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ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE DETERMINATION OF TOTAL PROSTATE-SPECIFIC ANTIGEN

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Prostate-specific antigen (PSA) remains the most widely used biomarker for prostate cancer diagnostics and monitoring. The development of highly informative, sensitive, and reproducible immunoassays for PSA determination is essential for improving diagnostic accuracy. The aim of the study was to develop and optimize a non-competitive "sandwich" ELISA for the determination of total PSA, using a panel of monoclonal antibodies (mAbs) of different specificity groups and epitopes. "Sandwich" ELISA configurations were designed using different capture and detecting antibody pairs. Antibody sorption conditions, reagents working concentrations, incubation parameters and buffer composition were optimized. Analytical performance was evaluated using PSA preparations standardized against the WHO International Standard (96/670). The most effective antibody combinations were found among mAbs targeting epitopes P2 (capture: 21B7, 11G5, 26B9) and P3 (detection: 21F4, 23B4, 27C10), with the high-affinity pair 26B9-27C10 showing the best sorption–detection properties. The use of mixed conjugates did not improve sensitivity in the range of 1-10 ng/ml PSA. Hydrophilization of polystyrene plates surface increased the ELISA signal up to 1.39-fold, depending on the antibody isotype and origin. The developed optimized "sandwich" ELISA demonstrates high specificity and sensitivity for total PSA determination, with analytical characteristics suitable for potential clinical diagnostic applications.

K e y w o r d s: prostate-specific antigen, monoclonal antibodies, ELISA, immunoassay, assay optimization, conjugates, sensitivity, hydrophilization, diagnostics.

Prostate-specific antigen (PSA, also referred to as gamma-seminoprotein or kallikrein-3, KLK3) is a glycoprotein enzyme with a molecular mass of approximately 34 kDa, comprising 237 amino acid residues. It belongs to the family of serine proteases (EC 3.4.21.77) and is encoded by the KLK3 gene located on the long arm of human chromosome 19 (19q13). Within the seminal plasma, PSA exerts its physiological function through the proteolytic cleavage of semenogelins, the major structural components of the seminal coagulum. The concentration of total PSA in blood serum is elevated under various pathological conditions, including prostatitis, benign prostatic hyperplasia (BPH), and prostate carcinoma, thereby rendering PSA an essential biomarker for the risk stratification and monitoring of prostatic disease.

In the circulation, protease inhibitors, most notably α_1-antichymotrypsin (α_1-ACT), α_2-macroglobulin (α_2-M), and other acute-phase proteins, form irreversible complexes with PSA, effectively abrogating its enzymatic activity. Consequently, in seminal plasma as well as in serum, PSA can be detected either in its free, enzymatically inactive form or as part of stable complexes with serine protease inhibitors. Approximately 75% of PSA released into the peripheral bloodstream is neutralized via complex formation with α1-ACT (~90 kDa), while less than 1% binds to α_2-M, generating a high-molecular-mass complex of about 800 kDa [1].

Quantitative determination of serum PSA is a well-established clinical practice for the management of patients with both benign prostatic hyperplasia and prostate cancer. Current diagnostic algorithms assess total PSA, free PSA, and their ratio to enhance specificity [1,2]. A broad repertoire of analytical platforms has been devised for PSA detection, encompassing electrochemical assays, chemi-

luminescent and electrochemiluminescent methods, fluorescence-based detection, surface plasmon resonance, enzyme-linked immunosorbent assays (ELISA), immunochromatographic tests, and mass spectrometry [3-5].

Among these modalities, ELISA can be regarded as the most practicable and, simultaneously, the most informative approach for routine clinical laboratory diagnostics. This methodology is widely implemented across healthcare systems of varying levels of resource availability, owing to its advantageous balance of analytical sensitivity and specificity compared with alternative clinical assays [6-9]. The continuous development and refinement of serological immunoassays for the detection of clinically relevant analytes remain a cornerstone of modern analytical biochemistry.

The enzyme-linked immunosorbent assay (ELISA) developed for the quantitative determination of prostate-specific antigen (PSA) concentrations in human serum should be designed and validated in accordance with the requirements of EU Regulation (EU) 2017/746 on *in vitro* diagnostic medical devices (IVDR/MDR) [10].

The overarching objective of the study was to design and validate a high-sensitivity, high-informativeness enzyme-linked immunosorbent assay for the quantitative assessment of prostate-specific antigen concentrations in human serum.

Materials and Methods

PSA preparations. For the purposes of validation experiments, a preparation of total PSA was employed, obtained from human seminal plasma by means of immunoaffinity chromatography, a procedure that ensures a high degree of purification while preserving the native conformational and enzymatic properties of the protein. The isolated antigen was subsequently standardized (calibrated) against the First International Standard of the World Health Organization: WHO International Standard Prostate Specific Antigen (90:10), NIBSC code: 96/670. This reference preparation contains both molecular forms of PSA, namely, inhibitor-complexed and free antigen, in a physiologically relevant ratio of 9:1, corresponding to the proportions characteristic of PSA circulating in human serum. Utilization of such a calibrated standard guarantees a high level of reproducibility of the obtained results and enables reliable comparison with internationally established reference values.

Solid-phase ELISA procedure ("antibody sandwich" format). Sorption of anti-PSA monoclonal antibodies (mAbs) onto the solid phase was performed in 0.05 M carbonate-bicarbonate buffer (pH 9.6) at 4°C overnight, using an antibody concentration of 2 μg/ml (100 μl per well). Washing steps were carried out with phosphate-buffered saline containing 0.05% Tween-20 (PBST, pH 7.2). Aliquots of 100 μl of test serum or plasma samples, as well as standard PSA solutions, were dispensed into the wells, followed by incubation for 1 h at 37°C and subsequent washing with PBST. Detection of bound PSA within the immune complexes was achieved by means of a horseradish peroxidase (HRP)-labeled monoclonal antibody directed against a non-overlapping epitope. Incubation with the conjugate was performed for 1 h at ambient temperature, after which the plates were washed three times with PBST and once with distilled water. The substrate solution consisted of 0.003% hydrogen peroxide in 0.15 M citrate buffer (pH 5.0), with 3,3′,5,5′-tetramethylbenzidine (TMB) as the chromogenic indicator. The enzymatic reaction was terminated by the addition of 50 μl of 2 M sulfuric acid, and the optical density (OD) was subsequently measured at 450/620 nm.

Synthesis of peroxidase-antibody conjugates. Conjugation of mAbs with a horseradish peroxidase (HRP) was carried out at an antibody-to-enzyme mass ratio of 2:1, using the periodate oxidation method described by P. Tijssen [11] with modifications [5]. HRP (Sigma, USA) was dissolved in 0.1 M bicarbonate buffer (pH 8.3) at a concentration of 15 mg/ml and mixed with an equal volume of 14 mM sodium periodate solution. The oxidation reaction was allowed to proceed at room temperature for 2 h. In parallel, the mAb solution, pre-dialyzed against 0.1 M carbonate buffer (pH 9.2), was combined with the oxidized HRP. The mixture was applied to a chromatography column, supplemented with one-third of the volume of dry Sephadex G-25, and incubated at room temperature for 3 h. The resulting conjugate was eluted, and 1/20 volume of an aqueous sodium borohydride solution (5 mg/ml) was added. After 30 min of incubation, an additional 3/20 volume of sodium borohydride was introduced, and incubation was continued for 60 min. The final HRP-mAb conjugate was transferred into 0.02 M phosphate buffer containing 0.15 M NaCl by dialysis.

Chemical modification of polystyrene. Chemical modification of microtiter plate wells, aimed at enhancing their surface hydrophilicity, was per-

formed by immersion in a 0.2 M aqueous solution of ammonium perchlorate (AP). Such treatment induces partial structural degradation of polystyrene [12], accompanied by an increase in its hydrophilic properties. The degree of hydrophilization was controlled by varying the incubation time in the solution. Due to complexity of direct quantitative assessment of hydrophilicity by contact angle measurement [13] caused by the geometry of the wells, preliminary calibration was performed using Petri dishes made of chemically pure polystyrene. For the selected solution concentration at 38 ± 0.5°C, maximum hydrophilization was achieved after 4.8 days of exposure, at which point the contact angle decreased below 10°, making precise measurement technically challenging. In subsequent experiments, the hydrophilization degree of strips was conventionally estimated according to their exposure time in AP solution (one unit corresponding to one day of treatment). The process was carried out in a TA-50 dry-air thermostat, designed for microbial culture incubation. To prevent evaporation of solution from the wells, the strips were hermetically sealed with Parafilm.

Statistical analysis. Data were expressed as M ± SEM and analyzed by one-way analysis of variance (ANOVA), followed by Tukey's post hoc test, using OriginPro 7.5 software. Differences were considered statistically significant at $P < 0.05$.

Ethical considerations. The study involved only *in vitro* experiments using purified and standardized prostate-specific antigen (WHO International Standard, NIBSC 96/670) and previously characterized monoclonal antibodies. No human participants or animals were involved in this study. All procedures were conducted in compliance with the principles of the Declaration of Helsinki, OECD Good Laboratory Practice and Biosafety in Microbiological and Biomedical Laboratories.

Results and Discussion

Determination of the optimal monoclonal antibody combinations for the development of a non-competitive "sandwich" ELISA. The selection of antibody pairs was performed on the basis of our previously published findings [14], within the framework of which a panel of highly active and rigorously characterized monoclonal antibodies specific to PSA had been generated and subjected to detailed immunochemical profiling. Information concerning those antibodies deemed most promising for application in immunobiotechnology is summarized in Table 1.

Monoclonal antibodies belonging to specificity group I recognize epitopes of PSA that become sterically concealed upon complex formation with α_1-antichymotrypsin (α_1-ACT). Consequently, these antibodies are suitable exclusively for the detection of the free antigenic form of PSA. By contrast, mAbs assigned to specificity group II are directed against epitopes that remain accessible even after PSA binds α_1-ACT, thereby permitting their use in the detection of total PSA, comprising both the free antigen and its inhibitor-complexed counterpart (Fig. 1).

At the subsequent stage of the investigation, particular emphasis was placed on the selection of the most suitable pair of monoclonal antibodies for the construction of a non-competitive sandwich ELISA intended for the quantitative determination of total PSA. According to the results of detailed epitope mapping [14], the greatest spatial divergence was observed between epitopes P2-P4 and P2-P3. Taking into consideration the epitope specificity of antibodies belonging to distinct groups, the most promising candidates for the development of an immunoassay for total PSA detection were identified as those directed against the P2-P3 epitopes, namely clones 11G5, 23B4, 21B7, 21F4, 27C10, and 26B9. For the purpose of pairwise evaluation, peroxidase conjugates of these antibodies were synthesized, and their efficacy in detecting total PSA was assessed in a sandwich ELISA format by combining mAbs that recognize topographically distinct epitopes of the antigen.

To determine the optimal orientation of monoclonal antibodies (mAbs) within the test system, antibodies recognizing distinct epitopes were systematically evaluated both as immobilized capture reagents and as HRP-conjugated detection antibodies (Table 2). When mAbs directed against epitope P2 were employed in the form of enzyme conjugates and those specific for epitope P3 were immobilized as the capture component, the mean ELISA signal reached 1.182 OD units. Conversely, in the reciprocal configuration – where the P3-specific mAbs were conjugated with HRP and P2-specific antibodies were used as the immobilized sorbent – the mean signal intensity was substantially lower, amounting to 0.700 OD units. Among all combinations examined, the antibody pair 26B9 (P2)-27C10 (P3) demonstrated the most favorable performance.

Accordingly, for the development of a non-competitive "sandwich"-format ELISA intended for the determination of total PSA, the use of P2-specific antibodies (26B9, 21B7, 11G5) as immobilized

Table 1. Characteristics of monoclonal antibodies against PSA [14]

mAbs	Origin (inbred murine strain)	OD_1 in indirect ELISA (tested against …)			mAb isotype	Titer[1)]	Affinity constant[1)], 10^9 M^{-1}	Epitop	Epitope specificity group	Degree of inhibition of PSA enzymatic activity
		PSA	PSA– α_1-ACT	hK2						
14C8	Balb/c	2.141	0.015	0.039	IgG2a	1:800	16	P4	I	18.0
11G5	Balb/c	2.224	2.051	0.141	IgG2a	1:800	16	P2	II	-0.2
21D7	NZB	2.574	0.054	0.042	IgG1	1:1600	20	P4	I	-3.4
23B4	NZB	2.479	2.051	0.045	IgG2b	1:800	16	P3	II	92.4
21B7	NZB	2.158	2.054	0.066	IgG1	1:800	8	P2	II	98.7
21F4	NZB	2.469	2.603	0.057	IgG1	1:1600	16	P3	II	60.3
27C10	NZB	2.425	2.197	0.032	IgG2a	1:1600	20	P3	II	26.2
26B9	NZB	1.984	1.935	0.074	IgG1	1:800	16	P2	II	3.6

Note. *Data represent mean values obtained from hybridoma supernatant assays performed in four replicates ($P < 0.05$)

capture reagents, in conjunction with P3-directed mAbs (21F4, 23B4, 27C10) as enzyme conjugates, appears to be the most rational strategy. It should be emphasized that prior experimental evidence from analogous ELISA systems indicated the feasibility of enhancing the analytical signal, without concomitant elevation of background noise, through the employment of mixtures of antibody conjugates exhibiting closely related epitope specificities [14]. On this basis, we investigated the efficacy of mixed conjugates comprising 27C10 + 21F4 and 27C10 + 23B4. However, the results (Fig. 2) clearly demonstrated that, in the context of total PSA determination, these combinations failed to yield any statistically significant improvement in assay signal.

In conclusion, the comparative analysis of alternative antibody pairings identified the combination 26B9-27C10 as the most promising configuration for the construction of a non-competitive sandwich ELISA system for quantifying total PSA.

Optimization of monoclonal antibody adsorption conditions via modulation of the hydrophilic properties of the solid phase. For the construction of enzyme immunoassay kits, polystyrene microplates with variable sorption characteristics are employed as the solid phase. The selection of a particular plate type is dictated by the physicochemical properties not only of the analyte under investigation but also of the biomolecules intended to be immobilized onto the solid surface.

The adsorption of any molecule onto a solid surface is governed by intermolecular interactions and ionic bonding. In this context, intermolecular forces are primarily mediated by the polarity of molecules, which can manifest as either variable (dynamic) or constant (stationary) polarity. In the case of variable polarity (VP), molecules bearing heterogeneous surface charges are capable of interacting through mutual orientation. Moreover, the magnitude of VP correlates with molecular dimensions analogous to the manner in which the melting and boiling points of nonpolar hydrocarbons increase with the number of carbon atoms in the chain. Variable and stationary polarities exhibit distinct distance-dependent behaviors: whereas the strength of VP interactions decreases with increasing intermolecular separation, the opposite trend is characteristic of stationary polarity (SP). It should be emphasized that among all SP-mediated interactions, hydrogen bonding is by far the most prominent, its strength being at least an order of magnitude greater than that of other intermolecular forces; consequently, its contribution

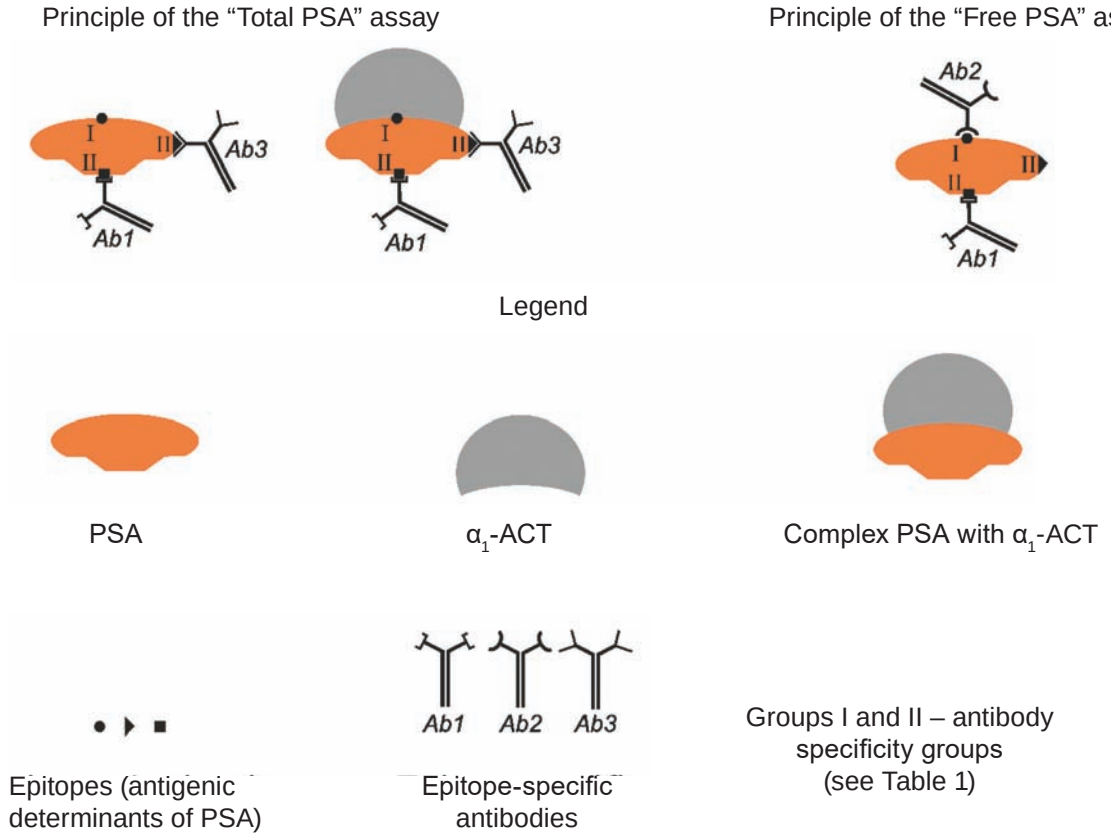

Fig. 1. Schematic representation of the sandwich ELISA for the determination of different PSA forms [5]

Table 2. Efficacy of total PSA detection by mAbs directed against distinct epitopes in a sandwich ELISA

Sorbtion			Conjugate					
			Epitopes and mAbs					
			P2			P3		
			21B7	11G5	26B9	21F4	23B4	27C10
Epitopes and mAbs	P2	21B7	-	-	-	1.205	0.927	1.114
		11G5	-	-	-	1.105	0.998	1.220
		26B9	-	-	-	1.118	1.429	**1.524**
	P3	21F4	0.651	0.591	0.459	-	-	-
		23B4	0.574	0.748	0.534	-	-	-
		27C10	0.999	0.817	0.928	-	-	-

Note. The table summarizes mean OD values from ELISA performed with a 10 ng/ml PSA standard in quadruplicate;$P < 0.05$

to the processes of biomolecular adsorption on solid substrates is the most significant. [15]

According to our experimental observations, the signal intensity in immunoenzymatic assays is determined by several interdependent factors. Foremost among these is the amount of biomolecule immobilized on the solid phase, in the present case anti-PSA monoclonal antibodies. It should be emphasized that once a certain threshold concentration of adsorbed protein is surpassed, a saturation phenomenon may emerge, and the identification of such concentration ranges is generally achieved through

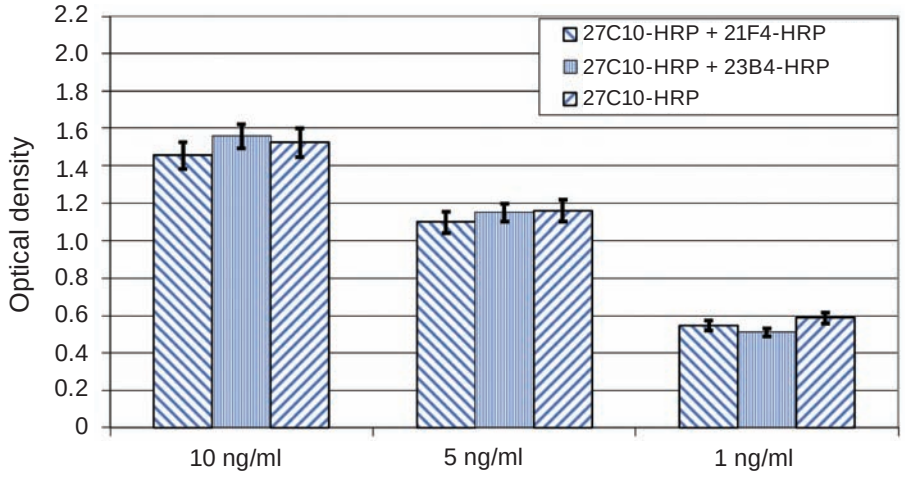

Fig. 2. Outcomes of the sandwich-type ELISA for the determination of various concentrations of PSA employing the 27C10 mAb conjugate, as well as its binary mixtures with HRP-conjugated mAbs 21F4 and 23B4

empirical procedures rather than theoretical prediction. At the same time, within specific intervals of surface coverage, an increase in signal intensity may be observed concomitantly with an augmentation of surface hydrophilicity. The extent of protein adsorption from solution is strongly dependent on its hydrophilic or hydrophobic character. Considering that antibodies, both polyclonal and monoclonal, possess carbohydrate moieties that are inherently variable in terms of their degree of hydrophilicity, such structural features may substantially influence the efficiency of adsorption as well as the spatial orientation of the immobilized molecules.

It is well established that antibodies synthesized by animal cells represent typical glycoproteins, which may contain no fewer than ten monosaccharide residues and are classified into two principal categories, namely N-glycoproteins, in which the carbohydrate moiety is covalently linked to the protein backbone through the nitrogen atom of an asparagine residue, and O-glycoproteins, where the carbohydrate chain is attached to the oxygen atom of serine or threonine residues of the polypeptide chain. At present, N-glycans have been investigated in greatest detail [16, 17]. The glycans of Fc and Fab fragments of human and animal IgG, while sharing a common biantennary structure consisting of a branched heptasaccharide core and two disaccharide antennae, differ considerably with respect to their specific monosaccharide composition. In healthy individuals, approximately 15-25% of IgG Fab fragments are glycosylated [18]. N-glycosylation of the Fab fragment may either decrease or enhance the antigen-binding capacity of the immunoglobulin, its serum half-life, and its propensity for aggregation and immune complex formation [19, 20]. The most frequently invoked explanation for these phenomena is the hypothesis that carbohydrate residues exert their effect through modulation of the conformation of the antibody variable region [17].

Although the glycosylation of Fc fragments of IgG has been studied far more extensively than that of Fab regions, the majority of these investigations have been directed toward the potential modulation of the functional activity of therapeutic antibodies. Consequently, given that the present study is focused on the *in vitro* application of monoclonal antibodies, a detailed discussion of Fc glycosylation data lies beyond the scope of our work. Nevertheless, it has been demonstrated recently [21] that the pattern of N-glycosylation of murine IgG Fc fragments varies between distinct antibody isotypes and across inbred mouse strains, including Balb/c, C57BL/6, CD-1, and Swiss Webster. This consideration is of particular relevance for the present study, as the antibody panel employed for the development of the two-site immunoenzymatic assay comprises monoclonal antibodies of different isotypes (namely IgG2a, IgG1, and IgG2b) obtained from animals of distinct inbred strains (Balb/c and NZB) [14].

When considering antibodies of biotechnological origin, it is essential to emphasize the pronounced variability in both the quantitative and qualitative composition of their glycan moieties. As noted by [17], the vast majority of monoclonal antibodies are produced either through expression

and cultivation in Chinese hamster ovary (CHO) cells or via hybridoma technology employing murine myeloma-derived cell lines such as Sp2/0 and NS0. Although both biotechnological platforms are based on mammalian cells belonging to the same taxonomic class, comparative analyses of the glycosylation profiles of the resulting antibodies reveal substantial differences. These discrepancies are attributable to the divergent intracellular glycosylation environments, which include variations in the pools of nucleotide sugar precursors, in the activities of specific synthetases, glycosyltransferases, and glycosidases, as well as in the abundance and specificity of nucleotide sugar transporter proteins [22-24]. Consequently, the composition of the culture medium in the case of *in vitro* hybridoma cultivation, and, analogously, the physiological status of animals in the case of *in vivo* antibody accumulation, exert a profound influence on the structural characteristics of antibody glycosylation.

A critical factor in the development of a highly informative antibody-based sandwich-type ELISA is the concentration of monoclonal antibodies used for solid-phase immobilization and the concentration of the antigen to be detected, as well as in their relative ratios and the geometrical dimensions of these molecules. The establishment of optimal antibody concentrations for adsorption is essential not only from the standpoint of achieving superior analytical performance but also with regard to the techno-economic feasibility of the assay design. According to the manufacturer's specifications for polystyrene microtiter plates [25], the maximum sorptive capacity of the polystyrene surface for monomeric IgG molecules is approximately 650 ng/cm^2, with an average value of 400 ng/cm^2 when considering the steric dimensions of the immunoglobulin molecule. Given that total PSA represents a combination of free PSA, with a molecular mass of 37 kDa, and PSA complexed with α1-antichymotrypsin (α_1-ACT), with a molecular mass of approximately 90 kDa, the mass ratio (and consequently, the relative molecular dimensions) between different PSA forms and immunoglobulins amounts to (1.9-4.6):1. Such a ratio may be regarded as relatively favorable in comparison with high-molecular-weight antigens. Nevertheless, it is evident that the spatial dimensions of PSA, and particularly of the PSA–α_1-ACT complex, preclude the maximal realization of antigen-antibody binding potential. The putative steric constraints inherent to the sandwich-type ELISA format for the determination of total PSA are schematically depicted in Fig. 1. It becomes apparent that all of the aforementioned factors influencing the analytical parameters of the sandwich-type ELISA can only be empirically established, including the optimal concentrations of capture and detection antibodies, the dynamic range of analyte concentrations, and the idiosyncratic characteristics of each monoclonal antibody employed. As part of the overall ELISA development process, the optimal incubation conditions – both in terms of temperature and duration – for each analytical step were also systematically determined.

Given the available evidence regarding differences in the degree of glycosylation across antibody isotypes and sources of origin, including distinct inbred animal strains, the subsequent stage of our study was devoted to establishing the optimal sorption conditions in terms of both the concentration ranges of antibodies and the presence of analyte (PSA) in model test samples, as well as to comparing these conditions for three distinct monoclonal antibodies employed in the composition of the immunosorbent. Based on our experimental experience, the signal intensity in the sandwich-type ELISA is largely governed by the amount of immobilized biomolecule, in this case anti-PSA monoclonal antibodies. It must be emphasized that exceeding a certain threshold concentration of the sorbed protein may result in a saturation effect, the definition of which is typically determined empirically within a specific concentration range.

In the present study, we employed Nunc MaxiSorp™ 96-well polystyrene plates (Thermo Fisher Scientific, USA), the surface of which exhibits enhanced protein-binding capacity due to proprietary chemical modifications introduced during the manufacturing process. These plates are specifically recommended for immunoglobulin adsorption, for which the manufacturer reports a sorptive capacity of approximately 600-650 ng IgG/cm^2. Nevertheless, the efficiency of analyte detection in sandwich-type ELISA is determined not solely by the binding capacity of the sorbent but also by the spatial orientation of IgG molecules on the solid surface. An optimal orientation is expected to avoid steric hindrance of antigen interaction with the Fab fragments of IgG, which harbor the antigen-binding sites specific for PSA. Although sensitization of wells through passive antibody adsorption does not generally permit direct control of molecular orientation, this parameter can be indirectly modulated by altering the physico-

chemical properties of the sorbent surface. Given that antibodies – whether polyclonal, monoclonal, or recombinant – possess carbohydrate moieties of variable hydrophilicity, an increase in surface hydrophilicity may, within a certain concentration range, yield an enhancement of the analytical signal. This effect arises from the dual influence of surface hydrophilicity on both the extent of protein adsorption from solution and the spatial arrangement of the adsorbed immunoglobulins.

In the course of our study, we compared the performance of the sandwich-type ELISA using plates with their wells in the native state of surface modification and those subjected to additional chemical treatment intended to enhance surface hydrophilicity. The latter approach was based on the partial disruption of the superficial polystyrene layer with an aqueous solution of polyacrylic acid, as described above. Following such treatment, the well surfaces exhibited no visible evidence of structural degradation, irrespective of the degree of hydrophilicity attained.

The proposed ranges of concentrations were selected on the basis of our own empirical experience in conjunction with data reported in the literature [26-32]. Monoclonal antibodies were immobilized within the concentration interval of 0.625–5 μg/ml (with a sorption volume of 100 μl per well). Model PSA solutions prepared in physiological saline were employed at concentrations of 1, 5, and 10 ng/ml, which correspond to levels typically observed in healthy individuals as well as in patients with prostatitis, benign prostatic hyperplasia, and prostate carcinoma. For detection, a horseradish peroxidase-conjugated monoclonal antibody 27C10 was utilized. A series of three analogous experiments was carried out with different monoclonal antibodies incorporated into the immunosorbent, namely 26B9, 21B7, and 11G5 (Fig. 3-5).

Two of the monoclonal antibodies investigated for immobilization, namely 26B9 and 21B7, demonstrated a pronounced enhancement of the ELISA signal against the background of increased hydrophilicity of the polystyrene plates. Specifically, for different PSA concentrations in the sample, this enhancement ranged from 1.26 to 1.34 and from 1.35 to 1.39, respectively. In contrast, antibody 11G5 produced only a modest increase, with values confined to the range of 1.13-1.23. For all monoclonal antibodies used within the immunosorbent, we observed a consistent trend of only minor or negligible signal elevation at relative degrees of surface hydrophilization between 0.1 and 0.3, where the optical density in ELISA did not exceed a 1.09-fold increase. The most pronounced enhancement of the analytical signal relative to the baseline method (i.e., without modification of the physicochemical properties of polystyrene) was observed at relative hydrophilization levels of 2 and 4.8.

It is noteworthy that the pattern of signal modulation across the range of hydrophilization values differed somewhat depending on the antibody employed. For immunosorbents based on 26B9 and 11G5, the relationship between polymer hydrophilization and ELISA response (at varying PSA concentrations) was characterized by a relatively gradual increase, whereas in the case of antibody 21B7, a stepwise transition was evident at a relative hydrophilization degree of 0.8. It can further be concluded that at low immobilization concentrations of all tested monoclonal antibodies (0.625 and 1.25 μg/ml), no appreciable changes in ELISA signal were observed, irrespective of the hydrophilization level. Another interesting observation should be emphasized: when comparing the signal-dependence profiles for antibody concentrations of 5 and 2.5 μg/ml, a convergence of values was apparent at relative hydrophilization degrees of 2 and 4.8.

The two monoclonal antibodies, 26B9 and 21B7, whose incorporation into the immunosorbent was associated with a more pronounced increase in the ELISA signal, were generated from splenocytes of NZB mice and belong to the IgG1 isotype. In contrast, antibody 11G5, which exerted only a negligible effect on the signal intensity, was derived from splenocytes of Balb/c mice and is classified as an IgG2a immunoglobulin. Although the small number of monoclonal antibodies examined in this study precludes the formulation of any definitive conclusions regarding potential correlations between antibody isotype or origin on the one hand, and their ability to adsorb onto polystyrene surfaces on the other, the data unequivocally demonstrate that even antibodies of identical specificity, produced by the same hybridoma technology under comparable conditions, may exhibit marked differences in physicochemical properties that govern their adsorption efficiency.

These findings bear significant implications for the development of immunosorbent production protocols by biotechnology companies engaged in the design and manufacture of ELISA-based diagnostic reagents. Specifically, they underscore the potential for substantial reductions in the quantity of monoclonal antibodies required for efficient adsorption, a

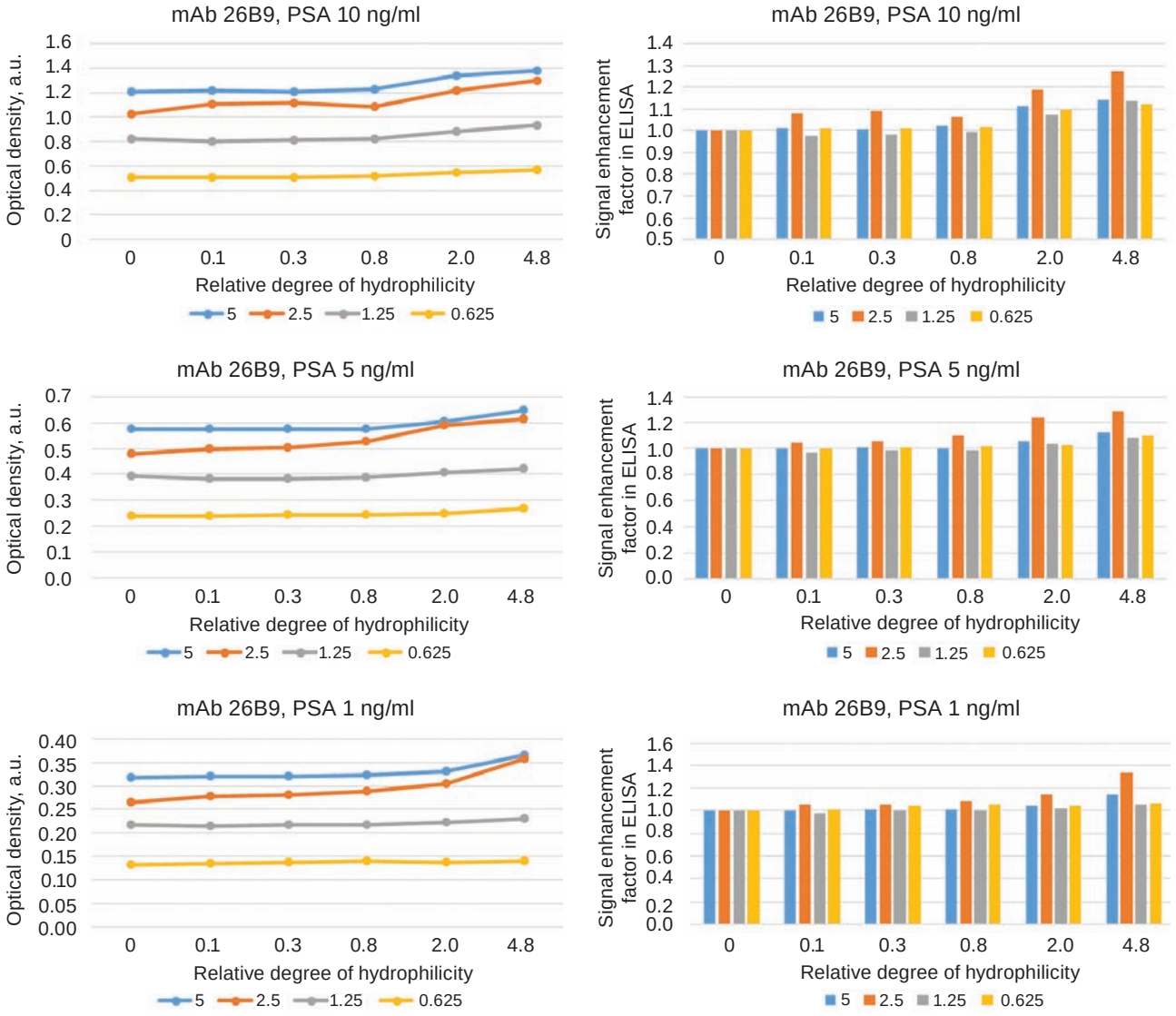

Fig. 3. Results of the experiment with an immunosorbent incorporating monoclonal antibody 26B9

factor of particular importance when the biomolecular components involved are of high economic value, thereby directly influencing the cost-effectiveness and scalability of the production technology.

Development of the quantitative ELISA protocol and its validation. Following the identification of the optimal monoclonal antibody pair for the "sandwich" ELISA format and the optimization of their adsorption conditions, the subsequent stage involved fine-tuning the analytical parameters of the assay. This optimization included determination of the working concentrations of capture and detection antibodies, adjustment of the concentration of the enzyme conjugate, evaluation of incubation conditions (time and temperature), selection of the sample volume, and optimization of the reaction buffer composition. The final protocol, derived from these studies and presented below, represents an optimized "sandwich" ELISA configuration for which the analytical performance characteristics were established.

The assay development was initiated with a systematic analysis of the biochemical properties of the mAb panel [14]. For selection of an effective antibody pair in the sandwich format, the primary criteria included epitope specificity and assignment to the appropriate specificity group. In accordance with the principles of ELISA design for total PSA determination (Fig. 1), the optimal antibody combination must comprise members of Group II specificity recognizing distinct epitopes. Based on this rationale, antibodies targeting epitope P2 (21B7, 11G5, 26B9) and those targeting epitope P3 (21F4, 23B4,

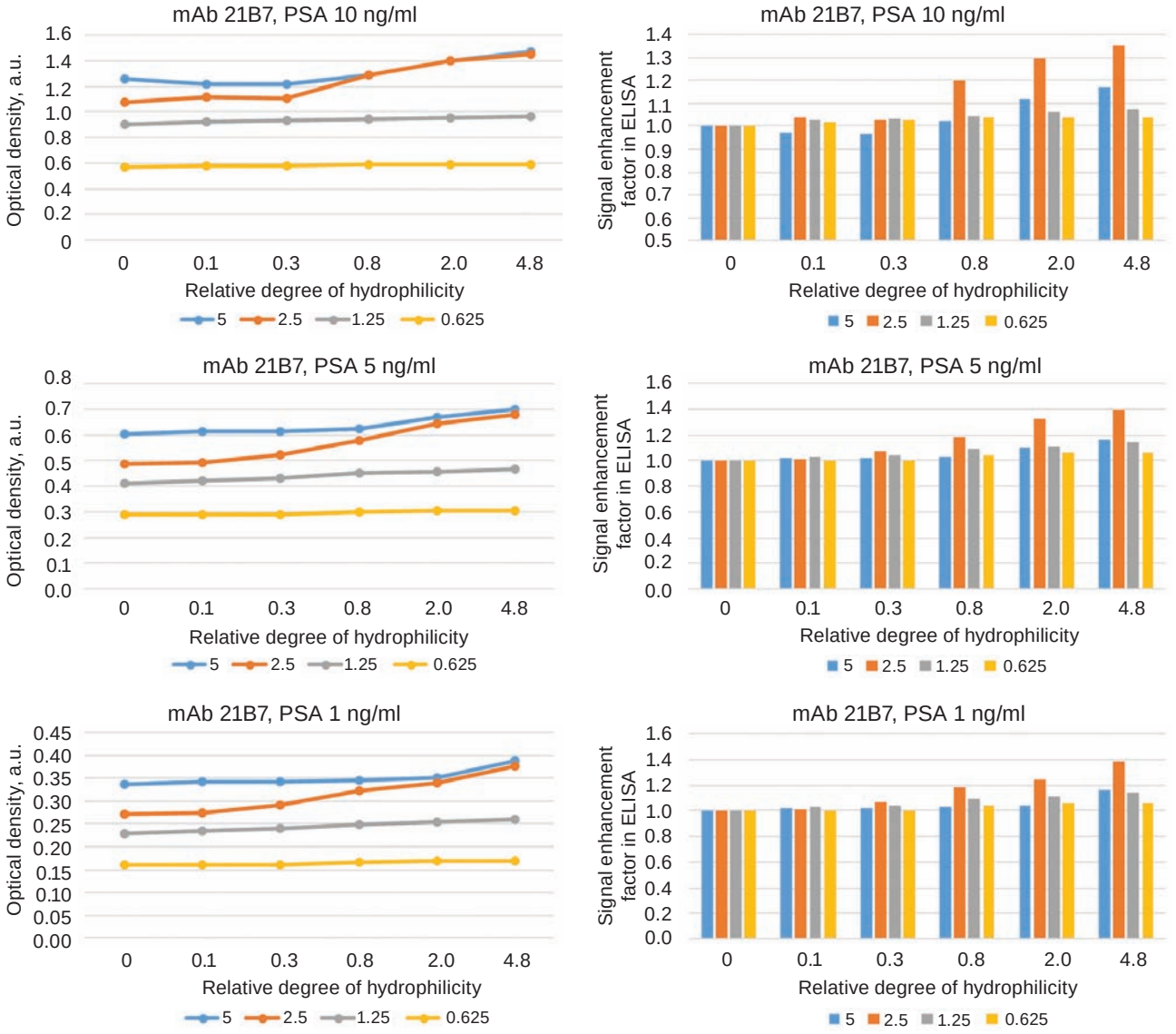

Fig. 4. Results of the experiment with an immunosorbent incorporating monoclonal antibody 21B7

27C10) were selected for evaluation in different assay configurations (either immobilized on the solid phase or used as enzyme conjugates).

A separate aspect of the optimization process was the assessment of the feasibility of employing antibody mixtures. Previous reports have indicated that combining conjugates with closely related epitope specificities may occasionally enhance signal intensity [5, 14]. In this study, we examined the performance of mAb conjugate mixtures (27C10 + 21F4 and 27C10 + 23B4) relative to the single conjugate mAb 27C10, which had demonstrated the highest individual activity (Table 2). The expansion of this comparison to include additional combinations was limited by the availability of suitable antibodies. Ultimately, the results confirmed that the use of conjugate mixtures did not improve assay sensitivity in PSA detection and, therefore, was not considered a practical strategy.

Conclusions. A comprehensive characterization of anti-PSA monoclonal antibodies demonstrated that antibodies directed against epitopes P2 and P3 (belonging to the same specificity group) represent the most suitable “sandwich” combination for the development of a non-competitive ELISA. The sorption-detection performance of different antibody pairs correlated with their binding affinity. The most effective antibodies for immobilization on the solid phase were mAbs 21B7, 11G5, and 26B9 (epitope P2, specificity group II), whereas the most effective de-

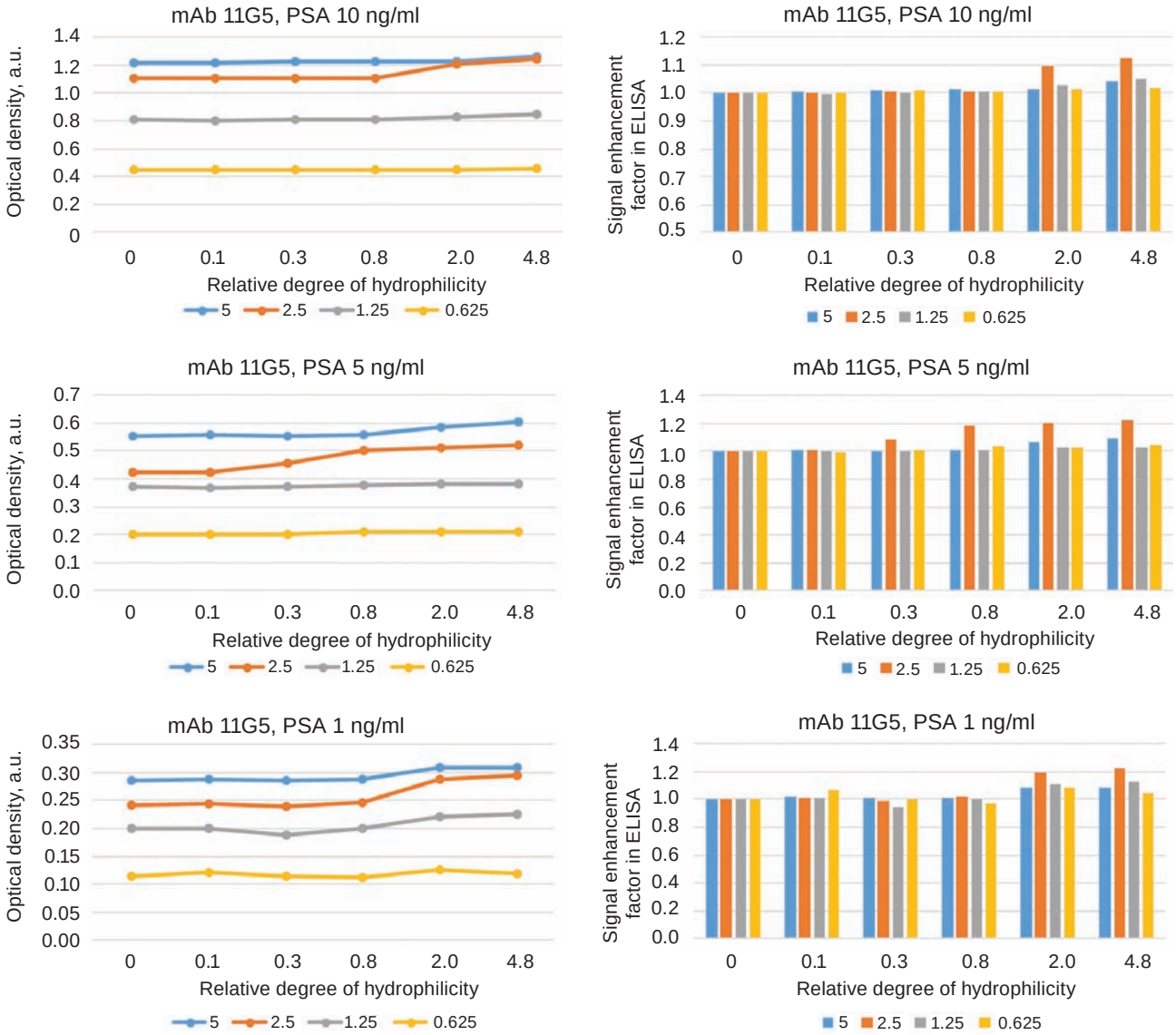

Fig. 5. Results of the experiment with an immunosorbent incorporating monoclonal antibody 11G5

tection antibodies (used as enzyme conjugates) were mAbs 21F4, 23B4, and 27C10 (epitope P3, specificity group II). Among these, the high-affinity pair 26B9-27C10 exhibited the strongest sorption-detection properties. In contrast, the use of conjugate mixtures (27C10 + 21F4 or 27C10 + 23B4) did not enhance the sensitivity of PSA detection within the concentration range of 1-10 ng/ml.

The influence of hydrophilization of the polystyrene surface on ELISA signal intensity was also investigated. The most pronounced increase in assay signal (1.26-1.39-fold) following surface hydrophilization was observed with mAbs 26B9 and 21B7. The maximum increase in optical density was noted at relative hydrophilization levels of 2-4.8. This effect is likely attributable to differences in the carbohydrate composition of antibodies of distinct isotypes (IgG1 vs. IgG2a) and different origins (Balb/c vs. NZB mouse strains).

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ІМУНОЕНЗИМНИЙ АНАЛІЗ ДЛЯ ВИЗНАЧЕННЯ ЗАГАЛЬНОГО ПРОСТАТ-СПЕЦИФІЧНОГО АНТИГЕНУ

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Простатоспецифічний антиген (ПСА) залишається найпоширенішим біомаркером для діагностики та моніторингу раку простати. Розробка високоінформативних, чутливих та відтворюваних імуноензимних аналізів для визначення ПСА є важливою для підвищення точності діагностики. Метою дослідження було розробити та оптимізувати неконкурентний «сендвіч»-ELISA для визначення загального ПСА із використанням панелі моноклональних антитіл (mAb) різних груп специфічності та епітопів. Конфігурації «сендвіч»-ELISA було сконструйовано з використанням різних пар антитіл для захоплення та виявлення. Оптимізовано умови сорбції антитіл, робочі концентрації реагентів, параметри інкубації та склад буфера. Аналітична ефективність оцінювалася з використанням препаратів ПСА, стандартизованих відповідно до Міжнародного стандарту ВООЗ (96/670). Найефективніші комбінації антитіл виявлено серед моноклональних антитіл, спрямованих на епітопи P2 (захоплення: 21B7, 11G5, 26B9) та P3 (детектування: 21F4, 23B4, 27C10), причому високоафінна пара 26B9-27C10 демонструвала найкращі сорбційно-детекторні властивості. Використання змішаних кон'югатів не покращило чутливість у діапазоні 1-10 нг/мл ПСА. Гідрофілізація поверхні полістирольних пластин підвищувала сигнал ІЕА до 1,39 раза, залежно від ізотипу та походження антитіла. Розроблений оптимізований «сендвіч» ІЕА продемонстрував високу специфічність та чутливість для визначення загального ПСА, з аналітичними характеристиками, придатними для потенційного застосування в клінічній діагностиці.

Ключові слова: простат-специфічний антиген, моноклональні антитіла, ІЕА, імуноаналіз, оптимізація аналізу, кон'югати, чутливість, гідрофілізація, діагностика.

References

1. Hollenberg MD. Hypothetical structure of prostate-specific antigen. *Clin Chem*. 2014; 60(4): 702.
2. Galkin OYu, Komar AG, Besarab OB. Different mice inbred strains humoral immune response against human prostate-specific antigen. *Ukr Biochem J*. 2019; 91(1): 30-37.
3. Yasnikova M, Kudriavtsev Y, Ponyrko A, Malyuk A, Riabenko T, Romaniuk A. Comparative analysis of MRI, morphological and biochemical features of malignant neoplasm of the prostate gland. *East Ukr Med J*. 2024; 12(2): 369-378.
4. Tkachenko NO, Protsenko OS, Remnyova NO, Chumak LI, Smolienko NP, Bielkina IO, Marakhovskyi IO, Korenieva YM, Bondarenko VO. Morphometric features of the prostate gland in rats with sulpiride-induced hyperplasia after combined administration of vitamin D and saw palmetto fruit extract. *J V N Karazin Kharkiv Natl Univ Ser Med*. 2025; 33(1(52)): 19-32.
5. Komar A, Kozerecka O, Besarab O, Galkin A. Development and validation of a highly informative immuno-enzymatic analysis for the determination of free prostat-specific antigen. *Innov Biosyst Bioeng.* 2019; 3(4): 220-231.
6. Hladkykh FV, Liadova TI. Analgesic potential of cryoextracts of biological tissues and conditioned media of mesenchymal stem cells in the treatment of experimental autoimmune arthritis. *Odesa Med J*. 2024; (1): 35-41.
7. Kulaiets NM. A differentiated approach to the assessment of biomarkers depending on the phenotype of heart failure. *Ukr J Cardiovasc Surg.* 2025; 33(1): 39-47.
8. Tytov EV, Yakovtsova II, Ivakhno IV, Nehoduiko VV, Makarov VV, Panasenko SI. Prognostic value of a panel of immunohistochemical markers for determining the risk of recurrence and progression of non-invasive bladder cancer. *Ukr J Radiol Oncol*. 2023; 31(4): 391-403.
9. Shevchuk K, Baranovska A, Chernetskyi A, Shchotkina N, Besarab A. Biosafety aspects of hybridoma technology: nature of risks and

approaches to their management. *Innov Biosyst Bioeng.* 2025; 9(2): 29-41.
10. Dmytrenko O, Golembiovska O, Pashuk V, Zgonnyk S. Problems of classification, safety assessment and risk management of medical devices with biologically active substances. *Sci Rise Pharm Sci.* 2025; (2(54)): 86-104.
11. Practice and theory of enzyme immunoassays. In: Laboratory techniques in biochemistry and molecular biology. Ed. P. Tijssen. Elsevier, 1985. 549 p.
12. Gorsuch TT. The destruction of organic matter. Pergamon, Oxford, 1970.
13. Prydatko AV, Myroniuk AV, Svyderskyi VA. Analysis of approaches to mathematical description of the characteristics of materials with high hydrophobicity. *East Eur J Enterp Technol.* 2015; 5(5(77)): 30.
14. Galkin A, Komar A, Gorshunov Y, Besarab A, Soloviova V. New monoclonal antibodies to the prostate-specific antigen: obtaining and studying biological properties. *J Microbiol Biotechnol Sci.* 2019; 9(3): 573-577.
15. Israelachvili JN. Intermolecular and Surface Forces (3rd ed.). Academic Press, 2011.
16. Lin Z, Lo A, Simeone DM, Ruffin MT, Lubman DM. An N-glycosylation Analysis of Human Alpha-2-Macroglobulin Using an Integrated Approach. *J Proteomics Bioinform.* 2012; 5: 127-134.
17. Wang T, Liu L, Voglmeir J. mAbs N-glycosylation: Implications for biotechnology and analytics. *Carbohydr Res.* 2022; 514: 108541.
18. van de Bovenkamp FS, Hafkenscheid L, Rispens T, Rombouts Y. The Emerging Importance of IgG Fab Glycosylation in Immunity. *J Immunol.* 2016; 196(4): 1435-1441.
19. Schneider D, Dühren-von Minden M, Alkhatib A, Setz C, van Bergen CA, Benkißer-Petersen M, Wilhelm I, Villringer S, Krysov S, Packham G, Zirlik K, Römer W, Buske C, Stevenson FK, Veelken H, Jumaa H. Lectins from opportunistic bacteria interact with acquired variable-region glycans of surface immunoglobulin in follicular lymphoma. *Blood.* 2015; 125(21): 3287-3296.
20. Courtois F, Agrawal NJ, Lauer TM, Trout BL. Rational design of therapeutic mAbs against aggregation through protein engineering and incorporation of glycosylation motifs applied to bevacizumab. *MAbs.* 2016; 8(1): 99-112.
21. de Haan N, Reiding KR, Krištić J, Hipgrave Ederveen AL, Lauc G, Wuhrer M. The N-Glycosylation of Mouse Immunoglobulin G (IgG)-Fragment Crystallizable Differs Between IgG Subclasses and Strains. *Front Immunol.* 2017; 8: 608.
22. Butler M, Spearman M. The choice of mammalian cell host and possibilities for glycosylation engineering. *Curr Opin Biotechnol.* 2014; 30: 107-112.
23. Kaverinska A, Shevchenko N, Osetsky A, Sukhodub L, Lazurenko V, Zhelezniakov O, Prokopiuk V. The effect of lyophilized and frozen umbilical cord cryoextract on L929 cell culture. *Innov Biosyst Bioeng.* 2025; 9(1): 3-12.
24. Bila G, Vovk V, Utka V, Grytsko R, Havrylyuk A, Chopyak V, Bilyy R. NET-inducing diamond nanoparticles with adsorbed hydrophobic SARS-CoV-2 antigens serving as vaccine candidate. *Ukr Biochem J.* 2024; 96(4): 95-105.
25. Esser P. Effects of enlarged surface/volume ratio in solid phase assays. Technical Bulletin: 12a. Thermo Fisher Scientific Inc. 2010. Available from: https://www.thermofisher.co.nz/Uploads/file/Scientific/Applications/Lab-Plasticware-Glassware-Supplies/Effects-of-Enlarged-Surface-Volume-Ratio-in-Solid-Phase-Assays-Documented-on-the-basis-of-Nunc-StarWell-Modules.pdf.
26. Galkin OYu, Savchenko AA, Nikitina KI, Dugan OM. Obtaining and study of properties of new monoclonal antibodies against human IgE. *Ukr Biochem J.* 2013; 85(5): 81-87.
27. Galkin OuY, Besarab AB, Lutsenko TN. Characteristics of enzyme-linked immunosorbent assay for detection of IgG antibodies specific to Chlamydia trachomatis heat shock protein (HSP-60). *Ukr Biochem J.* 2017; 89(1): 22-30.
28. Acevedo B, Perera Y, Ruiz M, Rojas G, Benítez J, Ayala M, Gavilondo J. Development and validation of a quantitative ELISA for the measurement of PSA concentration. *Clin Chim Acta.* 2002; 317(1-2): 55-63.
29. Gutiérrez-Zúñiga GG, Hernández-López JL. Sandwich-type ELISA impedimetric immunosensor for early detection of prostate-specific antigen (PSA) in human serum. *Procedia Chem.* 2014; 12: 47-54.
30. Mashkoor FC, Al-Asadi JN, Al-Naama LM. Serum level of prostate-specific antigen (PSA)

in women with breast cancer. *Cancer Epidemiol*. 2013; 37(5): 613-618.
31. Khudetskyy I, Antonova-Rafi J. Human influence and changes in nature on biological security (overview of the problem). *Phytotherapy J*. 2023; (2): 26-34.
32. Labunets I, Toporova O, Panteleymonova T, Dovbynchuk T, Kyryk V, Kashchuk O, Kordium V. Comparative effects of human umbilical cord-derived mesenchymal stromal cells and their extracellular vesicles in a mouse model of parkinsonism. *Cell Organ Transpl*. 2025; 13(1): 46-53.