

PROSTATE-IC

**ELISA kit for the
assessment of
Prostate Specific
Antigen (PSA)
Immune Complexes
(PSA-IgM) in
Prostate Cancer
(PCa)**

LIFE NANOTECHNOLOGY
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PROSTATE-IC

PRODUCT PROFILE

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code XG007

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PRODUCT PROFILE

| | |
|---|---|
| Prostate Cancer: characteristics, causes and prevention | 4 |
| Prostate Cancer Diagnosis..... | 4 |
| Prostate-IC ELISA Kit..... | 5 |
| References | 6 |
| Prostate-IC - Product Data Sheet..... | 8 |

Prostate Cancer: characteristics, causes and prevention

Prostate cancer (PCa) is the most common cancer and the main cause of death due to cancer among men. Even if it does not have a particularly high fatality rate compared to other tumours, showing after a positive diagnosis a probability of survival higher than 90%, the steady increase in the incidence rate, in either population at higher risk or at lower risk, could explain the observed rising number of deaths. Most of these deaths depend on late diagnosis, and early diagnosis of prostate cancer may determine the difference in terms of healing. Over 95% of prostate cancers are adenocarcinomas, which originate in glandular secretory cells and their evolution is influenced by patient hormonal status. The remaining 5% of prostate cancer types are sarcomas, lymphomas, squamous cell carcinomas and metastases from other tumour sites. The originating area of many prostate cancers is the peripheral region of the prostate gland. The peripheral zone is not in direct contact with the urinary tract and that is why the symptoms are almost absent in the early stages of cancer growth and they may worsen only in advanced stages of disease, when other organs are involved (1).

The prostate cancer is classified according to the TNM system (Tumour Node Metastasis), but frequently the prostate cancer is staged according to the Gleason's score. The TNM system can only be used in bioptic materials and it is the sum of two different tumor growth indexes each of these indexes ranges from 1 to 5, indicating with 1 the most differentiated tumor and with 5 the least one. The Gleason's score is determined on a scale from 2 to 10, where 2 represents the least aggressive tumor type and 10 the most one.

It is estimated that a man out of five could develop prostate cancer during his life time; this percentage may change in several cases:

- the risk doubles in the event of a tight relative who has manifested the disease, became five-time higher with two tight relatives having cancer, with three the risk is about 97%.
- the black population have a greater incidence rate of 60% compared to white population and the mortality rate is two-fold higher.
- men with an index of body mass higher than 32,5 (indicating an obesity state) have a greater risk of 33% compared to the "normal" population.

The advanced age (PCa mainly affects men aged over 55 years), hereditary factors and the occurrence of biologically active androgens in the bloodstream and in the prostatic tissue represent the most important risk factors of prostate cancer.

Undoubting, the aetiology of prostate cancer is multifactorial and it is a resultant of a complex interaction between genetic and environmental factors with the age and the hormonal state of subjects at risk.

Testosterone is closely linked to the development of the prostate gland and it seems that its elevation in the bloodstream of prostate cancer patients may promote the transition from the histological phase to a clinical phase of tumor (2).

Clinical studies have demonstrated that patients previously submitted to vasectomy or with a clinical history of benign prostatic hyperplasia have an higher risk of tumor development. Moreover, several studies on risk factors have been accomplished to find out the aetiological agents of the prostate cancer; pollution, dietetic factors, smoking, alcohol intake, risky sexual activity, venereal diseases and hormonal factors are all involved in the development of neoplasm (3-8).

Although it is not possible to change the genetic risk factors, it's absolutely possible to reduce the exposure to the other risk

factors of prostate cancer, decreasing therefore the incidence of this disease.

The increased risk caused by an elevated lipid assumption is probably associated to the rise of production of testosterone and to the lessening of vitamin A uptake. The vitamin A has a conflicting role in prostate cancer development: it seems that vitamin A of vegetable origin lowers the tumour risk, while that of animal origin increases it. This is in agreement with the differences of incidence rate of prostate cancer between Europe or America and the Asian continent, where the uptake of vitamin A is essentially of vegetable origin. The low incidence rate of prostate cancer in the Asian populations therefore could be related to a low lipid content diet and high content in fibres and fitoestrogens, which could play a protecting role (3-9).

Prostate Cancer Diagnosis

Currently, all diagnostic methods proposed for prostate cancer have low specificity. This lack of specificity prevents effective screening programmes of male population that could reduce deaths caused by this disease.

In clinical practice several tests are being used for prostate cancer diagnosis, including digital rectal examination (DRE), transrectal prostatic ultrasound and the assessment of serum levels of prostate-specific antigen (PSA).

A positive outcome of one or more of these tests arise a suspect of neoplasm leading to perform a prostate biopsy intervention, the most used analysis for diagnostic confirmation of cancer.

Digital rectal examination

Digital rectal examination (DRE) is the main tool for studying any prostate disease. It's the first approach in dealing with patients with urinary disorders caused by prostate troubles.

DRE is a step of the regular patient evaluation, the obtained its results, together with PSA assessment, define the criterion for deciding on biopsy necessity (10).

Sensitivity and specificity values are not appropriate mainly because DRE is able to appreciate cancers that originate in the peripheral zone of the gland, which represent only 70-80% of total carcinomas (11).

In 18% of cases, prostate cancer is detected only through a suspect digital rectal exploration, regardless the level of PSA.

In conclusion, DRE is simple to perform and is non-invasive, while the major disadvantages are its operator dependent nature and the inability to appreciate the full prostate gland.

Transrectal prostatic ultrasound

Trans-rectal prostatic ultrasound (TRUS) is a simple ultrasound exam that allows a detailed study of the prostate. TRUS allows to evaluate the volume of the prostate to view the morphology of the gland and structures next to prostate. It is able to document gland's increasing volume, presence of intra-prostatic adenoma, signs of both acute and chronic inflammation and any suspicious areas for prostate cancer development (12).

Currently, because of its low sensitivity and specificity, TRUS has a marginal role both in the diagnosis of cancer and in monitoring the advanced disease. Its role, on the contrary, is indispensable in performing biopsies to obtain histological confirmation of suspected prostate cancer.

Biopsy

Biopsy is performed when DRE, TRUS or changing PSA levels suggest the presence of prostate cancer. Usually it is performed by transperineal or transrectal way, under ultrasound guidance. Prostate biopsy is recommended, even in

cases where the diagnosis of cancer is obvious (typical case history of palpation with extracapsular extension, PSA elevated, with or without bone metastases), for obtaining histological definition and grading, necessary for prognosis and prediction of hormone sensibility (13).

Biopsy, however, is a highly invasive procedure that can cause many complications (13). Therefore it is essential to improve the accuracy of non-invasive tests, such as the assessments of tumour markers, to reduce the number of biopsies.

PSA test

The PSA test assesses circulating levels of total prostate specific antigen. Glandular cancer cells produce much more PSA than normal cells inducing an increase of PSA levels in the blood stream. A PSA value around 4 nanograms per milliliter (ng/mL) represents an alarm bell for likely presence of prostate cancer (14-17).

The PSA assay was a landmark in prostate cancer diagnosis, but has also increased overdiagnosis problems because of its poor diagnostic accuracy. In fact, PSA is an organ specific marker but it is not a specific marker of neoplasm, since increased circulating PSA levels may also be found in patients with benign prostatic hypertrophy (BPH), bacterial prostatitis as well as after prostatic manipulations or trauma.

To improve the diagnostic accuracy of PSA test, in particular for PSA values between 4 and 10 ng/mL (gray zone) where the outcome of this test is most equivocal and unpredictable due to an overlap between cancer and BPH patients (14), several other parameters related to PSA have been proposed. These are PSA density, PSA velocity, free/total PSA ratio and age-specific and race-specific reference ranges, but the introduction of these variants of PSA parameters did not lead to a significant improvement in the diagnosis of prostate cancer and they do not have allowed a reduction of diagnostic biopsies (15-17).

PSA density

The PSA density test indicates the relationship between the level of serum PSA and the size of the prostate gland measured by ultrasound. Since cancer cells of glandular tissue produce much more PSA than in hypertrophy state, the concentration of PSA per gram of glandular tissue increase. However, its interpretation is complicated by several variables: the different ratio between glandular tissue (which produces PSA) and stroma (that does not produce PSA), the possible error in prostatic volume determination and different age-related increases of PSA and gland size (14, 15).

PSA velocity

Another variant of PSA test developed for prostate cancer diagnosis is the increasing rate in PSA serum level over time. It seems, indeed, that the quantitative modification on annual basis between serial samples from the same patient is more significant than the absolute values of PSA in differential diagnosis between cancer and hypertrophy. In prostate cancer, the PSA raise usually exceeds 0.75 ng/mL per year or, at least, shows annual increases of 20% compared to baseline.

It should, however, be reminded that this approach requires a good control of variability of analytical methods and knowledge of intra-individual fluctuations of the marker not linked to the presence of disease. PSA velocity is therefore an interesting diagnostic tool, but requires careful standardization before using it in clinical routine. Quarterly intervals repeated determinations of PSA, for a minimum period of one year, but preferably for several years, are required to adopt this practice. Obviously this principle does not allow conclusions in a short time (15).

Free/total PSA values

It is believed that different production of free PSA (fPSA) by prostatic epithelial cells and cancer epithelial cells is the cause of differences between serum levels of fPSA of men with BPH and men with cancer. A high total PSA level, associated to a low percentage of free PSA, is a clear indication of a more aggressive tumor.

The use of fPSA has led to an improvement of specificity in the range 4-10 ng/mL with a reduction in dispensable biopsies. The fPSA cut-off used is about 25%.

Since commercial assessments of the fPSA show a still not satisfactory standardization level, the results are method-dependent.

Must also keep present that free PSA can't be valued separately, but it must be valued in association with total PSA and expressed in relation thereto. The value given from this ratio must be used only for diagnostic approach; it can't be used in stadiation, postoperatively long deadline monitoring and in therapeutic monitoring (radiotherapy endocrine-therapy, chemotherapy) since there aren't evidences supporting its efficacy in such clinicians backgrounds. In the end, the free/total PSA ratio must be used when total PSA values are included between 2.5 and 20.0 ng/mL. Indeed, in cases with total PSA < 2.5 or > 20 ng/mL the diagnosis is clearer, but free/total PSA ratio beyond these values lack of diagnostic accuracy (16,17).

PSA-IgM

Recently it was discovered that in serum of patients with several cancers, tumor markers can be detected not only in free form, but even in the form of immune complexes associated with immunoglobulin M (IgM) (18).

IgM are considered the main component of innate immunity, since they are able to bind to a broad spectrum of tumour antigens. It is well known that IgM antibodies play an important role in the first line of defence against infectious antigens, immune cells regulation and proliferation and immunoeediting.

In the serum of many patients with prostate cancer has been detected the presence of both PSA in free form and in form of immune complexes with IgM (PSA-IgM). The assessment of circulating levels of PSA-IgM immune complexes for the diagnosis of prostate cancer has recently been suggested, since it has shown increased accuracy compared to analysis of total PSA (18-23).

Prostate-IC ELISA Kit

ELISA kit for the assessment of Prostate Specific Antigen (PSA) Immune Complexes (PSA-IgM) in Prostate Cancer

Xeptagen has developed Prostate-IC, an ELISA kit for the determination of PSA-IgM immune complexes.

Prostate-IC kit was used in a clinical study to analyze serum levels of PSA-IgM in 50 specimens from patients with prostate cancer (PCa), 51 sera from patients with benign prostatic hyperplasia (BPH) and 15 sera from healthy donors used as control population (18). A commercial kit was used to assess in parallel the total PSA serum levels.

Comparative analysis of PSA-IgM and PSA has shown the advantage in terms of diagnostic sensitivity (SE) and specificity (SP) of PSA-IgM for the diagnosis of prostate cancer (Table 1, figure 1).

In the group of healthy donors the PSA-IgM serum levels were always negative (cut-off = 145.1 AU/mL), while in the group of patients with cancer, PSA-IgM levels were above the cut-off in 20 cases out of 50 (SE = 40%) and in the group of patients

with BPH, PSA-IgM was above the cut-off only in 6 out of 51 patients achieving a higher specificity (SP = 88%) compared to that attained with PSA using cut-off levels of either 4 ng/mL or of 10 ng/mL (SP = 4% or SP = 71%).

Serum levels of PSA-IgM were above the cut-off in 43% (13/30) of those cases in which cancer patients had a PSA value between 4 and 10 ng/mL; this did not occur in the hyperplastic group, where only 12% (4/34) of patients with BPH and PSA values between 4 and 10 ng/mL were positive to PSA-IgM assay (Table 1).

Moreover, the combination of PSA and PSA-IgM levels significantly enhanced the number of patients with prostate cancer correctly identified, which increased from 22% of the PSA 10 to 60% attained with the combination of the two tests (PSA 10 or PSA-IgM) without compromising significantly the diagnostic specificity (Table 1). The results of the study demonstrated that PSA-IgM is a complementary serological marker of PSA for prostate cancer detection suggesting that the approach of assessing PSA-IgM and PSA might be useful in clinical practice.

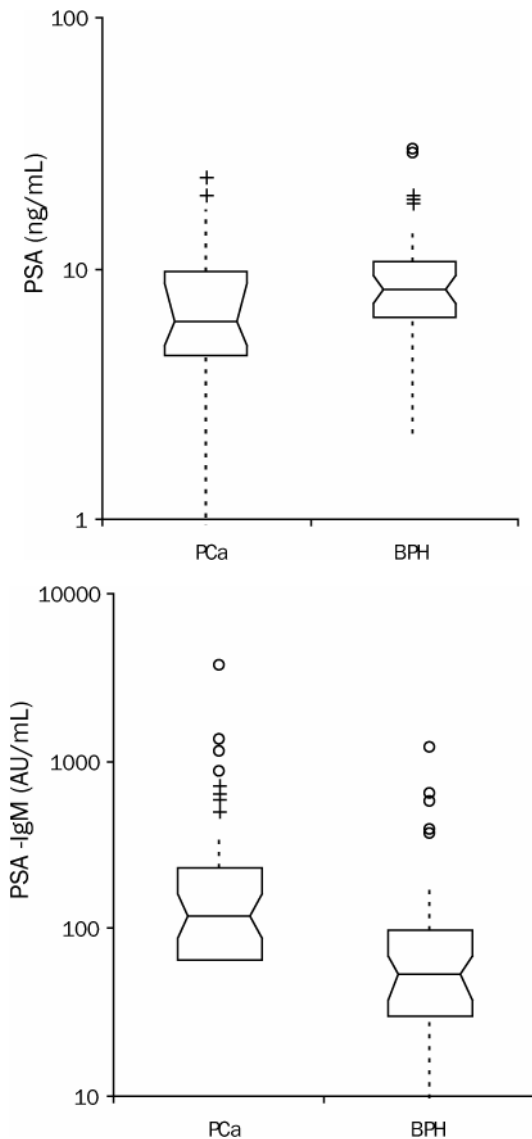


Figure 1: Box plot for serum levels of PSA and PSA-IgM in the patients with prostate cancer (PCa) and benign prostatic hyperplasia (BPH). The box indicates the lower and upper quartile, and the middle line is the median. A dashed line connects the observations within 1.5 interquartile ranges (IQRs). Crosses (1.5-3IQRs) and circles (>3 IQRs) are the far outliers.

| Biomarker | Sens | Spec | PPV | NPV |
|---|------|------|-----|-----|
| PSA-IgM 145,1 [AU/mL] | 40% | 88% | 77% | 60% |
| PSA 4 [ng/mL] | 84% | 4% | 46% | 20% |
| PSA 10 [ng/mL] | 22% | 71% | 42% | 48% |
| PSA 10 [ng/mL] or PSA-IgM 145,1 [AU/mL] | 60% | 63% | 61% | 62% |
| 4 ng/mL < PSA PSA < 10 ng/mL e PSA-IgM | 43% | 88% | 76% | 55% |

Table 1: Comparison of sensitivity (sens), specificity (spec), positive predictive value (PPV), negative predictive value (NPV) of PSA-IgM and PSA in differentiating patients with prostate cancer (n = 50) from patients with benign prostatic hyperplasia (n = 51).

The combination of serum PSA and PSA-IgM has been analyzed in depth in validation studies conducted in several Italian hospitals. The PSA-IgM assessment was always more accurate for the detection of prostate cancer in the organ-confined disease compared to the analysis of circulating levels of PSA. Particularly, in these studies for the discrimination of patients with organ-confined tumours from patients with benign prostatic hyperplasia, values of sensitivity and specificity of PSA-IgM and PSA were confirmed. Combination of both tests was always the best diagnostic approach compared to single test, allowing an enhanced identification of patients with cancer with improved diagnostic specificity up to 90% and, consequently, reducing the number of negative prostatic biopsies (20-23).

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Prostate-IC - Product Data Sheet

INTENDED USE

Prostate-IC is an enzyme linked immunosorbent assay (ELISA) for the quantitative measurement of Prostate Specific Antigen (PSA) immune complexes (PSA-IgM) in serum samples.

SUMMARY AND EXPLANATION OF THE TEST

Prostate-IC belongs to a novel generation of in vitro diagnostic methods based on the detection of PSA as circulating Immune Complexes. Prostate-IC is a highly specific and sensitive ELISA for Prostate Cancer detection designed to measure PSA-IgM in patient sera. The amount of PSA-IgM is expressed in Arbitrary Units (AU), using a specific calibrator as reference. Studies have demonstrated that the measurement of PSA-IgM levels in Prostate Cancer patients detects cancer with higher sensitivity with respect to PSA, the well-established serum cancer biomarker, without compromising specificity (1-3). Furthermore, the occurrence of the free and IgM-complexed form of the circulating PSA does not overlap, indicating that PSA-IgM is complementary to PSA and the use of both markers can increase the diagnostic accuracy (1-5).

PRINCIPLE OF THE TEST

Standard Calibrators and specimens are simultaneously incubated with anti-PSA antibodies coated to the wells of a microtiter plate. The immune complexes PSA-IgM are detected by the addition of an enzyme conjugated secondary antibody and an enzyme substrate (TMB). The developed color is proportional to the amount of the analyte in the sample.

REAGENTS AND MATERIALS PROVIDED

XG007-PL: 96 wells multi-strip Assay-Plate, pre-coated with affinity purified rabbit anti-PSA.

XG007-Calibrator: Two vials of calibrator lyophilized from PBS. White powder. Exact concentration on label. Totally soluble.

XG-EA: Two vials of 200 µL of Enzyme-conjugated goat anti-human IgM secondary antibody (Green cap) 50-fold concentrate in stabilizer solution. The solution contains 0.015% Proclin as preservative.

XG-CH3: 10 mL of TMB (3,3',5,5'-Tetramethylbenzidine) chromogen solution ready to use.

XG-ST3: 10 mL of 1N HCl Stop solution ready to use.

XG-DB5: 10 mL of concentrated Dilution Buffer solution 5X. Once diluted, the working solution contains 1% BSA and 0.05% Tween 20 in PBS. The solution contains 0.25% Proclin as preservative.

XG-WB2: Two tablets of lyophilized Washing Buffer. White powder. Once diluted, the working solution contains 0.05% Tween 20 in PBS. Totally soluble.

MATERIAL AND EQUIPMENT REQUIRED

Precision pipettes with disposable tips

Microplate washer

Microplate readers with a 450 ± 20 nm or 650 ± 20 nm filter

Distilled or deionized water

STORAGE CONDITIONS

Storage at 4 °C:

XG007-PL, XG-CH3, XG-ST3, XG-DB5*, XG-WB2*

Storage at -20 °C:

XG-EA, XG007-Calibrator[§].

Avoid repeated freezing and thawing cycles

(*) Must be used within one month of reconstitution

(§) Must be reconstituted just before the use

EXPIRATION DATE

Expiration date printed on the kit indicates limits of stability.

WARNINGS - POTENTIAL BIOHAZARDOUS MATERIALS

The standard calibrator XG007-Calibrator is of human origin. The reference material was tested using an approved method of evaluation for the presence of the antibodies to HIV, antibodies to the hepatitis C virus and hepatitis B surface antigens, and found to be negative. **Since no test method can offer complete assurance that HIV, hepatitis B virus, hepatitis C virus, or other infectious agents are absent, all human sourced materials should be considered potentially infectious.** It is recommended that these reagents and human specimens be handled in accordance with the OSHA Standard on Bloodborne Pathogens (6). Biosafety Level 2 (7) or other appropriate biosafety practices (8, 9) should be used for materials that contain or are suspected of containing infectious agents.

SPECIMEN COLLECTION AND PREPARATION

The use of serum samples are recommended for the Prostate-IC assay.

Serum specimens should be collected aseptically, avoiding hemolysis if possible.

Specimens should be stored at 2-8 °C if the assay will be performed within 24 hours after collection. Specimens should be stored frozen if testing will occur after 24 hours.

If frozen, specimens should be mixed thoroughly after thawing to ensure consistency in the results. Avoid repeated freezing and thawing. Specimens showing particulate matter, erythrocytes, or turbidity must be clarified by centrifugation before testing.

INSTRUCTIONS FOR USE

PROCEDURAL NOTES

- Allow samples and reagents to reach room temperature prior to testing. Do not use water baths to thaw samples or reagents.
- Mix samples and all reagents thoroughly before use.
- Avoid excessive foaming of reagents. Also avoid exposure of reagents to excessive heat or light during storage and incubation.
- Avoid handling the tops of the wells both before and after filling.
- Standards and samples should be assayed in duplicate.
- Run a separate standard curve for each assay.
- Use only coated wells from the same reagent batch for each assay. Also do not mix reagents from different kit lots.
- Perform incubations in a sealed box containing a wet paper towel in order to prevent evaporation.

REAGENTS PREPARATION

- Reconstitute XG007-Calibrator with 440 µL of deionized water for each calibrator vial.
- Prepare the required amount of XG-DB5 dilution buffer by diluting 5-fold the concentrated solution in deionized water. If crystals appear upon refrigeration, warm the bottle to 37 °C with mixing to dissolve.
- Reconstitute 1 tablet of XG-WB2 washing buffer in 500 mL of deionized water.
- Prepare the required amount of XG-EA enzyme-conjugated secondary antibody solution diluting 50-fold in reconstituted XG-DB5 dilution buffer.

ASSAY PROTOCOL

1. Prepare assay reagents as described above.
2. Set up the microtiter plate with sufficient wells to enable the running of all required standards and samples.
3. Remove excess microtiter plate strips from the frame and store in the re-sealable foil bag with the desiccant provided.

4. Wash the microtiter plate strips three times with XG-WB2 washing buffer (300 μ L/well).
5. Dispense 100 μ L/well of standard calibrators (in duplicate) starting from the reconstituted solution and performing in-plate 2-fold serial dilutions in order to obtain a seven-point calibration curve. Use XG-DB5 dilution buffer as diluent. For exact concentration of the reconstituted calibrator please refer to the concentration value (AU/mL) indicated on the XG007-Calibrator vial. Also dispense 100 μ L/well of XG-DB5 dilution buffer as blank, in duplicate.
6. Dispense 100 μ L/well of a 50- or 100-fold diluted sample (in duplicate). Use XG-DB5 dilution buffer as diluent.
7. Incubate 1 hour at room temperature.
8. Wash six times with XG-WB2 washing buffer (300 μ L/well).
9. Add 100 μ L/well of diluted XG-EA enzyme-conjugated secondary antibody solution.
10. Incubate 1 hour at room temperature.
11. Wash six times with XG-WB2 washing buffer (300 μ L/well).
12. Apply 100 μ L/well of XG-CH3 chromogen solution.
13. Allow color to develop for 10-15 min at room temperature in the dark and measure OD values of each well using an ELISA plate reader with a 650 nm filter or, alternatively, apply 100 μ L/well of XG-ST3 Stop Solution and measure OD values of each well using an ELISA plate reader equipped with a 450 nm filter. Stopped reaction should be read within 1 hour.
14. Plot the standard curve Δ OD values as described in the next section: Processing of the results.

PROCESSING OF THE RESULTS

Average the duplicate readings for each standard calibrator and sample and subtract the zero standard optical density. The standard calibrators may be used to construct two distinct standard curves with values reported in AU/mL. Plot on a semi-logarithmic graph the OD readings corresponding to the reconstituted standard calibrator and those corresponding to 1:2, 1:4, 1:8 dilutions. Plot on a linear graph the OD readings corresponding to the PSA-IgM titers obtained diluting 1:8 to 1:64 the reconstituted standard calibrator. Data deduction may be performed through computer methods using curve fitting routines or may also be manually deduced on paper.

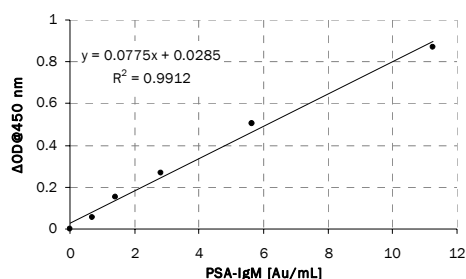


FIG. 1A: range of linearity (0 to 11.25 AU/mL) of a typical standard curve for PSA-IgM after 15 minutes of substrate incubation at room temperature and addition of stop solution

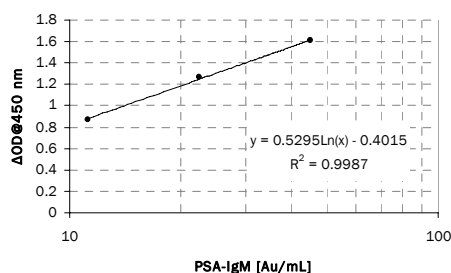


FIG. 1B: range of linearity (from 11.25 to 45.0 AU/mL) of a typical semi-logarithmic standard curve for PSA-IgM after 15 minutes of substrate incubation at room temperature and addition of stop solution

The immune complexes concentration in the biological sample can be calculated directly from the appropriate standard curve, depending on the sample absorbance value, by interpolation. The value obtained has to be multiplied by the dilution factor of the sample. Samples with OD values exceeding the upper calibration limit should be further diluted and re-measured.

QUALITY CONTROL

The intra- and inter-assay coefficients of variation were determined on 4 typical standard curves and the results were less than 10%.

It is recommended that each laboratory assays appropriate quality control samples in each run to ensure that all reagents and procedures are correct.

INTERPRETATION

The PSA-IgM cut-off value was 145 AU/mL for differentiating Prostate Cancer from Hyperplasia (2,3).

SPECIFIC PERFORMANCE CHARACTERISTICS

The linear range of the assay is 0.7-45 AU/mL. The sample with values above 45 AU/mL should be further diluted and re-measured.

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