Colorimetric detection of immobilized horseradish peroxidase based on the co-oxidation of benzidine derivatives and 4-chloro-1-naphthol

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Enzymatic co-oxidation of benzidine derivatives and 4-chloro-1-naphthol with hydrogen peroxide was studied for the colorimetric detection of horseradish peroxidase using the DNA microarray on glass surface technology. The co-oxidation of *o*-dianisidine and 4-chloro-1-naphthol affords a new product with the intense violet color characterized by a high rate of accumulation, good adsorbability on the glass surface, and stability. This reaction can be used as a detection system for the identification of β-lactamase genes on DNA microarrays. The DNA microarray technique using horseradish peroxidase and colorimetric detection is characterized by high sensitivity and reproducibility comparable to the characteristics of the microarrays with fluorescence detection.

Key words: horseradish peroxidase, 4-chloro-1-naphthol, benzidine derivatives, substrate—substrate activation, DNA microarrays, class A β-lactamases.

Oxidation reactions of a series of electron-releasing substrates with hydrogen peroxide catalyzed by peroxidase from horseradish roots (HP) are widely used as detection systems in analytical biotechnology and clinical medicine.[1] Specific features of HP are the high catalytic activity and stability and a low specificity toward an electron-releasing substrate. Therefore, various substituted aromatic hydrocarbons can be used as chromogenic substrates of HP and serve as a basis for detection systems. Depending on the type of analysis, HP detection is carried out by either the accumulation of a colored product in solution or the accumulation of a colored product adsorbed on a support surface. The latter detection type is characteristic of histochemical, immunoblotting, and membrane immunoenzymatic analytical methods.[2]

In the recent time, one of intensely developed directions of analytical biotechnology is the use of protein and DNA microarrays. Microarrays represent a matrix of microcells arranged on a support surface. Specific probes capable of recognizing biologically active molecules are immobilized in each microcell. Such probes can be oligonucleotides, DNA and RNA fragments, proteins, *etc*. The main advantage of the microarray method is the possibility of performing multiparametric analysis of a sample at minimum consumptions of the analyzed material and reagents.[3—5]

Glass slides with modified surface for covalent immobilization of probes are generally used as supports for microarrays.[3,4] The operation of all bioarrays is based on the principle of molecular recognition, due to which specific biological complexes are formed in microarray cells. A tag is introduced into molecules under study to detect these complexes. Modern diagnostic microarrays are often based on the use of various fluorescent labels.[6] The main advantages of this detection type are high sensitivity and simplicity of analysis; however, high cost of reagents and, especially, detecting devices prevent wide use of fluorescence microarrays in clinical diagnostics. From this point of view, it is promising to use an alternative tag, biotin, which can further be detected by a streptavidin conjugate with an enzyme followed by colorimetric quantitation of the latter. In this case, analytical signals can be detected with a cheap optical scanners or even visually (for low-density microarrays).

Many chromogenic substrates have been developed for the detection of immobilized HP; however, only 4-chloro-1-naphthol, 3,3′-diaminobenzidine, 3-amino-9-ethylcarbazole, and *o*-dianisidine form water-insoluble products and, thus, are suitable for solid-phase analytical methods, where detection is carried out by the accumulation of the colored product on the support surface at the localization site of enzyme molecules.[7,8] In the area of search for new substrate systems, it is interesting to consider the reactions of co-oxidation of two substrates with HP affording insoluble intensely colored products. The systems using 4-chloro-1-naphthol with *N*,*N*′-diethylphenylenediamine, *N*,*N*′-dimethylphenylenediamine, or 3-methyl-2-benzothiazolinone hydrazone as substrates were proposed[9] for the detection of HP on the surface of membrane supports. It was found that the peroxidase-induced

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co-oxidation of 4-chloro-1-naphthol with *N*,*N*′-diethylphenylenediamine or *N*,*N*′-dimethylphenylenediamine generated intensely blue-colored compounds insoluble in water and in buffer mixtures, whereas red-colored compounds formed in the case of the oxidation of 4-chloro-1-naphthol with 3-methyl-2-benzothiazolinone hydrazone. A comparison of the proposed substrate systems with the earlier known systems showed that they made it possible to substantially decrease the limit of enzyme detection at a lower background signal. The use of co-oxidation of 3-methyl-2-benzothiazolinone hydrazone and various methoxyphenols with hydrogen peroxide in the presence of HP also showed a considerable increase in the sensitivity of enzyme determination in solid-phase analytical methods.[10]

The purpose of this work is to study the reactions of peroxidase-induced co-oxidation of substituted benzidines and 4-chloro-1-naphthol with hydrogen peroxide for the construction of highly sensitive systems of colorimetric detection in the area of microarray technology.

Experimental

A set of primers for the polymerase chain reaction (PCR) and amino-modified oligonucleotide probes were synthesized by the Sintol company (Russia). Nucleotide sequences of primers and probes were published.[11] DNA samples isolated from clinical cultures of enterobacteria producents of class A β-lactamases were the gift from the Research Institute of Antimicrobial Chemiotherapy at the Smolensk State Medical Academy of the Ministry of Health Protection and Social Development of the Russian Federation (Smolensk, Russia).

The amplification of class A β-lactamase genes with the simultaneous inclusion of biotin as a tag was performed by a procedure described earlier.[11]

Glass slides Nexterion E (2.5×7.5 cm) with surface epoxy groups (Schott, Germany) were used as supports for DNA microarrays. For immobilization, oligonucleotides were dissolved in a salt buffer (160 *mM* Na_2SO_4, 130 *mM* Na_2HPO_4) to a concentration of 20 μmol L^{-1} and spotted on glass slides using a XactII™ Arrayer robot (LabNEXT Inc., USA) with needles 200 μm in diameter, after which the microarrays were incubated at 60 °C for 30 min.

Prior to hybridization, the microarrays were washed at room temperature (for 5 min with a 0.1% solution of Triton X-100, for 4 min with 2 *mM* HCl, and for 10 min with 100 *mM* KCl) and then free DNA and protein binding sites were blocked (incubation of the microarrays in a 25% solution of ethylene glycol and a 1% solution of bovine serum albumin at 37 °C for 30 min). The necessary amounts of tagged DNA (the concentration was estimated spectrophotometrically at λ = 260 nm on a Shimadzu spectrophotometer, Japan) were dissolved in a hybridization buffer (2×SSPE: 0.3 *M* NaCl, 0.02 *M* NaH_2PO_4, 0.002 *M* EDTA, pH = 7.4) containing 1.6 pmol mL^{-1} of biotin-tagged reference oligonucleotide (positive hybridization control). The hybridization mixture (70 μL) was applied on each DNA microarray, which was covered with a frame, and incubated for 1 h at 45 °C. Then the microarrays were washed with an PBST buffer (0.01 *M* K_2HPO_4, 0.15 *M* NaCl, 0.05% Tween 20, pH 7.0) two times for 15 min at room temperature. After this, the microarrays were incubated for 30 min at room temperature in a solution of the streptavidin—HP conjugate (Imtek, Russia) in an PBST buffer (1 μg mL^{-1}). Then the slides were washed (PBST, 2×5 min) and placed in substrate solutions containing 1 *mM* H_2O_2 and one substrate or a combination of the following substrates: 4 *mM* of 4-chloro-1-naphthol in a 0.1 *M* K-phosphate buffer, pH 7.0; 4 *mM* of *o*-dianisidine in a 0.1 *M* K-citrate buffer, pH 4.7; 4 *mM* of 3,3′-diaminobenzidine in a 0.1 *M* K-phosphate buffer, pH 7.0; 4 *mM* of 3,3′,5,5′-tetramethylbenzidine in a 0.1 *M* Na-acetate buffer, pH 5.5. Then the microarrays were washed with distilled water, dried, and scanned on a Perfection V750 Pro scanner (Epson, Germany) with a resolution of 4800 dpi. The resultant images (in the tiff format) were quantitatively processed using the Scan Array Express program (PerkinElmer, version 3.0, Germany).

After colorimetric detection, photographs of microarray cells were obtained using an Axio Imager A1m optical microscope (Carl Zeiss).

The diffuse reflectance spectra of peroxidase oxidation products of *o*-dianisidine and 4-chloro-1-naphthol were obtained using a reflectance densimeter (CS-1000, Shimadzu, Japan).

After the enzymatic reaction has occurred in solution ($1 \cdot 10^{-9}$ *M* HP, 1 *mM* H_2O_2, fixed concentrations of *o*-dianisidine and 4-chloro-1-naphthol in a potassium-citrate buffer, pH 4.7), the products were separated by centrifugation, and the precipitate was dissolved in DMF and analyzed by TLC in $CHCl_3$ using Sorbfil plates with microfractionated silica gel (Fizlabpribor, Russia).

Mass spectra of peroxidase oxidation products of *o*-dianisidine and 4-chloro-1-naphthol were obtained using a MALDI-TOF mass spectrometer (Bruker Daltonics, Germany).

Results and Discussion

HP detection on the glass surface using the enzymatic co-oxidation of benzidine derivatives and 4-chloro-1-naphthol. We performed a comparative study of the sensitivity of the detection systems based on the co-oxidation of substrates of the benzidine series (3,3′-diaminobenzidine, *o*-dianisidine, and 3,3′,5,5′-tetramethylbenzidine) with 4-chloro-1-naphthol catalyzed by HP immobilized on the glass surface. For this purpose, model DNA microarrays were prepared. They represented glass slides with the surface modified by epoxy groups on which oligonucleotide probes were covalently immobilized. The diameter of each microarray cell was 200 μm. The microarrays were hybridized with tagged (biotinylated) oligonucleotide, whose sequence matched completely to the immobilized probes. Biotinylated DNA duplexes formed on the microarray surface were detected using the streptavidin—HP conjugate followed by the colorimetric quantitation of the the enzyme judging from the accumulation of insoluble colored oxidation products of the corresponding substrates on the support. The color intensity of the microarray cell was directly proportional to the amount of DNA duplexes on the support and, correspondingly, to the concentration of the

tagged oligonucleotide in the solution. The optimum conditions for the peroxidase-induced co-oxidation of benzidine derivatives and 4-chloro-1-naphthol have been found, namely, the concentration of the substrate, pH of the buffer solution, and the time of product accumulation.

The calibration curves for the quantitation of biotinylated oligonucleotide in the hybridization mixture on the glass microarrays using both one- and two-substrate enzymatic reactions for HP detection are shown in Fig. 1. All one-substrate systems under study, *viz.*, 4-chloro-1-naphthol (CN), 3,3′-diaminobenzidine (DAB), *o*-dianisidine (OD), exhibited the low sensitivity of determination of HP and, correspondingly, the biotin-tagged oligonucleotide in the hybridization mixture. The lowest detection limit for the tagged nucleotide among the one-substrate systems was found for the use of 4-chloro-1-naphthol, being 0.80±0.16 nmol L^{-1}. The reaction product was well adsorbable on the glass surface; however, its pale blue color was unstable. In addition, its accumulation rate was low, because 4-chloro-1-naphthol slowly undergoes peroxidase-induced oxidation. The enzymatic oxidation products of 3,3′-diaminobenzidine and *o*-dianisidine, which are intensely brown-colored, poorly adsorbed on the glass surface forming diffuse cells with high background coloration (the background coloration intensity was up to 30% of the maximum analytical signal).

For the co-oxidation of all studied substrates of the benzidine series with 4-chloro-1-naphthol, the products formed had a darker shade compared to the oxidation products of particular substrates (black for 3,3′-diaminobenzidine with 4-chloro-1-naphthol, dark violet for *o*-dianisidine and 4-chloro-1-naphthol, and gray for 3,3′,5,5′-tetramethylbenzidine and 4-chloro-1-naphthol), they well adsorbed on the glass at the site of HP localization, and the background coloration did not exceed 4% of the maximum analytical signal. The time of accumulation of the amount of the products sufficient for optical detection did not exceed 10 min. The use of all substrate systems made it possible to determine the analyte in a wide concentration range, which is important for quantitative analysis. The co-oxidation of *o*-dianisidine and 4-chloro-1-naphthol showed the highest sensitivity of biotin-tagged oligonucleotide determination. The detection limit was 0.03±0.005 nmol L^{-1}, which is more than an order of magnitude higher than that for the one-substrate reactions.

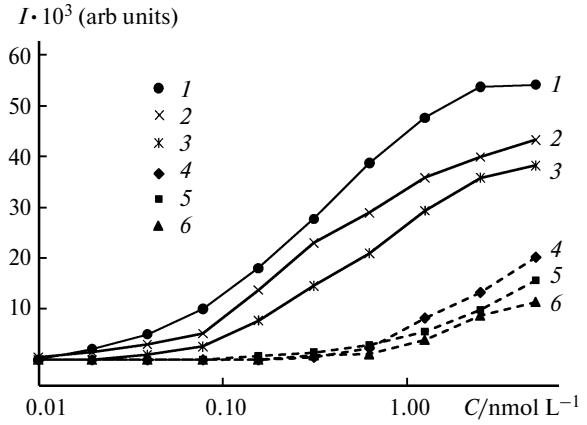


Fig. 1. Calibration curves for the determination of biotinylated oligonucleotide in a hybridization mixture on glass microarrays with colorimetric detection based on the enzymatic oxidation of benzidine derivatives and 4-chloro-1-naphthol (CN): *1*, *o*-dianisidine—CN; *2*, 3,3′-diaminobenzidine—CN; *3*, 3,3′,5,5′-tetramethylbenzidine—CN; *4*, CN; *5*, 3,3′-diaminobenzidine; and *6*, *o*-dianisidine. *I* is the color intensity, and *C* is the concentration of the tagged nucleotide.

The morphology of coloration of microarray cells is an important parameter for the quantitative analysis on colorimetric microarrays. Optical spectroscopy was used for the determination of uniformity of coloration of the microarray cell. It was shown that the peroxidase-induced oxidation products of benzidine derivatives with 4-chloro-1-naphthol uniformly adsorbed over the overall cell surface forming distinct boundaries between the analytical zone and background (Fig. 2). This favorably distinguishes the enzymatic microarray detection method from the colorimetric detection microarray based on gold nanoparticles followed by silver strengthening, which often results in the nonuniform distribution of the tag over the microcell surface and duffusion of the colored zone to increase the background signals.[12,13]

Thus, the HP-catalyzed co-oxidation of *o*-dianisidine and 4-chloro-1-naphthol with hydrogen peroxide was chosen for further investigation as a detection system providing the high sensitivity of determination of the result of DNA hybridization on the glass surface.

Study of the HP-catalyzed co-oxidation products of *o*-dianisidine and 4-chloro-1-naphthol with hydrogen peroxide. We recorded the diffuse reflectance spectra of the individual and co-oxidation products to study the composition of the enzymatic co-oxidation products of *o*-dianisidine and 4-chloro-1-naphthol (Fig. 3). The spectrum of the products of co-oxidation of *o*-dianisidine and 4-chloro-1-naphthol does not coincide with the spectra of oxidation products of particular substrates and represents, most likely, the superimposition of the diffuse reflectance spectra of the individual oxidation products (450 nm for *o*-dianisidine and 550 nm for 4-chloro-1-naphthol) and new products (520 and 590 nm). It was difficult to characterize the composition of the reaction products in more detail because of the superimposition of the spectra.

The composition of the reaction products was studied by TLC. For this purpose, the enzymatic co-oxidation of *o*-dianisidine and 4-chloro-1-naphthol was carried out in a solution with variable ratio of substrate concentrations (Fig. 4). Both the individual oxidation products of *o*-dianisidine (brown) and 4-chloro-1-naphthol (blue) and two

Fig. 2. Microarray cell after colorimetric detection based on the enzymatic oxidation of *o*-dianisidine and 4-chloro-1-naphthol (the photograph was made on an Axio Imager A1m optical microscope (Carl Zeiss)).

new dark violet products formed upon the peroxidase-induced co-oxidation of *o*-dianisidine and 4-chloro-1-naphthol. It is most likely that it is these the products that enable highly sensitive detection of HP judging from the adsorption on the glass surface. The amount of each product depended on the ratio of substrate concentrations in the reaction mixture. The maximum amount of new products (compared to the individual oxidation products of the substrates) formed at the equimolar substrate ratio.

The mass spectrum of the co-oxidation products of *o*-dianisidine and 4-chloro-1-naphthol (MALDI-TOF) contains one intense peak with $m/z = 384.854$ along with a series of minor peaks characteristic of the initial substrates and individual oxidation products. This peak value is very close to the calculated value of $M = 384.427$ for compound with the composition $C_{24}H_{20}N_2O_3$.

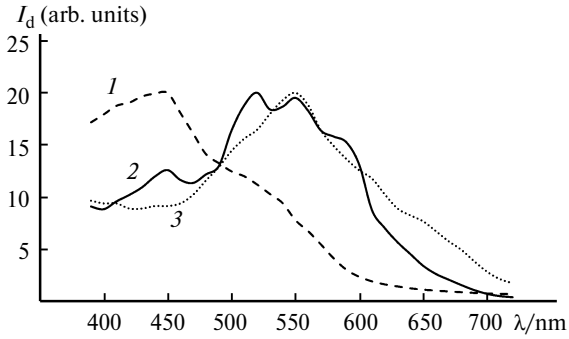


Fig. 3. Diffuse reflectance spectra of the enzymatic oxidation products of *o*-dianisidine (OD) and 4-chloro-1-napthol (CN) for individual oxidation and co-oxidation: *1*, 4 *mM* OD; *2*, 4 *mM* OD—4 *mM* CN; and *3*, 4 *mM* CN. I_d is the diffuse reflectance intensity.

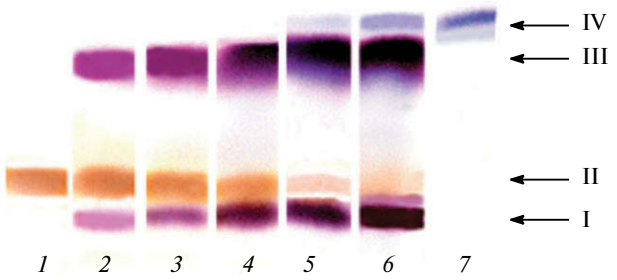
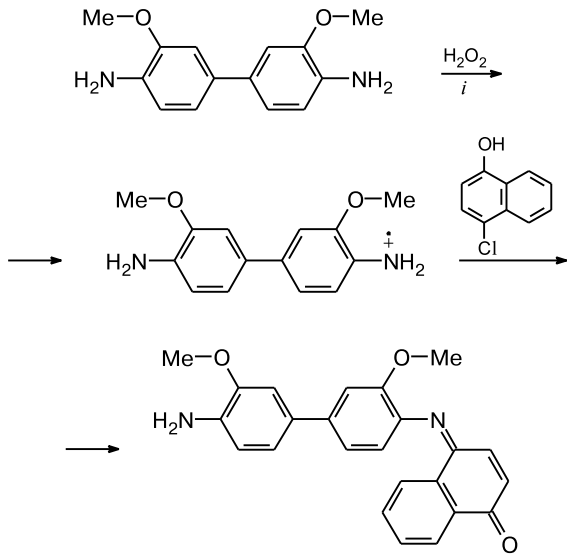


Fig. 4. Separation of the enzymatic co-oxidation products of *o*-dianisidine and 4-chloro-1-naphthol by TLC (chloroform): *1*, 4 *mM* OD; *2*, 4 *mM* OD + 0.5 *mM* CN; *3*, 4 *mM* OD + 1.0 *mM* CN; *4*, 4 *mM* OD + 2.0 *mM* CN; *5*, 4 *mM* OD + 4.0 *mM* CN; *6*, 4 *mM* OD + 6.0 *mM* CN; and *7*, 6 *mM* CN; I is the co-oxidation product of OD and CN (violet), II is the oxidation product of OD (brown), III is the co-oxidation product of OD and CN (violet), and IV is the oxidation product of CN (dark blue).

Analysis of the published data[9—10,14] and our results suggest the following mechanism of co-oxidation of *o*-dianisidine and 4-chloro-1-naphthol depicted in Scheme 1. It has earlier been found that activation or inhibition of one substrate by another is often observed for the peroxidase-induced oxidation of two substrates with different reactivities.[15] It can be assumed that substrate—substrate activation occurs in the co-oxidation of *o*-dianisidine and 4-chloro-1-naphthol: the enzymatic transformation of the rapidly oxidizable substrate (*o*-dianisidine) results in a highly reactive radical cation, which oxidizes the slowly oxidizable substrate, 4-chloro-1-naphthol to form the product with the composition $C_{24}H_{20}N_2O_3$ in which electrophilic substitution occurs at position 4 of the 4-chloro-1-naphthol molecule.

Scheme 1



i. HR is horseradish peroxidase

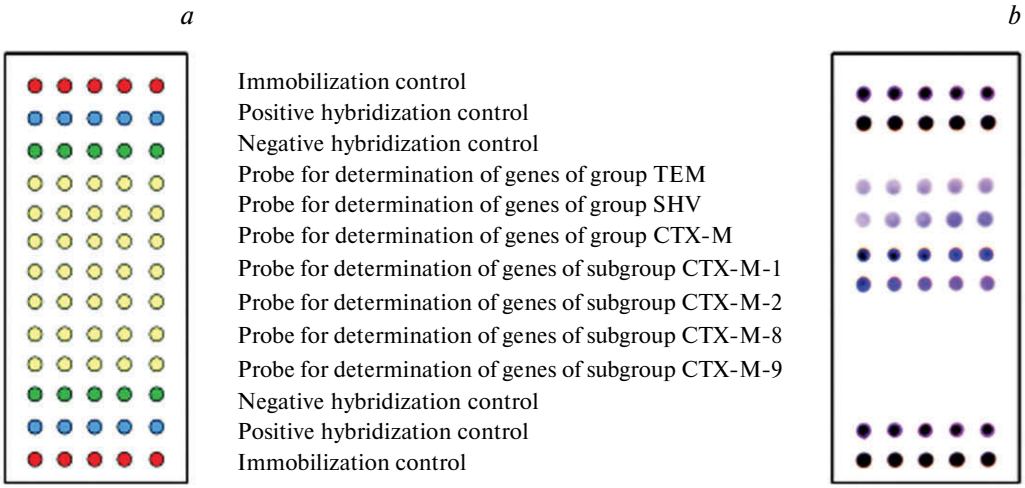


Fig. 5. (*a*) Arrangement of oligonucleotide probes on the DNA microarray for the identification of class A β-lactamase gene types: (*b*) results of testing a sample containing β-lactamase genes of the types TEM, SHV, and CTX-M on the DNA microarray with colorimetric detection based on the co-oxidation of *o*-dianisidine and 4-chloro-1-naphthol (*b*).

The use of the detection system based on peroxidase-induced co-oxidation of *o*-dianisidine and 4-chloro-1-naphthol in the microarray technology. The detection system developed on the basis of co-oxidation of *o*-dianisidine and 4-chloro-1-naphthol was applied for the DNA hybridization technique on glass microarrays. For this purpose, we used DNA microarrays developed earlier for genotyping of class A β-lactamases.[11] Identification of genes encoding these enzymes is topical due to widespread bacterial infections resistant to the action of β-lactam antibiotics.[16] Enzymatic hydrolysis of the β-lactam ring of antibiotics is the main mechanism of microorganism resistance to penicillins and cephalosporins.

The DNA microarray for genotyping class A β-lactamases is a matrix 3.5×9.0 mm in size with immobilized nucleotide probes. The matrix is spotted on a glass support. The sequences of oligonucleotide probes are specific to various regions of β-lactamase genes of types TEM, SHV, and CTX-M beloging to molecular class A.[17] Each oligonucleotide probe is immobilized on the microarray surface (Fig. 5, *a*). Each microarray also contained three types of control oligonucleotides: immobilization control (biotin-tagged oligonucleotide), positive hybridization control (oligonucleotide, whose nucleotide sequence is complementary to the biotin-tagged oligonucleotide added to the mixture), and negative hybridization control (oligonucleotide with a random sequence of bases). The determination of β-lactamase genes in the samples by the hybridization assay method on the glass DNA microarray consisted of the following stages: (1) amplification of β-lactamase gene from clinical material by multiplex PCR with the simultaneous introduction of the biotin tag; (2) hybridization of biotin-tagged DNA with oligonucleotide probes on the microarray surface; and (3) detection of biotin in the DNA complexes on the support by the streptavidin—HP conjugate followed by colorimetric quantitation of the enzyme. The image of the microarray after hybridization of β-lactamase genes of groups TEM, SHV, and CTX-M-1 is shown in Fig. 5, *b*.

The detection limit of genes of the main types of β-lactamases of molecular class A was estimated on the DNA microarray with colorimetric detection based on the peroxidase-induced co-oxidation of *o*-dianisidine and 4-chloro-1-naphthol. For this purpose, we studied the dependences of the color intensity of certain microarray cells on the concentration of the corresponding β-lactamase gene in the hybridization mixture in a series of experiments. The detection limits of β-lactamase genes varied from 0.20 to 0.92 ng μL^{-1} of tagged DNA, and the variation coefficients ranged from 4 to 11% depending on the type of the determined gene (Table 1). The analytical characteristics of the identification method for β-lactamase genes on the microarray with fluorescence detection are

Table 1. Analytical characteristics of the DNA hybridization technique for class A β-lactamase genes on microarrays with colorimetric detection based on *o*-dianisidine and 4-chloro-1-naphthol and with fluorescence detection

Type of β-lactamase	Colorimetric detection*		Fluorescence detection	
	C_{min}/ng μL^{-1}	k (%)	C_{min}/ng μL^{-1}	k (%)
TEM	0.88	11	0.75	10
SHV	0.92	7	0.90	8
CTX-M-1	0.15	4	0.15	5
CTX-M-2	0.40	6	0.35	6
CTX-M-8	0.35	4	0.35	5
CTX-M-9	0.20	5	0.15	5

* C_{min} is the detection limit, and k is the variation coefficient.

given for comparison in Table 1 (the streptavidin conjugate with the fluorescent dye Cy3 was used to detect hybridization). It was demonstrated that the detection limits of genes on the microarrays with HP-based colorimetric detection and fluorescence detection are comparable.

Thus, the study of the co-oxidation of *o*-dianisidine and 4-chloro-1-naphthol with hydrogen peroxide catalyzed by horseradish peroxidase showed its high efficiency for colorimetric detection of enzyme on the surface of glass DNA microarrays. It was found that the reaction affords a new product with intense violet color characterized by the high accumulation rate, sorbability well on the glass surface, and high stability. The system developed for HP-based colorimetric detection provides high sensitivity, and the reproducibility of hybridization assay on DNA microarrays is comparable in microarray characteristics with that of fluorescence detection.

The assay using colorimetric microarrays does not require expensive reagents and equipment and can be performed with an optical scanner or visually, which allows the DNA microarray technique to become more available for use in clinical laboratories.

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