preparation of Purified PSA-ACT Complex

PSA-ACT is prepared using the method of Chen et al.[43] Typically, 2.5 mg of PSA [0.68 mL of a stock PSA primary standard at 3.7 mg/mL in 10 mM PBS, pH 7.4 is incubated with 10 mg of ACT (3.7 mL of a stock ACT at 2.7 mg/mL in 20 mmol/L Tris-HCl, 0.15 mol/L NaCl, adjusted to pH 7.4 using concentrated HCl) at 37 °C for 3 hours. This results in a molar ratio of PSA to ACT of approximately 1:1.7. Then, concentrate the reaction mixture to 0.8 mL by ultrafiltration [PM or YM 10 membranes (Amicon)]. Apply the concentrated reaction mixture (0.8 mL) to a Sephacryl S-200 column (1.5 x 92 cm) that has been previously equilibrated with acetate buffered saline, pH 5.6 (ABS, pH 5.6) [prepared from 0.2M sodium acetate and 0.2M acetic acid, blended to pH 5.6, diluted with NaCl solution to a final concentration of 10 mmoles/L acetate in 0.15 mole/L NaCl]. Alternatively, a Superdex 200 resin may be used to enhance the resolution of peak separation. Develop the column at a flow rate of 0.1 mL/minute and collect the fractions (0.7 mL). Separate two peaks, with the first asymmetrical peak containing PSA-ACT complex plus excess ACT.

The second peak contains free PSA, with both residual enzymatically reactive and nonreactive free PSA forms present.[17] The elution profile of free and complexed PSA is determined by any PSA immunoassay that recognizes both forms of PSA. Those fractions that contain the PSA-ACT complexed peak (approximately 20 fractions) should be pooled and concentrated by diafiltration to 1 mL. To decrease the concentration of salt, add 4 mL of 10 mmol/L sodium acetate buffer, pH 5.6 (Ac, pH 5.6) [prepared from 0.2M sodium acetate and 0.2M acetic acid blended to pH 5.6 and diluted with water to a final concentration of 10 mmoles/L acetate] to the concentrated material and reconcentrate the mixture to 1 mL by ultrafiltration.

Removal of unreacted ACT from the PSA-ACT complex is accomplished by applying the 1-mL sample to a column of CM-cellulose (1 x 18 cm) equilibrated and washed with Ac, pH 5.6 at a flow rate of 0.15 mL/minute. The free ACT at pH 5.6 is not bound to the CM-cellulose and passes through the column. Then, elute the column with a linear gradient of sodium chloride, from zero to 1 mol/L in Ac, pH 5.6. Collect the full gradient (encompassed in ~60 mL) in 40 fractions of 1.5 mL each. A single peak, PSA-ACT complex elutes at about 0.r mol/L NaCl. Add additional Ac, pH 5.6 to the PSA-ACT fraction to bring the final salt content to 0.15 mol/L NaCl. Dilute the PSA-ACT complex to approximately 1 to 2 mg/mL as determined using absorbance at 280 nm. Each in-process lot should be stored in cryostorage vials that are resistant to desiccation and frozen at –70 °C. For short-term storage, PSA-ACT that is "in process" should be kept at 4 °C. To eliminate the possibility of complex dissociation, “in-process” PSA-ACT should be kept in an ice bath when removed from refrigeration.

Repeat this purification procedure, as described previously, using the same lots of PSA primary standard and ACT, until the targeted amount of PSA-ACT has been purified. Before pooling each preparation, evaluate the lot-to-lot variability of the immunological activity by comparing the concentration (defined by absorbance at 280 nm or quantitative amino acid analysis) with the results of each FDA-approved assay for PSA. This step is included to ensure that the immunoreactivity of various epitopes is reproducible from lot to lot during performance of the PSA-ACT purification procedure.

5.4 Pooling

Once sufficient PSA-ACT has been obtained, thaw the individual PSA-ACT pools in a water bath at 20 °C and consolidate them into a grand stock pool. Thawing should be carefully monitored to minimize excessive warming. Thawed PSA-ACT should be maintained in an ice bath when removed from refrigeration to eliminate the possiblity of complex dissociation. Centrifuge this stock pool (10,000 x *g* for 15 minutes), decant the supernatant, and filter it through a 0.22-μm filter under sterile conditions. Estimate the concentration of PSA-ACT in the pool based on absorbance at 280 nm. Following the submission of aliquots for biochemical analysis, flash freeze the stock pool in aliquots in a desiccation-resistant cryo-storage vial and store at –70 °C.[27] Avoid repeated freeze thaws of this material. The preferred long-term storage mode is as a lyophilized powder (see Section 6.3.1).

5.5 Ensuring Sterility

The PSA-ACT stock material should be cultured using media that are appropriate for ensuring sterility.

5.6 Biochemical Evaluation

The PSA-ACT stock material should be biochemically evaluated and it should meet certain criteria for acceptance as an RM.

5.6.1 SDS-PAGE and Western Blot

Subject the concentrated PSA-ACT fraction to SDS-PAGE (reducing and nonreducing conditions), use Coomassie Blue for visualization, then perform the Western blot using antibodies to PSA and to ACT. This evaluation is for qualitative and semiquantitative purposes, and it is used to confirm that the major band at 95 kDa contains both PSA and ACT. Western blots of pure ACT and pure free PSA should be run as controls. This preparation should be >95% pure complex by both protein and immunostaining techniques.

5.6.2 Enzyme Activity

Test the PSA-ACT stock material for enzymatic activity using a synthetic, chymotrypsin-like peptide and a synthetic, trypsin-like substrate, as previously noted in Section 4.6.6. The enzymatic activity of the PSA-ACT stock should be less than 5% of the activity of the parent PSA preparation. This will ensure that the PSA-ACT preparation is free of significant contamination with enzymatically active free PSA.

5.6.3 Amino Acid Analysis

Following demonstrated purity, submit a sample of PSA-ACT for quantitative amino acid analysis using vapor-phase acid hydrolysis at 24, 48, and 72 hours. Use reference purified amino acids for calibration. The concentration of the PSA-ACT stock material should be based on replicate runs (a minimum of three) at several sites (a minimum of three) and each run should include at least one pertinent control. The total number of residues for each amino acid (which is stable during analysis) per mole of complex should closely correspond to the sum of the known amino acid sequences for both PSA and ACT because they are known to react in equimolar ratios. Based on the theoretical sequence data (including generation of possible peptide fragments from cleavage of ACT), a molar concentration of the PSA-ACT stock material may be determined as described in Section 4.7. The molecular mass of the PSA-ACT complex has been assigned by calculating the mass of the peptide moiety of ACT (based on the cDNA sequence coding for 398 amino acids), adding 26% for the carbohydrate moiety, and 28,430 for the mass of PSA.[7,43] This yields a calculated mass for the PSA-ACT complex of 89,280.[43] The concentration of PSA-ACT in mg/mL may be converted to a PSA equivalent concentration [in milligrams per milliliter (mg/mL)] by multiplying the PSA-ACT concentration by the ratio of their molecular masses (28,430/89,280 or 0.318).

The absorbance of the PSA-ACT stock material should be determined by at least three different reference laboratories. The stock material should be diluted in the same manner by each laboratory into the linear range of the spectrophotometer (e.g., between approximately 0.1 and 0.7 absorbance units) before determination. Appropriate light scatter corrections and corrections for diluent (reference blanking) should be used [30]

The average absorbance and the average concentration of the PSA-ACT stock will be used to assign an absorptivity at 280 nm. This value has been previously determined to be 0.99 ± 0.05 for a 1 mg/mL solution of PSA-ACT. This value was based on amino acid analysis of 12 different lots of PSA-ACT.[43] The absorption coefficient is useful in quantifying the concentration of future preparations of PSA-ACT, after demonstration of equivalent purity.

5.6.4 Independent Confirmation of Molar Equivalence of PSA and PSA-ACT Standards

The molar equivalence of PSA in the two standards (PSA and PSA-ACT primary standards) should be independently confirmed. This can be performed most simply by testing solutions of PSA and PSA complexed to ACT, where the PSA molar concentration of each solution is determined by identical volumetric dilution from stock PSA primary standard solution of a known concentration. Identical dilutions of both solutions are tested by all current FDA-approved PSA assays. The ratios of free and complexed PSA in the complexed solution are determined by an assay for free PSA and an assay for total

PSA. This independent test allows identification of significant discrepancies in value assignment (i.e., >5%) to the two primary PSA standards (PSA and PSA-ACT primary standards).

5.7 Aliquoting and Storage of PSA-ACT Primary Standards

Following the establishment of a reference ultraviolet (UV) spectrophotometric absorptivity at 280 nm for the PSA-ACT complex, thaw the stock PSA-ACT material (in a water bath at 20 °C) and dilute it in Ac, pH 5.6 to a final concentration of 35.2 μmol/L (1 mg/mL PSA mass equivalent). The mixture containing diluted reference protein is subjected to ultrafiltration under sterile conditions through a 0.22 μm filter. To minimize excessive warming, thawing should be carefully monitored. To eliminate the possibility of complex dissociation, thawed PSA-ACT should be maintained in an ice bath when removed from the freezer. Prepare aliquots in cryostorage vials that are impervious to desiccation, and flash freeze and store them frozen at −70 °C. Confirm the concentration assigned to the final PSA-ACT primary standard by an absorbance measurement at 280 nm obtained by at least three different laboratories, as previously noted.

Recent studies indicate that PSA-ACT complex tends to dissociate during prolonged storage in aqueous solution. This instability can be counteracted by storing the PSA-ACT at low temperatures at a pH of between 7.8 and 7.4, and by the addition of a 10–1000-fold molar excess of native ACT.[31] Additional studies, however, have shown that PSA-ACT complex in aqueous solution is stable with regard to dissociation when maintained at 4 °C and that it will resist dissociation for at least 8 hours at 45 °C. Ongoing accelerated, real-time and temperature studies have shown that PSA-ACT/PSA standards (currently stored for 4 months at 25 °C) lyophilized in 1% BSA in 20 mM PBS, pH 7.4 show no dissociated free PSA when tested within 8 hours of reconstitution (Dr. Zuxiong Chen, personal observations). These results suggest that when working with reconstituted PSA-ACT standards, materials should be used within 1 working day following reconstitution or else maintained under refrigeration when not in use. Further studies of the stability of the pure PSA-ACT complex and mixtures of free PSA and PSA-ACT are recommended.

The preferred mode of storage of the PSA-ACT primary standard is as a lyophilized powder. The PSA-ACT stock material (1 mg/mL, PSA mass equivalent) should be thawed in a water bath at 20 °C (as described above), then cooled to 4 °C and pooled. Aliquots of this PSA-ACT stock material (1 mg/mL, PSA mass equivalent) should be saved for production of the 90:10 primary standard described in Section 6. The PSA-ACT stock should then be diluted in 1% BSA in 20 mM PBS, pH 7.4, to a final concentration of 500 ng/mL (PSA nanogram equivalents). The molar equivalence of this PSA-ACT primary standard and the PSA primary standard (Section 4) should be confirmed as described in Section 6.2.1. Refer to Section 6.3 and 6.3.1 for general procedures for the aliquoting, lyophilization, and reconstitution of this material.

6 Primary Standard of 90% PSA-ACT and 10% PSA: Preparation and Value Assignment

Calibration of assays for total PSA using a mixture of 90% PSA-ACT and 10% PSA (90:10) has been shown to minimize differences in PSA measurements between assays with differing formats and molar response ratios to the two forms of PSA.[43] This 90:10 mixture represents the average proportion of PSA-ACT and PSA in sera of patients with cancer of the prostate.[44]

6.1 Description

A primary standard composed of 90% PSA-ACT and 10% PSA (molar percentages) can be used either independently or in parallel with the PSA and PSA-ACT primary standards for calibration. Diluted solutions of this 90:10 primary standard can be used to calibrate PSA assays that measure total PSA. They may also be used to calibrate reference equimolar-response assays to assign values to serum-based secondary PSA standards (see Figure 1).

6.2 Preparation and Value Assignment of the 90:10 Primary Standard

The 90:10 primary standard is formed by combining the appropriate volumetric proportions of purified PSA-ACT and PSA from solutions containing equal molar concentrations of PSA.

This standard is prepared from the same unblended materials as used in the preparation of the PSA and PSA-ACT primary standards (Sections 4.8 and 5.7). The purification methods and concentration assignments are described in previous sections of this document (PSA in Section 4 and PSA-ACT in Section 5). The 90:10 primary standard is prepared in the same buffer used for dilution of the other primary standards, consisting of 20 mmol/L PBS buffer, pH 7.4, with 1% BSA. The standard is stored at -70 °C in a lyophilized state and has a final concentration of 500 ng/mL (PSA nanogram equivalents) after it is reconstituted with 2 mL of H_2O.

6.2.1 Confirmation of Molar Equivalence of PSA, PSA-ACT and 90:10 Primary Standards

The molar equivalence of the PSA-ACT and the PSA primary standard dilutions should be confirmed. This is done by testing the two preparations using an FDA-approved PSA assay that measures PSA and PSA-ACT with an equimolar response. The assay chosen for this testing should be known to have an equimolar response to PSA and PSA-ACT before testing. An assay specific for free PSA is used to confirm that no free PSA exists in the preparation of PSA-ACT.

In a similar fashion, the molar equivalence of the final concentrations of the 90:10 primary standard and the primary standards for free PSA (Section 4.8) and PSA-ACT (Section 5.7) should be confirmed by an FDA-approved equimolar-response PSA assay. The final concentration of each should be 500 ng/mL (PSA nanogram equivalents) before aliquoting, lyophilization, and storage as described in the following sections.

6.3 Aliquoting and Storage of the Primary Standards

The primary standards are separated into 2-mL aliquots and lyophilized for long-term storage. Lyophilization takes into account the gravimetric mass of the standard. The standards can be restored to a concentration of 500 ng/mL (PSA nanogram equivalents) by addition of a volumetrically determined 2 mL of distilled H_2O. For long-term storage, the lyophilized standards should be frozen at –70 °C.

6.3.1 Lyophilization and Reconstitution of the Materials

Lyophilized standards are prepared and filled based on weight. The specific gravity (*g*/mL) of the diluent used for dilution of the three standards should be determined. This is accomplished by weighing multiple aliquots of a fixed volume of diluent on a calibrated balance with accuracy to three decimal places. Fill volumes are determined using calibrated Class A pipets. Before final lyophilization, an additional weight factor is determined to account for the displacement volume of the dry solids in the matrix diluent. This represents an additional 4% of the target fill volume, based on the specific gravity of 1.025 for the 1% BSA, 20-mmol PBS buffer used as a diluent for the primary standards.

6.3.2 Establishing a Sampling Pattern

After the filling equipment is calibrated, a sampling pattern is established to verify the uniformity of filling according to accepted tolerance specifications (± 1% of the target weight). Final set up of filling equipment is performed by adjusting to the target mass (*g*) as determined by multiplying the desired fill volume (adjusted for the displacement of dry solids) by the specific gravity. To account for freezing cycles, drying temperatures, and pressure evacuation, lyophilization is performed under controlled conditions. After freeze drying, sampling is performed to verify uniformity of drying by assessing residual moisture content via Karl Fischer titration.

Vials are reconstituted with distilled water to the initial target fill volume (without adjustment for volume of solids).

7 Summary and Next Steps

I/LA19-A describes the purification, characterization, biochemical definition, and storage of three primary standards: PSA, PSA-ACT, and a mixture of 90% PSA-ACT with 10% free PSA (molar percentages). Additional standards can be prepared using the primary standards. For example, the primary standards may be diluted in defined synthetic matrices to concentrations representative of clinical specimens. These dilutions may then be used for calibration of assays for free PSA, PSA-ACT, or total PSA (see

Figure 1). Different primary standards may be better suited for calibration of certain types of assays, e.g., the PSA primary standard for free PSA assays,[31] or the 90:10 primary standard for skewed-response assays for total PSA.[21, 43] The use of dilutions of the primary standards in various types of assays is referred to as assay-independent standardization.

Alternatively, the primary standards can be used to calibrate FDA-approved equimolar-response kits for the transfer of value to PSA-containing serum pools with ratios of PSA and PSA-ACT representative of the range of clinical samples (assay-dependent standardization). Figure 1 presents options for the production of secondary standards with value assignment by either assay-independent (reference material) or assay-dependent (reference assay plus reference material) methods.

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Summary of Comments and Subcommittee Responses

I/LA19-P: *Purification and Characterization of Prostate Specific Antigen (PSA) and PSA Complexed to Alpha-1-Antichymotrypsin (PSA-ACT) for Use as Primary Standards*; *Proposed Guideline*

General Comments

1. The procedure and techniques presented for isolation and preparation of both PSA and PSA-ACT seem both clear and adequate for manufacturers. This standard is important for manufacturers to implement and it is designed to eliminate one source of the discordant PSA values between different assays.

   It is in the best interest of patient care to have manufacturer's evaluate and publish how their assay is standardized. Does their assay respond equivalently to both forms of PSA? This is important if PSA is to be used as a screening test. One commercial assay has a 1:1 molar equivalency between these two forms of PSA. Some of the other FDA-approved assays do not have 1:1 molar equivalency.

- **Although the subcommittee believes that information on assay standardization is important, requiring that this information be published by manufacturers is not within the purview of NCCLS. Mandating this practice is a regulatory issue.**

  Note that the development and characterization of PSA and PSA-ACT primary standards of equivalent molarity, as described in this guideline, will help manufacturers determine the molar response ratios of their assays to the two forms of PSA.

2. This is a good and timely guideline. The guideline should consider future possibilities, such as PSA-alpha-2-macro globulin complex (which is believed to be inert).

- **The subcommittee believes that adding a discussion of PSA-alpha-2-macro globulin complex is not appropriate at this time. However, the guideline is expanded to include a new Section 6, which addresses the preparation of a primary calibrator.**

3. I believe that there are a few technical points (extinction coefficients, etc.) that are now available. If possible, these should now be added.

- **Section 4.7 is revised to include supporting references.**

4. Please provide better directions for the preparation of buffers used in the document and, where applicable, indicate how the pH can be adjusted to the required value.

- **Additional information was added to the buffer descriptions as part of the revision process.**

5. The document uses both the antiquated term “optical density” and the correct term “absorbance” rather loosely throughout the document.

- **Appropriate changes have been made throughout the guideline.**

6. I suggest that the following disclaimer be added to anticipate the eventuality that a different method of ACT purification is accepted by WHO: "In the event that a different purification method for human ACT is adopted by WHO for the creation of a primary ACT reference material, this section (describing purification and characterization of ACT) will be revised in accordance with new WHO guidelines."

- **The subcommittee revised Section 5.2 but cautions that the ACT prepared by an alternate procedure should be verified by enzymatic and immunologic methods to be fully equivalent to that of the ACT described in this guideline.**

7. There is some concern that the procedures for purification of ACT are "antiquated." Is there another procedure that is well documented and "newer"?

- **See the response to Comment 6.**

8. NCCLS document I/LA19-P is a proposed national guideline for the production and characterization of PSA standards. This is needed not only for regulation of proficiency testing but also for good laboratory practices. The document meets these needs and will allow inter-laboratory and inter- and intra-manufacturer comparisons. Production and characterization of these standards is tedious and time consuming, and thus expensive. Improved and simpler methods of producing the standards may be possible, and NCCLS should not be so dogmatic about the production of these products. Characterization must be vigorous. Some terms need to be defined in this document. Otherwise, this is a good start to enlightening a gray area.

- **This document was developed as an NCCLS guideline and is intended to provide guidance based on proven methods. The subcommittee recommends that modifications to the methods be verified because of the potential differences in assay endpoints.**

9. This document describes procedures for the purification, characterization, and concentration value assignment for PSA and for the complex of PSA with alpha-1-antichymotrypsin (PSA-ACT). It is targeted toward national developers and manufacturers.

   The document fulfills the need for preparation of primary standards for assays to detect PSA and PSA-ACT in blood. The availability of high-quality primary standards prepared according to good manufacturing practices is critical to the goal of standardization of these assays, which are widely used to aid in diagnosis or monitoring of prostate carcinoma, a leading cause of morbidity and mortality in men in the United States.

   I/LA19 does not appear to be too restrictive. Developers and manufacturers should be expected to meet and adhere to the preparation standards described in this document.

   The document provides guidance for the preparation of primary standards for use with FDA-approved medical diagnostic kits. In this capacity, the availability of primary standard addresses method validation requirements and quality control considerations promulgated by CLIA '88.

   The document also describes standard laboratory techniques that should not present difficulties to developers and manufacturers committed to the production of high-quality reagents.

- **The subcommittee appreciates the comment.**

10. This document appears to be useful for the formulation of PSA/PSA-ACT clinical laboratory standards. This document is straightforward and gives useful information to be used in establishing and assaying a production PSA standard. It gives definitions and is well written. It also includes references and addresses details necessary for reproduction of the laboratory methods.

- **The subcommittee appreciates the comment.**

Section 4.7

11. I recommend changing the term "extinction coefficient" to the preferred term(s), "absorption coefficient" and "(molar) absorptivity."

- **This change has been made throughout the guideline.**

Section 5.6.3

12. This section should be deleted because it is not possible to determine a precise molecular mass for PSA-ACT based on mass spectrometry. Furthermore, I recommend revising Section 4.6.4 with the information presented below which has been received in a draft manuscript from Stanford summarizing the conference proceedings. It will be published in the journal, *Urology*, in 1995. Additionally, I would insert this publication as a reference for quantitation and analytical characterization of PSA-ACT. It should be cited as follows: Stamey TA. Second Stanford Conference on International Standardization of Prostate Specific Antigen (PSA) Immunoassays, September 1 and 2, 1994. *Urol* 1995 (in press).

- **Section 5.6.3 was deleted from the guideline. Section 6 was added to discuss the preparation of a 90:10 blended primary standard, which was recommended as a result of the Stanford Conference.**

Section 5.6.4

13. This section should be revised to read as follows:

"Amino Acid Analysis

Following demonstrated purity, a sample is submitted for quantitative amino acid analysis using vapor phase acid hydrolysis of PSA-ACT at 24, 48, and 72 hours. Reference purified amino acids are used for calibration. The concentration of the PSA-ACT stock material should be based on replicate runs (minimum of three) at several sites (minimum of three) and each run should include at least one pertinent control. The total number of residues for each amino acid per mole of complex should closely correspond to the sum of the known amino acid sequences for both PSA and ACT since they are known to react in equimolar ratios. Based on the theoretical sequences data, a molar concentration of the PSA-ACT stock material may be determined in a manner described in Section 3.7. The molecular mass of the PSA-ACT complex has been assigned by taking the cDNA of ACT (plus 26% carbohydrate) added to the mass of PSA as determined by mass spectroscopy (28,430) (for a total mass of 89,280).[Stamey,Urol,24] This concentration, determined by amino acid analysis, may be converted to a PSA equivalent concentration (mg/mL) by multiplying the PSA-ACT concentration (converted to mg/mL) by 28,430/89,280 or 0.318. Amino acid analyses on 12 different lots of PSA-ACT have demonstrated an extinction coefficient of 0.99 ± 0.05 at 280 nm (mL x mg^{-1} x cm^{-1}) for PSA-ACT.[Stamey, Urol] These analyses were based on 9 amino acids representing 67% of the total amino acids in the PSA-ACT complex.

The optical density of the PSA-ACT stock material should be determined by at least three different reference laboratories. The stock material should be diluted in the same manner by each laboratory into the linear range of the spectrophotometer (e.g., between approximately 0.1 and 0.7 absorbance units) before determination. Appropriate light scatter corrections and corrections for diluent (reference blanking) should be used.

The average optical density and the concentration of the PSA-ACT stock will be used to assign an extinction coefficient at 280 nm. This value has previously been determined to be 0.99 ± 0.05 for a 1 mg/mL solution of PSA-ACT. Multiplication by 0.318 converts PSA-ACT concentration to a PSA equivalent value.[Stamey, Urol] This value will be useful to quantify the concentration of future preparations of PSA-ACT, after demonstration of equivalent purity."

- **Section 5.6.4 has been revised as part of the guideline revision.**

Related NCCLS Publications[†]

M29-T2 **Protection of Laboratory Workers from Infectious Disease Transmitted by Blood, Body Fluids, Tissue—Second Edition; Tentative Guideline (1991).** M29-T2 provides guidance on the risk of the transmission of hepatitiis B virus and the human immunodeficiency virus in the laboratory. It gives specific precautions for preventing the transmission of bloodborne infection during clinical and anatomical laboratory procedures.

NRSCL13-P **The Reference System for the Clinical Laboratory: Criteria for Development and Credentialing of Methods and Materials for Harmonization of Results; Proposed Guideline (1995).** NRSCL13-P provides procedures for developing and evaluating definitive methods, reference methods, designated comparison methods, and reference materials to provide a harmonized clinical measurement system.

[†]Proposed- and tentative-level documents are being advanced through the NCCLS consensus process; therefore, readers should refer to the most recent editions.