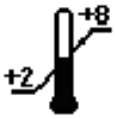
BIOGENIX
INC. PVT. LTD.

Free PSA ELISA

Enzyme Immunoassay for Quantitative Determination of Free Prostate-Specific Antigen in Human Serum

Instructions for use

1. SYMBOL LEGEND

IVD	In vitro diagnostic medical device	RCNS	Reconstitute with specified volume of liquid
REF	Catalogue number	LOT	Batch code
	Use by		Manufacturer
	Date of manufacture		Consult operating instructions
	Temperature limitation		Biological risks
	Contains sufficient for n tests	CONJ	Conjugate
MP	Coated microplate (96 wells)	SUB	Substrate
WASH P 20X	Wash solution, 20X concentrated	STOP	Stop solution
CAL	Calibrators	H2O	Deionized or distilled water
CONTROL	Controls	OD	Optical density
TRIAL 5000X	Trial	ASSAYB	Assay buffer
	Irritant		

Warning

2. INTENDED USE

Free PSA kit is provided for the **quantitative** determination of free prostate-specific antigen (fPSA) in human serum.

PSA is a glycoprotein with a molecular mass of about 32 000 Da, that consists of one polypeptide chain. PSA is a serine protease that is produced only by human prostate epithelium. Normally PSA is secreted into seminal fluid in high concentrations. There it exhibits its enzymatic activity and is directly involved into liquefaction of seminal clot. In serum PSA persists in low concentrations. Increase of PSA concentration in serum is a sign of prostate pathologies, such as benign hyperplasia or malignant degeneration of prostate tissues. PSA determination is widely used for the revealing and monitoring of prostate cancer patients.

It was demonstrated that PSA makes stable complexes with different protease inhibitors. The main part of the serum PSA is a complex with α_1-antichymothrypsine (PSA-ACT). But there is a great difference in free PSA/PSA-ACT ratio in different groups of patients. In the case of benign hyperplasia the part of free PSA is higher than in the case of prostate cancer.

3. PRINCIPLE OF TEST

Free PSA kit is a “sandwich” type of solid-phase enzyme immunoassay, based on two monoclonal antibodies that are specific for different epitopes of free PSA. One of these antibodies is conjugated with horseradish peroxidase (HRP); the other is coated onto the inner surface of microwells. PSA molecules from the serum sample bound to both immobilized antibody and anti-PSA-peroxidase conjugate (Fig. 1).

Then the wells are washed with wash solution to remove any material not bound to the inner surface of the wells. Quantity of the bound conjugate is directly proportional to the PSA level in sample.

During incubation with substrate the colour is developing. The intensity of the colour is directly proportional to the concentration of PSA in specimens or calibrators. PSA concentration in the patient sample is read from a standard curve that is processed in each assay.

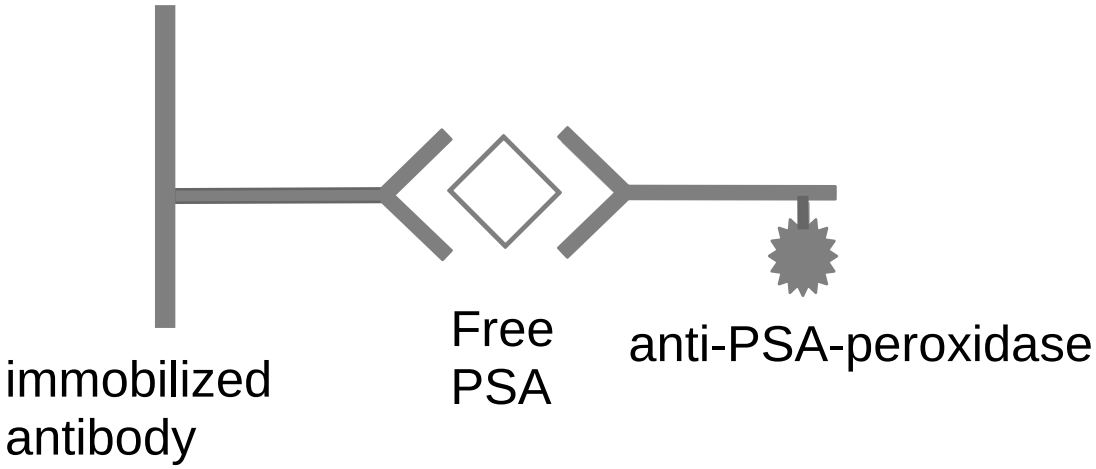


Fig. 1. Assay scheme

4. STORAGE AND STABILITY OF THE KIT

The expiration date of the kit is printed on the box label; the expiration date for each component is printed on the respective label.

Free PSA kit should be stored at +2…+8 °C upon receipt, preferably in the original kit box, until the expiration date. Storage at +25 °C is allowed but for no more than 5 days.

Shelf life of the kit is 18 months.

After initial opening the kit is stable upto expiration date if stored at +2…+8 °C.

If used in several separate experiments, kit contents should be stored as follows:

- unused strips: in a firmly closed resealable zipper bag at +2…+8 °C until the expiration date;

- opened vial with substrate: at +2…+8 °C for no more than 1 month after opening, protected from light;
- opened vials with concentrated Trial, wash solution, stop solution and assay buffer: at +2…+8 °C until the expiration date;
- wash solution prepared for use: at room temperature (+18…+25 °C) for no more than 5 days, in a firmly closed bottle.
- wash solution prepared for use: at room temperature (+18…+25 °C) for no more than 5 days or at +2…+8°C for no more 3 weeks, in a firmly closed bottle; wash solution prepared for use at 2-8 °C for no more than 4 weeks, in a firmly closed bottle.
- Trial solution prepared for use: at room temperature for no more than 5 days, in a firmly closed bottle; wash solution prepared for use at 2-8 °C for no more than 4 weeks, in a firmly closed bottle.

Damaged Test Kits

In case of any severe damage of the test kit or components, it has to be informed in writing, during one week after receiving the kit. Usage of severely damaged single components for a test run is not recommended.

5. SAMPLE COLLECTION AND STORAGE

Collect blood by venipuncture. After clotting, the serum is separated by centrifugation.

Do not use plasma, haemolyzed (bright red) or lipaemic (milky) serum samples as well as samples containing sodium azide as preservative.

Store serum samples at 2-8 °C for no more than 2 days. Aliquot and freeze samples for longer storage (-20 °C and lower). Avoid repeated freezing.

6. EXPECTED VALUES

The ratio of free PSA to total PSA concentration can be used for differentiation between benign prostate hyperplasia and prostate malignancies in the patients with moderately increased total PSA level. The ratio is calculated as follows:

$$\frac{\textit{Free PSA concentration}}{\textit{Total PSA concentration}} \times 100\%$$

Ratio below certain threshold limit (14-16 %, according to most publications), indicates the high probability of prostate malignancy.

It is highly recommended for each laboratory to determine its own reference range of free PSA concentrations.

7. QUALITY CONTROL

It is recommended to use control samples according to the state and federal regulations. The use of control samples is advised to assure the day to day validity of results.

8. REAGENT PREPARATION

Allow all the reagents to reach room temperature, and then thoroughly stir.

MP Keep **microplate** at room temperature (+18…+25 °C) for at least 30 minutes before opening the bag. Place required number of strips into strip holder. Place unused strips onto the resealable zipper bag and reseal duly.

CAL **CONTROL** **Calibrators and Control**

Liquid calibrators and control are ready to use.

Prepare lyophilized calibrators and control as follows. Gently tap on the vial caps to knock off all the dry matter.

Open the vials and carefully place the caps upside down on the clean dry surface.

Add 0.5 mL of distilled or deionized water to each vial with lyophilized calibrators and control, close vials with the corresponding caps and leave for 10 min at room temperature without stirring. Then stir gently avoiding foaming, until the dry matter is completely dissolved. Leave for another 10 minutes at room temperature stirring gently periodically. Make sure that no dry matter is left on the caps and walls of the vials.

WASH P Prepare required volume of **wash solution** by dilution of the concentrate 20-fold with distilled or deionized water. For example:

5 mL of **WASH P** **20X** + 95 mL of water.

Mix thoroughly, avoid foaming.

SUB Protect **substrate** from direct light.

9. SAMPLE PREPARATION

Allow samples to reach room temperature. Stir samples gently in order to ensure homogeneity.

10. PERFORMANCE CHARACTERISTICS OF THE ASSAY

10.1. Calibration-Traceability

Free PSA test kit was calibrated against the WHO Reference Standard 96/668 (100 % free PSA).

10.2. Analytical Sensitivity

Analytical sensitivity of **Free PSA kit** i.e. concentration that can be distinguished from zero calibrator, is 0.08 ng/mL. It is defined as mean OD of 10 replicates of calibrator 0 plus 2SD.

10.3. Specificity

No cross-reaction between used anti-PSA monoclonal antibodies and PSA-ACT complex was detected.

10.4. Measurement Range

Free PSA kit was validated for measurement of PSA concentration within the concentration of 0.08 - 10 ng/mL.

10.5. Intra- and Inter-Assay Variation (Precision)

a. Intra-assay CV

Sample	Mean free PSA concentration, ng/mL	Intra-assay CV	
		SD	CV, %
HS 1	0.16	0.012	7.7
HS 2	0.39	0.030	6.9
HS 3	0.63	0.049	4.8
HS 4	0.92	0.071	3.9
HS 5	1.51	0.116	4.0
HS 6	1.96	0.151	5.4
HS 7	2.07	0.159	7.1
HS 8	4.89	0.377	7.5

For **intra-assay CV** determination 8 serum samples were run, each in 9 replicates. The results are shown below.

b. Inter-assay CV

For **inter-assay CV** determination, 7 serum samples were assayed 9 times by different operators with 1-week interval. Each specimen was run in 2 replicates. The results are shown below.

Sample	Mean free PSA concentration, ng/mL			Inter-assay precision	
	1 assay	2 assay	3 assay	SD	CV, %
HS 1	0.42	0.39	0.36	0.030	7.7
HS 2	0.59	0.63	0.60	0.021	3.4
HS 3	1.03	0.92	0.98	0.055	5.6
HS 4	1.41	1.51	1.39	0.064	4.5
HS 5	1.82	1.96	2.03	0.107	5.5
HS 6	1.97	2.07	2.16	0.095	4.6
HS 7	5.01	4.89	4.80	0.105	2.2

11. LIMITATION OF THE METHOD

Any clinical diagnosis should not be based on the results of in vitro diagnostic method alone. For diagnosis establishment, a physician is supposed to consider all available clinical and laboratory findings.

12. SAFETY PRECAUTIONS

- **This kit is for in vitro diagnostic use only.** Operator should follow the manual closely in order to ensure reliable data. The manual is valid for the present kit only, within the listed composition. Any substitution of kit components is not allowed by CE regulations.

- Do not use kit or its components after the expiration date indicated on the label. Take into account stability period for reconstituted reagents.
- Do not mix or use together reagents from different lots of the kit except substrate, stop solution and wash solution.
- Do not use substrate, stop solution and wash solution supplied by other vendors.
- Use only “P”-labeled wash solution.
- Note that stop solution is 1 N HCl solution. Avoid contacts with skin and mucosa. In case of contact rinse affected area thoroughly with plenty of water and seek medical advice.
- Take into account the following common procedural notes:
    - always pipette reagents into wells immediately after washing procedure;
    - avoid contamination of the solutions;
    - in case of partial use of the kit, dispense only required volume of the reagent into the tray;
    - do not pour unused reagents back into the original vials;

avoid exposure to direct sunlight during incubations;

- always pipette reagents in the same order to minimize reaction time differences between wells; the total dispensing time for the calibrators, control and samples must not exceed 15 min;
- the incubation temperature for all the immunological reactions must be kept at +37 °C;
- do not touch the bottom of the wells.
- calibrators should be measured in each separate assay. It is also recommended to measure each time PSA concentration in the control.

- TMB solution should be colourless. Light colouring of solution is admissible. Avoid direct exposure of substrate to sunlight.
-  Source materials of human origin used for kit components preparation were tested and found negative for HBsAg, anti-HIV and anti-HCV antibodies. However, none of known laboratory test guarantees absence of these viral agents. Therefore, all kit components and patient's samples should be handled as potentially hazardous.
-  After usage strips, calibrators, controls, specimens and all consumables which contacted with specimens during handling, storage or assay (tubes, vials, gloves, pipette tips etc.) should be collected separately and sterilized by autoclaving. Instead of autoclaving pipette tips may be sterilized by disinfectant treatment. After sterilization all components and expendable materials may be utilized as non-dangerous garbage. Other components of the kit should be discarded into conventional garbage.

-  During manual washing procedure do not discard the contents of the wells directly to drainage. Use a container with disinfectant solution.
-  As the kit contains potentially hazardous material, the following precautions should be taken:
    - do not smoke, eat or drink while performing the assay;
    - always use protective gloves;
    - never pipette material by mouth;
    - in case of spilling, wipe up the spills promptly and wash affected area thoroughly using decontaminant.

-  As the kit contains irritant (CONJ, CAL, CONTROL, DIL), the following precautions should be observed:
    - P261 - Avoid breathing spray;
    - P272 - Contaminated work clothing should not be allowed out of the workplace;
    - P280 - Wear protective gloves/protective clothing/eye protection;
    - P302+P352 - IF ON SKIN: Wash with plenty of soap and water;
    - P333+P313 - If skin irritation or rash occurs: Get medical advice/attention;
    - P363 - Wash contaminated clothing before reuse;
    - P501 - Dispose of contents/container in accordance with national regulation.
- GLP including all general and individual regulations should be applied for the kit usage.

13. MATERIAL PROVIDED

13.1 Material Provided

MP	**Microplate:** 12 breakable 8-well strips (total 96 wells) coated with anti-PSA monoclonal antibodies	1 pcs
CONJ	**Conjugate:** solution contains anti-PSA monoclonal antibodies conjugated with HRP	14 mL, ready to use
0–5 CAL	**Calibrators:** protein-based solution or lyophilized preparations containing known fPSA concentrations - 0; 0.2; 0.5; 2; 5; 10 ng/mL. The concentrations of calibrators may be different for schemes with or without shaking. For exact fPSA concentrations, see vial labels	6 vials, 0.5 mL each; ready to use or lyophilized preparations
CONTROL	**Control:** protein-based solution or lyophilized preparation containing known fPSA concentration. The range of AFP concentration may be different for schemes with or without shaking. For exact range of fPSA concentration see vial label.	0.5 mL, ready to use or lyophilized preparation
WASH P 20X	**Wash solution P, 20X concentrated:** surfactant in buffered saline, sufficient for preparation of 840 mL of solution	3 vials, 14 mL each concentrated
SUB	**Substrate (TMB solution):** 3,3’,5,5’-tetramethylbenzidine solution in citrate buffer containing hydrogen peroxide	14 mL, ready to use
STOP	**Stop solution:** 1 N HCl solution	14 mL, ready to use
ASSAYB	**Assay buffer**	14 mL, ready to use

13.2. Equipment and Materials Required but not provided

- 1-channel calibrated variable precision pipettes, with disposable tips;
- 8-channel calibrated variable precision pipette, with disposable tips;
- microplate incubator (+37 °C) or microplate incubator/shaker (+37 °C, shaking speed 500–800 rpm);
- manual or automatic equipment for rinsing wells;
- calibrated microplate reader (450 nm);
- vortex tube mixer;
- deionized or distilled water;
- graduated beaker and cylinder of appropriate volume;
- latex or plastic gloves;
- trays for pipetting reagents with 8-channel pipette;
- disinfectant;
- absorbent material (for manual wash).

13.3. Test Procedure

Free PSA kit is designed for 96 tests. This is sufficient for 40 unknowns, 6 calibrators, 1 control and 1 blank (OD of TMB solution) in duplicates, provided that all the strips are used simultaneously.

13.3.1. Assay Procedure

13.3.1.1. Protocol with shaking

(See assay scheme, section 13.5.)

A. Pipette**:**

- **100 µL** of **assay buffer** **ASSAYB** into each well,

Leave wells A1-A2 empty for blank!

B. Pipette**:**

- **50 µL** of calibrators **CAL**, control **CONTROL** and patient's samples in duplicates**;**

except wells A1-A2.

Note: total time of dispensing must not exceed 15 minutes, otherwise the test result may be unreliable, because the time of incubation will substantially vary for different samples.

C. Incubate for **60 minutes at +37 °C while shaking (500–800 rpm)**.

D. Wash 5 times, as described in section 13.3.2.

E. Pipette **120 µL** of **conjugate CONJ** into each well;

F. Incubate for **60 minutes at +37 °C while shaking (500–800 rpm)**.

G. Wash 5 times, as described in section 13.3.2.

H. Pipette **100 µL** of **substrate SUB** into each well (including blank); incubate strips **at room temperature** (+18…+25 °C) **in the dark for 15-30 minutes**, depending on the colour intensity, or **10 minutes while shaking (500-800 rpm) at +37 °C**.

I. Pipette **100 µL** of **stop solution STOP** to all the wells in the same sequence and at the same speed as used for dispensing TMB substrate. Shake for 1–2 min at room temperature.

J. Read OD at **450 nm within 20 min**.

13.3.1.2. Protocol without shaking

(See assay scheme, section 13.6.)

A. Pipette**:**

- **100 µL** of **assay buffer ASSAYB** into each well,

Leave wells A1-A2 empty for blank!

B. Pipette**:**

- **50 µL** of calibrators **CAL**, control **CONTROL** and patient's samples in duplicates**;**

except wells A1-A2.

Note: total time of dispensing must not exceed 15 minutes, otherwise the test result may be unreliable, because the time of incubation will substantially vary for different samples.

C. Incubate for **90 minutes at +37 °C** (pre-shake for 1-2 minutes at room temperature).

D. Wash 5 times, as described in section 13.3.2.

E. Pipette **120 µL** of **conjugate CONJ** into each well;

F. Incubate for **90 minutes at +37 °C**.

G. Wash 5 times, as described in section 13.3.2.

H. Pipette **100 µL** of **substrate SUB** into each well (including blank); incubate strips **at room temperature** (+18…+25 °C) **in the dark for 15-30 minutes**, depending on the colour intensity.

I. Pipette **100 µL** of **stop solution STOP** to all the wells in the same sequence and at the same speed as used for dispensing TMB substrate. Shake for 1–2 min at room temperature.

Read OD at **450 nm within 20 min**.

13.3.2. Wash Procedure

It is advisable to use an automatic microplate washer set at 5 wash cycles and a volume of 300 µL of wash solution per well per cycle.

If an automatic washer is not available, the wash procedure can be carried out manually as follows:

- remove the contents of the wells into a container with disinfectant;
- dispense 300 µL of wash solution, prepared according to section 8, into each well, shake the plate carefully for 5–10 sec and remove the contents of the wells; repeat 5 times;
- strike the wells sharply on absorbent material to remove any liquid residue.

13.4. Data Processing

If the reader cannot be adjusted to zero using the substrate blank in wells A1-A2, subtract mean OD value of wells A1-A2 from all OD values before further calculations.

Example:

OD (Cal 5) measured = 2.28 and OD (blank) = 0.06;

OD (Cal 5) calculated = 2.28 – 0.06 = 2.22

13.4.1. Data Reliability (for OD Measured at 450 nm)

The data should meet the following criteria:

- average blank OD (in wells A1-A2) ≤0.100;
- average OD of Cal 5 ≥1.5 (after blank subtraction);
- control’s concentration must fall within the acceptability range that are shown on the vial label.

If the data obtained do not meet the criteria, the results are considered unreliable and the test should be repeated.

13.4.2. Quantitative Determination

Specialized software for quantitative determination is recommended. Plot mean OD values of the calibrators at 450 nm versus their respective PSA concentrations using 4PL or 5PL fit (see typical standard curve, fig. 2). Calculate concentration of PSA in samples using standard curve.

Any extrapolation of the standard curve to PSA concentration above the nominal value of the calibrator 5 (approximately 10 ng/mL) is forbidden.

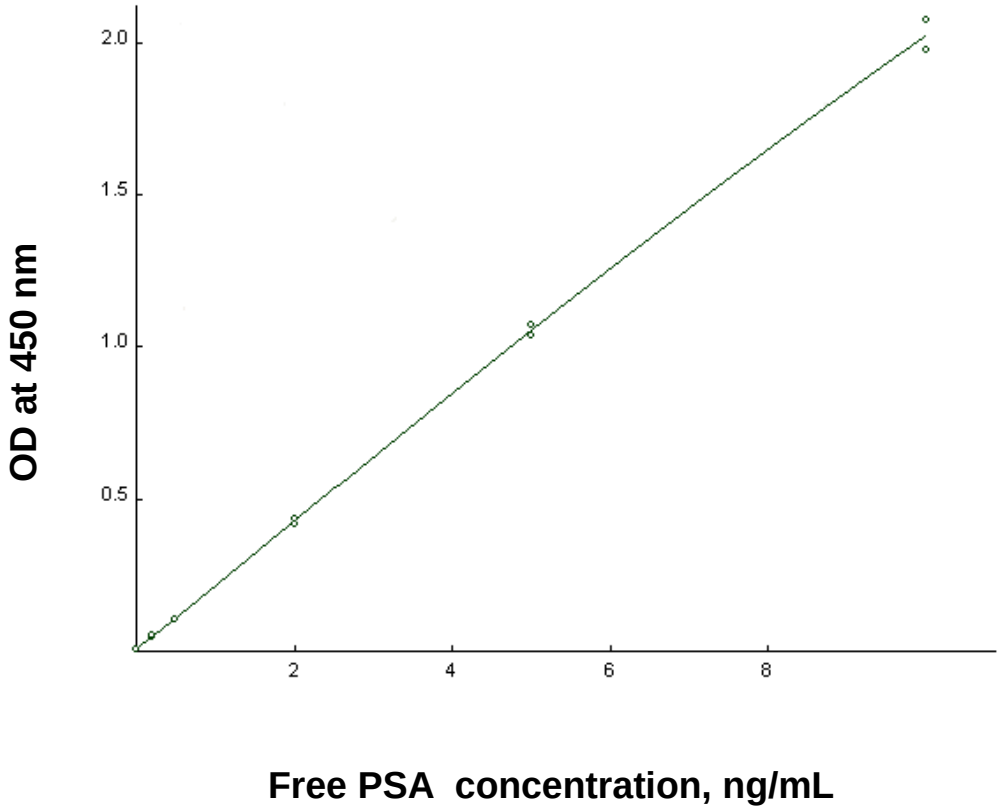


Fig. 2. Example of typical standard curve.
Do not use for evaluation of real assay data!

13.5. Assay scheme with shaking

Wells / Reagents	«Blank»	**CAL** **CONTROL**	Samples
ASSAYB	–	100 µL	100 µL
CAL **CONTROL**	–	50 µL	–
Samples	–	–	50 µL
Incubation No.1	60 min, +37 °C, 500–800 rpm		
WASH P (diluted)	5 x 300 µL		
CONJ	–	120 µL	120 µL
Incubation No.2	60 min, +37 °C, 500–800 rpm		
WASH P (diluted)	5 x 300 µL		
SUB	100 µL	100 µL	100 µL
Incubation No.3	15–30 min, +18…+25 °C, in the dark		
	10 min, +37 °C, 500–800 rpm		
STOP	100 µL	100 µL	100 µL
Stirring	1–2 min, +18…+25 °C		
OD measuring	450 nm		
Calculations	Corresponding software		

13.6. Assay scheme without shaking

Wells / Reagents	«Blank»	**CAL** **CONTROL**	Samples
ASSAYB	–	100 µL	100 µL
CAL **CONTROL**	–	50 µL	–
Samples	–	–	50 µL
Incubation No.1	90 min, +37 °C (pre-shake for 1-2 minutes at room temperature)		
WASH P (diluted)	5 x 300 µL		
CONJ	–	120 µL	120 µL
Incubation No.2	90 min, +37 °C		
WASH P (diluted)	5 x 300 µL		
SUB	100 µL	100 µL	100 µL
Incubation No.3	15–30 min, +18…+25 °C, in the dark		
STOP	100 µL	100 µL	100 µL
Stirring	1–2 min, +18…+25 °C		
OD measuring	450 nm		
Calculations	Corresponding software		

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