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(54) Title: SIMULTANEOUS ENZYMATIC DETECTION OF TWO ANALYTES

(57) Abstract: The invention provides a new method for simultaneous detection of two or more analytes in a sample. In one aspect, a first enzyme, such as a hydrolase, is associated with a first analyte; a second enzyme, such as an oxidase, is associated with a second analyte; and substrates are provided so that the product of the two resulting enzymatic reactions chemically combine to form a third reaction product. Detection of the third reaction product is correlated with the presence of both the first analyte and the second analyte in the sample.

SIMULTANEOUS ENZYMATIC DETECTION OF TWO ANALYTES

FIELD OF THE INVENTION

This invention relates to new methods for detection of analytes in a sample, and finds application in medicine, histology, and cell biology.

BACKGROUND OF THE INVENTION

A variety of methods are known in the fields of histochemistry and immunohistochemistry for detecting proteins and other compounds in a sample, such as a tissue section. These methods typically rely on detectable labels, including radiolabels, fluorescent labels, and enzymatic labels. In some applications it is advantageous to simultaneously detect the presence of more than one species of molecule or epitope in a sample. Various approaches have been taken to accomplish this, such as sequential immunoenzymatic staining to produce precipitates of different colors. However, there is a need for simpler and improved methods for detection of multiple analytes in a sample.

BRIEF DESCRIPTION OF THE INVENTION

In one aspect, the invention provides a method for determining whether a first analyte and second analyte are co-localized in a sample by (a) associating a first enzyme with the first analyte if it is present in the sample; (b) associating a second enzyme with the second analyte if it is present in the sample; (c) providing a first substrate and a second substrate, wherein the first enzyme acts on the first substrate to produce a first reaction product, the second enzyme acts on the second substrate to produce a second reaction product; and the first and second reaction products chemically combine to form a third reaction product; (d) detecting the third reaction product; and, (e) correlating the presence of the third reaction product with the presence of co-localized first analyte and second analyte in the sample. In various embodiments, the first enzyme is a hydrolase, such as a phosphatase, an esterase, or an amidase, and the first enzyme acts on the first substrate by hydrolyzing the substrate. In various embodiments, the second enzyme is an oxidase, such as a peroxidase (e.g., horseradish peroxidase) or pseudoperoxidase, and the second enzyme acts on the second by oxidizing the substrate. In one embodiment of the invention, the sample is a tissue section.

In some embodiments of the invention, either the first or second enzyme is conjugated to an antibody. In some embodiments, the antibody directly binds either the first or second analyte. In some embodiments of the invention, the first substrate is a naphthol derivative, such as alpha-naphthol-phosphate. In an embodiment of the invention, the second reaction product forms a detectable product in the absence of the first reaction product.

In an embodiment of the invention, the method includes adding a compound, such as diazonium salt, that combines with the first reaction product to form a detectable colored product.

In one embodiment, the invention provides a method for determining whether an analyte and a protein with hydrolytic activity are co-localized in a sample by (a) associating an oxidase with the analyte if it is present in the sample; (b) providing a first substrate and a second substrate, wherein the hydrolase acts on the first substrate to produce a first reaction product, the oxidase acts on the second substrate to produce a second reaction product; and the first and second reaction products chemically combine to form a third reaction product; (c) detecting the third reaction product; and, (d) correlating the presence of the third reaction product with presence of co-localized first analyte and hydrolase in the sample. The protein with hydrolytic activity may be a phosphatase, esterase, galactosidase, lipase, glucuronidase, amidase, a sulfatase, or the like.

In one embodiment, the invention provides a method for determining whether an analyte and a protein with oxidase activity are both present in a sample by (a) associating a hydrolase with the analyte if it is present in the sample; (b) providing a first substrate and a second substrate, wherein the hydrolase acts on the first substrate to produce a first reaction product, the protein with oxidase activity acts on the second substrate to produce a second reaction product; and the first and second reaction products chemically combine to form a third reaction product; (c) detecting the third reaction product; and, (d) correlating the presence of the third reaction product with presence of co-localized first analyte and oxidase in the sample. In some embodiments, the method of claim 17 wherein the oxidase is a peroxidase or a pseudoperoxidase.

In one embodiment, the invention provides a method for determining whether a first enzyme and a second enzyme are co-localized in a sample by (a) providing a first substrate and a second substrate, wherein the first enzyme acts on the first substrate to produce a first reaction product, the second enzyme acts on the second substrate to produce a second reaction product; and the first and second reaction products chemically combine to form a third reaction product; (b) detecting the third reaction product; and, (c) correlating the

presence of the third reaction product with presence of co-localized first enzyme and second enzyme in the sample. In one embodiment, the first enzyme is a hydrolase and the second enzyme is an oxidase.

In another aspect, the invention provides kits useful in practicing the method of the invention.

DETAILED DESCRIPTION

I. Definitions

As used herein, the term "hydrocarbyl" refers to an organic radical comprised of carbon chains to which hydrogen and other elements are attached. The term includes alkyl, alkenyl, alkynyl and aryl groups, groups which have a mixture of saturated and unsaturated bonds, carbocyclic rings and includes combinations of such groups. It may refer to straight chain, branched-chain, cyclic structures or combinations thereof.

"Alkyl" refers to, a cyclic, branched or straight chain saturated hydrocarbon group. "Lower alkyl" refers to an alkyl with one to eight carbon atoms. This term is further exemplified by such groups as methyl, ethyl, n-propyl, i-propyl, n-butyl, t-butyl, i-butyl (or 2-methylproply), cyclopropylmethyl, i-amyl, n-amyl, and hexyl. "Substituted lower alkyl" refers to lower alkyl as just described including one or more functional groups such as lower alkyl, aryl, aralkyl, acyl, halogen, hydroxyl, amino, acylamino, acyloxy, alkoxyl, mercapto and the like. These groups may be attached to any carbon atom of the lower alkyl moiety.

"Alkenyl" refers to a cyclic, branched or straight chain group containing a carbon-carbon double bond, i.e., an unsaturated hydrocarbon. This term is further exemplified by such groups as ethylene, propylene, butene, 2-methylpropene, pentene, hexene and the like.

"Alkynyl" refers to a branched or straight chain group containing a carbon-carbon triple bond. This term is further exemplified by such groups as acetylene, propyne, butyne, pentyne, 3-methyl-1-butyne, hexyen and the like.

The term "aryl" refers to an aromatic carbocyclic radical having one (e.g., phenyl) or more condensed rings (e.g., naphthyl), which can optionally be mono-, di-, or tri-substituted, independently, with alkyl, lower-alkyl, cycloalkyl, hydroxylower-alkyl, aminolower-alkyl, hydroxyl, thiol, amino, halo, nitro, lower-alkylthio, lower-alkoxy, mono-lower-alkylamino, di-lower-alkylamino, acyl, hydroxycarbonyl, lower-alkoxycarbonyl, hydroxysulfonyl, lower-alkoxysulfonyl, lower-alkylsulfonyl, lower-alkylsulfinyl,

trifluoromethyl, cyano, tetrazoyl, carbamoyl, lower-alkylcarbamoyl, and di-lower-alkylcarbamoyl.

The term "optionally substituted" refers to optional mono-, di-, or tri-substitution, independently, with substituents including hydroxylower-alkyl, aminolower-alkyl, hydroxyl, thiol, amino, halo, nitro, lower-alkylthio, lower-alkoxy, mono-lower-alkylamino, di-lower-alkylamino, acyl, hydroxycarbonyl, lower-alkoxycarbonyl, hydroxysulfonyl, lower-alkoxysulfonyl, lower-alkylsulfonyl, lower-alkylsulfinyl, trifluoromethyl, cyano, tetrazoyl, carbamoyl, lower-alkylcarbamoyl, and di-lower-alkylcarbamoyl.

The term "lower" as used herein in connection with organic radicals or compounds respectively defines such with up to and including 6, preferably up to and in including 4 and more preferably one or two carbon atoms. Such groups and radicals may be straight chain or branched.

The term "effective amount" refers to the amount required to produce the desired effect. Thus, an effective amount of a substrate or enzyme refers to the amount of each which is needed to produce a detectable signal (e.g., color precipitate) in a sample containing the target analytes (i.e., co-localized analytes).

The terms "specific binding pair (SBP)" and "specific binding pair partners (SBP partners)" refer to pairs of molecules that specifically bind to each other. "Specific binding" of SBP partners to each other in a sample is evidenced by the binding of one member of the SBP pair to the other with a higher affinity and specificity than to other components in a sample, *i.e.*, without concurrent binding to other components in a sample. The binding between the SBP partners is typically non-covalent. Exemplary SBP pairs include any haptenic or antigenic compound in combination with an antibody or binding fragment thereof (e.g., antigen-anti-antigen immunoglobulin, digoxigenin and anti-digoxigenin; mouse immunoglobulin and goat anti-mouse immunoglobulin) and non-immunological binding pairs (e.g., biotin-avidin, biotin-streptavidin, hormone-hormone binding protein, IgG-protein A, lectin-carbohydrate, enzyme-enzyme cofactor, enzyme-enzyme-inhibitor, and complementary single-stranded polynucleotide pairs capable of forming nucleic acid duplexes) and the like. SBP pairs may also be referred to as "ligand-anti-ligand" pairs. No particular biological function, other than specific binding, is implied by use of the terms ligand and anti-ligand.

An "analyte" detected according to the present invention may be any compounds (e.g., biological molecule or region thereof) including, without limitation, an

antigen, an epitopic region of an antigen, a hapten, an antibody, a polypeptide, a polynucleotide (i.e., DNA or RNA) including a specific sequence, subsequence, fragment or region, an enzyme, adduct, carbohydrate and the like. According to the invention, the two "analytes" to be detected may be part of a single macromolecule, e.g., two different epitopes of a polypeptide, two different sequences or regions of polynucleotide, or the like, so long as the two analytes can be distinguished by distinct enzymatic activities or by specific binding to a distinct anti-ligand or SBP partner.

The term "sample," as used herein, may be a cell, a tissue section, a membrane, homogenate, body fluid (e.g., blood), cell or tissue extract, protein or polynucleotide preparation or the like suspected of containing the target analytes. Typically the analytes are immobilized at the time of detection, for example attached to a microscope slide, electrophoretic matrix, nitrocellulose or nylon membrane, bead (e.g., polystyrene, agarose, acrylamide), immobilized in single cells in suspension. The term "sample" does not imply homogeneity. Thus, collections of cells in a sample may not all contain the same analytes and the like.

II. Description

The present invention provides a method for determining whether two different analytes are co-localized in a sample, *i.e.*, both present in the same region (e.g., same cell or electrophoretic band) of the sample. The detecting occurs according to the basic scheme summarized in Table 1, where enzyme 1 is associated with one analyte, enzyme 2 is associated with the second analyte, and the reaction products 1 and 2 chemically combine to produce a detectable reaction product 3.

Table 1

(I) Substrate 1 $\xrightarrow{\text{enzyme 1}}$ Reaction Product 1

(II) Substrate 2 $\xrightarrow{\text{enzyme 2}}$ Reaction Product 2

(III) Reaction Prod. 1 + Reaction Prod. 2 $\xrightarrow{\text{chemical combination}}$ Reaction Prod. 3 (Detectable, e.g., colored precipitate)

As used herein, the terms "chemically combine," "chemical combination" and the like refer to the formation of a covalent chemical linkage between reaction product 1 and reaction product 2, resulting in a reaction product 3 that is detectable and distinguishable from reaction products 1 or 2. Typically, reaction product 3 forms a colored precipitate that remains localized in the support at the site of the production of reaction product 3. Thus, in some embodiments, the formation of reaction product 3 identifies not only the presence of the two co-localized analytes, but also their location (e.g., intracellular location, as described in Example 6, *infra*; location in an electrophoretic zone, as described in Example 8, *infra*; and the like). Reactions (I) and (II) as shown in Table 1 are sometimes referred to as "half reactions," referring to the fact that the products of these two reactions are both required for reaction (III), resulting in a detectable product.

According to the invention, the characteristic detectable signal of reaction product 3 is produced only if both analytes are present and co-localized in the sample, allowing both "half reactions" to occur, and allowing the products of both "half-reactions" to chemically combine to produce a distinct product. As noted, formation of the detectable reaction product 3 requires that reaction products 1 and 2 are produced in proximity to each other, i.e., sufficiently close to interact to product reaction product 3 before an alternative reaction occurs (e.g., within the same cell in a tissue section, in the same band in an electrophoretic matrix, in a specific anatomical region or cell cluster in a tissue sample, and the like). Examples of alternative reactions include autopolymerization of reaction product 1 or 2 to form a different color precipitate, or coupling to form a soluble or uncolored precipitate.

Sufficient proximity is usually within from about one micron to about 1 millimeter, typically within about 200 microns, depending on the method of immobilization of the analytes. For example, when cells are analyzed, the two analytes are detected when they are in the same cell. When the analytes are immobilized on a membrane (e.g., following electrophoresis) or in a gel matrix, they will be detected when the separated analytes (e.g., "bands") are within about 1 millimeter, more often within about 500 microns of each other. Two analytes that are within sufficient proximity may be referred to as "co-localized." In contrast to the detection of two co-localized analytes according to the method of the invention, if either of the analytes is not present in the sample, or is present only in a different region than the other analyte (e.g., a different cell, a different area or zone of a matrix such as an electrophoretic separation matrix or membrane, a different region of a tissue section) reaction product 3 will not form in sufficient quantities to detect. It will be apparent that two

target analytes may be present in the same sample (e.g., tissue section or electrophoretic strip) but not be co-localized (e.g., in the same cell or electrophoretic band).

In some embodiments of the invention, as described in greater detail *infra*, at least one of reaction products 1 and 2 is also detectable alone or in combination with additional reagents, e.g., by formation of a colored precipitate that can be distinguished from reaction product 3.

Either, or both, of the enzymatic activities catalyzing the half-reactions may be present as an inherent characteristic of the analyte(s) or may be associated (i.e., physically associated) with the analyte(s), e.g., via the interaction of a specific binding pair (e.g., an analyte/antigen and an enzyme-labeled antibody). Thus, in some embodiments, one or both of the analytes to be detected has an enzymatic activity. In other embodiments, one or both of the analytes comprises a recognition site that can be specifically bound by a specified SBP partner, and can thereby become physically associated with a specific enzyme directly or indirectly bound to the SBP partner. The "association" may be an immunological association (e.g., the analyte is bound by enzyme-linked antibody specific for the analyte), an association resulting from nucleic acid hybridization (a target nucleic acid sequence bound by an enzyme-linked complementary probe), or other ligand-anti-ligand- or SBP- mediated associations. As will be apparent to one of ordinary skill, the term "associated" in this context refers to a directly or indirectly linking the enzyme and the target analyte, as is common, for example, in immunohistochenistry.

In one embodiment of the invention, reaction product 1 is a "color coupler" produced by the action of a hydrolase on a substrate (hereinafter referred to as "substrate 1") and reaction product 2 is an "oxidized developer" produced by the action of an oxidase (e.g., a peroxidase) on a substrate (hereinafter referred to as "substrate 2"). Although for ease of reference the phrase "reaction product 2" is used throughout this disclosure, it will be appreciated that "reaction product 2" may be a highly reactive species or intermediate, e.g., a reactive free radical, and not necessarily a stable product.

(a) Color Couplers

The term "color coupler" refers to compounds such as those used in color photography ("photographic color coupler") to couple with an "oxidized developer" to produce a colored product, e.g., a precipitable product. Photographic color couplers are of several major classes. Those that contain open chain active methylene groups generally form yellow to orange dyes and are called yellow couplers; those that contain the active methylene

group in a heterocyclic ring generally form magenta dyes and are called magenta couplers; and those that contain an active methine group (e.g., the para position of a phenol or naphthol) generally form blue to cyan dyes and are termed cyan couplers.

Typical precursors of couplers (*i.e.*, substrates that produce yellow couplers) include amide derivatives such as R-CH(OR2)-C(O)-NH-R1, wherein R and R1 are optionally substituted hydrocarbyl groups, and OR2 is a hydrolyzable group such as an ester group (such as phosphate ester, acetate groups) which upon cleavage will result in a hydroxide or carbonyl functionality. Yellow couplers include acetanitide derivatives such as R2-CH(OR3)-C(O)-NH-Ar (I), Ar-CH(OR4)-C(O)-NH-Ar (II) and t-Bu-CH(OR5)-C(O)-NH-Ar (III), where Ar represents an optionally substituted aryl and OR3, OR4 and OR5 independently are hydrolyzable groups as described in the preceding sentence. See, e.g., U.S. Patent Nos. 2,186,849; 2,875,057; 3,265,506; 3,770,446; 3,778,277; and GB 808,276.

Yellow couplers include ß-ketoanilides substituted at the α-position with a leaving group. Such couplers are represented by R3-C(O)-CH(Z)-C(O)-NH-Ar, where R3 represents an optionally substituted hydrocarbyl group, Ar an optionally substituted aryl and Z is a leaving group (also termed a "coupling off group" in color photography). Coupling off groups include, halo, phthalimido, succinimido, 5,5-dimethylhydantoinyl and various other 5-membered heterocycles, acyloxy, sulfonyloxy, aryloxy, urethane, imido, pyridone, pyridazone and the like. See, U.S. Patent Nos. 2,278,658; 3,849,140; 3,277,155. Another family of yellow couplers, unusual in not containing an active methylene group, are the substituted indazolones and the benzisoxazolones (see e.g., GB 875,470 and GB 778,089). Other examples include pivaloylacetanilides (see Hamilton et al., supra).

Magenta couplers contain a heterocyclic active methylene structure, such as the 5-pyrazolones (U.S. Patent No. 1,969,479) and may be pyrazolines, pyrazolones, pyrazolobenzimidazoles, or pyrazolotriazoles. Particular magenta couplers are the 1-aryl-5-pyrazolones (G. Brown et al., J. Am. Chem. Soc., 73:919 (1951)). Many 5-pyrazolones contain a nitrogen containing substituent at the 3-position including carbonamido, sulfonamido, alkylamino, arylamino, heterocyclicamino, guanadino, ureido and the like. The 1-phenyl-5-pyrazolones can be substituted by up to three halogens in the phenyl ring. Other variations include the presence of a heterocyclic ring at the 1-position as in the benzimidazoyl pyrazolones. As with the yellow couplers, the magenta couplers can carry leaving groups at the coupling position giving 2-equivalent pyrazolone couplers. Related magenta couplers include pyrazolo[2,3-a]benzimidazoles, pyrazolo-(3,2-c)-5-triazoles and others (W. Pelz,

"Farbkuppler" in Mitteilungen aus den Forschung-laboratorien der Afga Leverkusen-Munchen, Vol. III pp. 111-175 (Springer-Verlag, Berlin 1961).

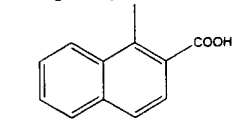
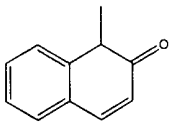
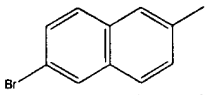
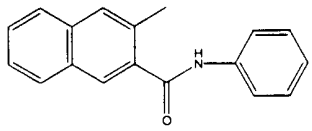
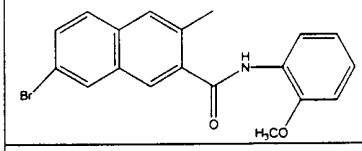
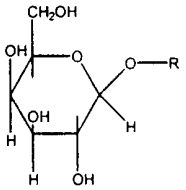
Cyan couplers are typically phenols or naphthols which may carry electron donating (e.g., alkyl, alkoxy, alkylamino) or electron withdrawing substituents (e.g., halo, cyano). Frequently, cyan couplers carry substituents, such as carbonamido, ureido, carbamyl, heterocyclic groups and the like, at the 2-position. In the naphthol family, cyan couplers include the substituted 1-naphthols, particularly derivatives of 1-hydroxy-naphthoic acids, such as, for example, the 1-hydroxy-2-naphthamides. Various coupling-off groups, such as halo, carboxy, sulfo, alkoxy, hydroxymethylene and alkylidene, may also be found attached to the 4-position of cyan couplers.

Photographic color couplers may also be functionalized with a combination of hydrophilic and hydrophobic groups to reduce water solubility and prevent excessive diffusion. Other exemplary compounds useful as couplers include those described in U.S. Pat. No. 4,978,612 and p-amino-N-dialylanalines such as those described by Bent et al. (1951) "Chemical Constitution, Electrochemical Photographic and Allergenic Properties of p-Amino-N-Dialylanilines" (communication no. 1385 of Kodak Research Laboratories).

According to the invention, "Substrate 1" is a compound that is converted to a color coupler by the action of an enzyme, typically a hydrolase, by removal of a blocking group. The presence of the blocking group prevents the chemical combination of Substrate 1 and Reaction Product 2 to produce Reaction Product 3. In exemplary embodiments, substrate 1 has the formula: "R---B" , where "R" is a color coupler moiety, such as described *supra*, "---B" is a blocking group such as a phosphate, a sulfate, an acetate or a butyrate, or the like, linked to the color coupler moiety by an enzyme-cleavable (e.g., hydrolase-cleavable) linkage such as an ester (including a phosphate ester or a sulfate ester) linkage or an amide linkage.

Hydrolases are enzymes that catalyze a hydrolysis reaction. Exemplary hydrolases include phosphatases (e.g., alkaline phosphatase), esterases (e.g., cholinesterases, carboxyl esterase), galactosidases (e.g., alphanaphthol betagalactosidase), lipases, glucuronidases, amidase, peptidases, and sulphatases. Substrates for hydrolases are, as noted, esters, amides, peptides, ethers, or any chemical compound having an enzymatically hydrolyzable covalent bond. The enzyme catalyzed hydrolysis reaction results in a hydroxy or an amine compound as one product (and free phosphate, acetate, etc. as a second product). Exemplary substrates (i.e., "substrate 1") include naphthol derivatives such as those listed for illustration in Table 2, *infra*.

Table 2

"substrate 1"	Enzyme	"Product 1"
R-phosphate, where R is: 1-naphthyl     	phosphatase	1-napthol 1-hydroxy-2-naphthoic acid 1-hydroxy-2-naphthone 6-bromo-2-naphthol Naphthol AS Naphthol AS BI
R-acetate, where R is: 1-naphthyl Other R groups have same structures as above.	esterase	Same as above
R-butyrate, where R is: 1-naphthyl Other R groups have same structures as above.	lipase	Same as above
R-galactopyranoside, represented by the formula.  where R is: 1-Naphthyl Other R groups have same structures as above.	β-galactosidase	same as above

Benzoyl-arginine-2-naphthylamide	trypsin	2-naphthol
Benzoyl-L-leucine-2-napthylamide	chymotrypsin	2-naphthol
R-glucopyranoside, where R is 1-naphthyl Other R groups have same structures as above.	beta-glucosidase	Same as above
R-sulfate, where R is 1-naphthyl Other R groups have same structures as above	aryl-sulphatase	Same as above
p-chloroanilidophosphonic acid chlorophenyldiamidophosphonic acid	phosphamidase	*p*-chloroaniline chlorphenylaniline

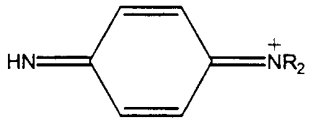
(b) Oxidized Developer

The "oxidized developer" of the present invention is the product of an enzyme catalyzed oxidation reaction. Generally, any oxidase substrate which is converted by an oxidase to an intermediate capable of coupling with a color coupler (e.g., an "oxidizable developer") may be used in the methods disclosed herein. Oxidases are enzymes that catalyze the oxidation of a substrate. The reaction product (Reaction Product 2) is an activated form of the substrate, for example, a free radical, which reacts quickly with Reaction Product 1 (or otherwise reverts to a stable compound, e.g., a homopolymer, couples with another compound, or returns to the unactivated state). Exemplary oxidases include cytochrome oxidases, monoamine oxidases, galactose oxidases, peroxidases (e.g., Horseradish peroxidase; myeloperoxidase), pseudoperoxidases (e.g., hemoglobin) and the like. It will be appreciated that, depending on the enzyme, certain cofactors may be required for the oxidation reaction. Thus, when a peroxidase enzyme is used, hydrogen peroxide is also used as a cosubstrate for the oxidation reaction. When certain oxidases are used, cytochrome C may be required as a cofactor.

Exemplary substrates (i.e., "substrate 2") include hydroxy- or amine-substituted aryl compounds, including without limitation p-phenylene diamines (e.g., N,N-dimethylparaphenylenediamine), and diaminobenzidines (e.g., 3.3'-diaminobenzidine). Other substrates include o-tolidine, o-dianisidine and. Representative substrates are disclosed in the references listed in the` WORTHINGTON MANUAL OF ENZYMES, following sections on peroxidase and lactoperoxidase, pages 66-73; and PRACTICE AND THEORY OF ENZYME IMMUNOASSAYS, P. Tijssen (Elsevier, 1985). Suitable substrates also include *p*-amino-N-dialkylanilines such as those listed in Bent et al., 1951, communication from Kodak Research

Labs "chemical constitution, electrochemical and allergenic properties of p-Amino-N-dialkylanilines 73:3100.

In addition to being a reactive free radical, the oxidized developer can also, depending on the nature of the substrate, be a charged species. For example, in one embodiment, when the substrate for oxidation is a paraphenylene diamine (including alkylated diamines), the oxidized developer is a quinonediamine. In one embodiment, the quinonediamine is represented by the structure



wherein each R independently is a lower alkyl group which may be same or different.

Table 3 shows exemplary substrates and enzymes that catalyze the oxidation of these substrates to form the oxidized developers.

Table 3

"substrate 2" (oxidizable developer)	enzyme
diamino benzidine o-tolidine 0-dianisidine	peroxidases, e.g., horseradish peroxidase, myelperoxidase, pseudoperoxidase (of hemoglobin or myoglobin)*
N,N-dimethyl-paraphenylene diamine N,N-dimethylnapthylamine p-aminophenol Quinol Tyramine	oxidases (e.g., cytochrome oxidases**, succinic acid dehydrogenase**, monoamine oxidase)

*peroxide is a required cosubstrate.
**These reactions require cytochrome C as a cofactor.

During the oxidation reaction, radicals are often generated which can couple with themselves (self-coupling) and/or polymerize. The radicals species are especially likely to self-couple or polymerize if no color coupler is present to react with the free radical oxidation product. The self-coupled or polymerized products may form detectable colored precipitates.

Photographic color couplers and oxidized developers are described in the photography literature, e.g., Hamilton, J.F. et al., THE THEORY OF THE PHOTOGRAPHIC PROCESS," (Macmillan, New York, 1977) particularly at Chapter 12.

(c) Reaction Product 3

As discussed *supra*, the reaction product of the oxidized developer and the color coupler can be any compound which is the result of a chemical combination of the developer and the coupler. In one embodiment, reaction product 3 is a dye molecule. Exemplary dye molecules include indoanilines, indophenols, indoamines, and azomethines In one embodiment, when the product for an oxidase catalyzed reaction is an aldehyde, and the product of a hydrolase catalyzed reaction is an amine, reaction product 3 is a Schiff base (having an imine functionality)

Table 4 shows exemplary specific combinations of reagents that may be used to detect a pair of analytes in a sample. It will be apparent to one of skill that, in some embodiments, other reagents will be included in the reaction mixture as a cosubstrate for the oxidation reaction (e.g., hydrogen peroxide or other peroxide), and that in addition to substrates and cosubstrates, various buffers will be provided that allow the enzymatic reactions to proceed. Exemplary buffers suitable for the practice of the invention include, without limitation, borate, TRIS, bis TRIS, acetate, caccodylate, barbiturate (barbitol or veronal) and the like. It will be recognized that phosphate buffers should not be used in combination with phosphatases, acetate should not be used with carboxylic esterases, etc. Typically both of the substrates (and the co-substrates) will be contacted with the sample at the same time. However, if, for example, the product of a hydrolysis reaction ("Reaction Product 1") is very poorly soluble, the oxidizable developer ("substrate 2") can be added subsequently after substrate 1.

(d) Association of Enzymatic Activity with Analytes

As discussed *supra*, the invention provides a method for determining whether two different analytes are co-localized in a sample based on the association with each analyte of an enzymatic activity (e.g., peroxidase activity and hydrolytic activity). As noted *supra*, the enzymatic activity may be associated with the analyte via a specific binding pair interaction or may be an inherent property of the analyte (e.g., the pseudoperoxidase activity of hemoglobin).

In certain embodiments of the invention, one or both of the requisite enzymatic activities is provided by the analyte itself. For example, the presence and co-localization in a tissue sample of both an enzyme with peroxidase activity (the second analyte) and an enzyme with phosphatase activity (the first analyte) may be evaluated by incubating the tissue sample with appropriate substrates as described *supra*. One example is the detection of both myeloperoxidase and alkaline phosphatase in a granulocyte from human blood. Other cell types in the same sample of blood (e.g., monocytes) may contain peroxidase but do not contain alkaline phosphatase.

In other embodiments at least one, and usually both, of the enzymatic activities are specifically associated with the analytes through an SBP interaction, in which one member of the pair is directly (by conjugation) or indirectly bound to an enzyme.

In one embodiment, the analytes and enzyme are associated via an antigen-antibody interaction. For example, the enzyme may be bound, directly or indirectly, to an anti-analyte antibody (anti-ligand) capable of binding the analyte in the sample. As used herein "antibody" refers to a polyclonal or monoclonal immunoglobulin, binding fragments thereof (e.g., Fab, Fv and F(ab')2, Fab') as well as chimeric or genetically engineered species.

The enzyme may be directly associated with the antibody, e.g., by covalent conjugation. Methods for conjugating enzymes to antibodies are well known (see, e.g., Harlow and Lane, 1988, ANTIBODIES: A LABORATORY MANUAL, Cold Spring Harbor Laboratory, New York at Chapter 9) and numerous enzyme-conjugated antibodies may also be obtained from commercial sources (e.g., Sigma Chemical Co. St. Louis, MO; Vector Inc., Burlingame, CA; Cortex Biochem, Inc., San Leandro CA). In alternative embodiments, the enzyme may be indirectly bound to an anti-analyte antibody (e.g., by conjugating the enzyme to a secondary antibody that binds the primary anti-analyte antibody) or by conjugating the enzyme to a second anti-ligand (e.g., biotin, Protein A) that binds the first antibody (e.g., for biotin, an avidin/streptavidin-conjugated first antibody). It will be recognized that when an enzyme is associated with analyte via an SBP interaction (e.g., an antibody-antigen

interaction) washing steps are carried out to remove any unbound antibody or other SBP partner (e.g., enzyme-conjugated-antibody) prior to addition of reagents for color development. Suitable washing steps are well known in the art and include, for example, washes with PBS, or similar buffers that do not disrupt the SBP interaction.

In one embodiment of the invention, each of the two analytes is bound by an anti-analyte-antibody conjugated to a specific enzyme. For example, to determine whether progesterone receptor, hereinafter "PR," and estrogen receptor, hereinafter "ER," are co-localized in a sample (e.g., both expressed in the same cell), the sample may be combined with anti-PR-IgG conjugated to a first enzyme and an anti-ER-IgG conjugated to a second enzyme. After suitable washing steps, the sample is contacted with suitable enzyme substrates as described *supra*. Notably, detection of the estrogen and progesterone receptors is of diagnostic significance, and may be predictive of success in treatment of certain breast and uterine cancers. The presence of both receptors indicates probable success of hormone-related therapies. The test may be performed on microscope samples on glass slides, and observed by conventional light microscopy.

In another embodiment, the analytes are first and second different nucleic acid sequences in a polynucleotide or polynucleotides. One enzyme (e.g., a hydrolase) is associated with a first polynucleotide probe that binds the first sequence and the second enzyme (e.g., a peroxidase) is associated with a second polynucleotide probe that binds the second sequence. A variety of methods are known for binding enzymes to specific polynucleotide probes. In one embodiment, for example, a first DNA probe is labeled with fluorescein and a second DNA probe is labeled with digoxigenin according to standard methods. The probes are hybridized to a polynucleotide or polynucleotides in a sample, such as membrane to which DNA is bound (e.g., a Southern blot) or a tissue section containing DNA, with appropriate washing steps to remove unbound material. The sample is then contacted with enzyme-conjugated antibodies, e.g., phosphatase-conjugated anti-fluorescein and peroxidase-labeled anti-digoxigenin, washed to remove unbound antibodies, and then contacted with a mixture of substrates as described *supra*. If the sample contained nucleic acid sequences for hybridization of both probes (e.g., co-localized in the same nucleus), a third reaction product will become detectable. This method may be used to distinguish cells that are heterozygous for a DNA sequence from those that are homozygous.

Reaction product 3 may be detected by any suitable means, including microscopy and direct visualization. As noted, Reaction product 3 is typically detectable as a

colored precipitate. Photography and photoscanning may be used to record the appearance and location of the product.

The analytes according to the invention are immobilized in the sample (e.g., in a cell, tissue section or Western blot). Methods for fixation of cells and tissues are well known in the histochemical arts. Suitable fixatives include formaldehyde-based fixatives (e.g., formaldehyde, formalin, buffered formalin, paraformaldehyde, and the like), glutaraldehyde, and others (e.g., precipitating or extractive fixatives such as acetone, methanol/acetone, methanol/acetic acid and the like. It will be appreciated that fixatives and fixation conditions are selected, in various embodiments, to retain the enzymatic activity of the target analyte, and/or to preserve the ability of the target analyte to be bound by a SPB partner (e.g., anti-analyte antibody). Methods of binding antibodies to cell or immobilized protein (e.g., Western Blots) are also known (see, e.g., Harlow, *supra*; Staines, 1988, *J. Histochem. Cytochem.* 36:145; Gillitzer et al., 1990, *J. Histochem. Cytochem.* 38:307; Wagner and Worman, 1988, *Stain Technology* 63:129; McGovern and Crocker, 1987, *Am. J. Clin. Pathol.* 88, 480; Graham et al., 1991, *J. Clin. Pathol.* 44:96; Ausubel et al., 1999, *Current Protocols In Molecular Biology*, Greene Publishing and Wiley-Interscience, New York, including *Supplement 46* (April 1999)). In some embodiments (see e.g., Example 9, *infra*), analyte subjected to electrophoresis is immobilized ("fixed") to a substrate using a fixative such as 2% trichloroacetic acid, 5% glacial acetic acid, or the like. It will be appreciated that the specific method of immobilization is not critical to the present invention.

According to normal protocols, following antibody binding to the sample, any unbound antibody is removed in a wash step using, e.g., PBS (phosphate buffered saline), Tris-based buffer, and the like, with or without non-ionic detergent, or other suitable washing solution.

(e) Detecting a second reaction product

In some embodiments, at least one of the reaction products 1 and 2 is also detectable and can be distinguished from reaction product 3. Consider, for example, the following assay for progesterone receptor or "PR" (analyte 1) and estrogen receptor or "ER" (analyte 2) in tissue section such as a breast cancer tissue (also discussed *supra*).

The tissue section is contacted with HRP-labeled anti-PR immunoglobulin and AP-labeled anti-ER immunoglobulin by standard methods (see e.g., Harlow and Lane, 1988, ANTIBODIES: A LABORATORY MANUAL, Cold Spring Harbor Laboratory, New York). After

washing to remove unbound immunoglobulin/enzyme, the following reagents are added in a suitable aqueous buffer (e.g., 50 mM Borax, 10 mM $MgCl_2$, pH 9.2):

1. alpha napthol-phosphate (substrate for AP which is associated with ER)
2. diaminobenzidine (substrate for HRP which is associated with PR)
3. hydrogen peroxide (cosubstrate for HRP)

If both ER and PR are present in a cell, alpha napthol and oxidized diaminobenzidine will be produced, and will chemically combine to produce a blue precipitate. However, if only PR is present, the action of HRP on diaminobenzidine will produce a yellow product resulting from the homopolymerization of oxidized diaminobenzidine. Thus, in this example, the presence of the two receptors can be determined as follows:

+PR, +ER	blue
+PR, -ER	yellow
-PR, +ER	no color
-PR, -ER	no color

In another embodiment, a second color coupler may be added to increase contrast if only one analyte is present. For example, given the ER/PR example above, the following reagents can be added:

1. naphthol AS BI phosphate (substrate for AP which is associated with ER)
2. diaminobenzidine (substrate for HRP which is associated with PR)
3. peroxide (cosubstrate for HRP)

After development, the sample, e.g., tissue section on slide, is washed and Fast Red (diazonium salt of 5-nitroanisidine) is added. If both ER and PR are present in the same cell, Napthol AS BI and oxidized diaminobenzidine will be produced, and will chemically combine to produce a blue precipitate. However, if only PR is present, the oxidized diaminobenzidine in the cell will homopolymerize. If only ER is in a cell, the cell will stain red as a result of coupling Naphthol AS BI (which is very poorly soluble) with the diazonium compound, Fast Red. Other reagents, including other diazonium salts (e.g., Fast Garnet, aminoazotoluene) may be used in place of Fast Red. Thus, in this example, the presence and co-localization of the two receptors can be determined as follows:

+PR, +ER	blue
+PR, -ER	yellow
-PR, +ER	red
-PR, -ER	no color

Using the present invention, it is possible to quickly and conveniently determine that single cells in the sample contained neither, both, or one of the two target analytes.

Advantages of the present invention over other detection methods include reduced non-specific backgrounds; a convenient one-step color reaction; the ability to detect the reaction product (and therefor confirm the presence of both analytes) using light microscopy, which is both inexpensive and subject to automation.

(f) Kits

The reagents useful for practicing the methods of the present invention are conveniently provided in kit form. In one embodiment, the kit comprises a container including separate vials of one or more of: (1) a hydrolase substrate that can be hydrolized to produce a color coupler (such as a specific color coupler listed herein); (2) an oxidizable developer (such as a specific oxidizable developer listed herein) (2) a hydrolase (such as a specific hydrolase listed herein) conjugated to antibody, biotin, avidin, streptavidin, or other SPB (3) an oxidase (such as a specific oxidase listed herein) conjugated to an antibody, biotin, avidin, streptavidin, or other SPB (4) a peroxidase, (5) a peroxide (e.g., hydrogen peroxide), (6) a buffer. Typically at least two or at least three of the above-listed reagents are included.

EXAMPLES

Reagents

The following reagents were used in the experiments described in this section:

(a) Hydrogen peroxide (3%).

(b) Borax buffer

170mM borax, 120mM sodium chloride, 50mM magnesium chloride, pH 9.2

(c) Bis TRIS buffer

150mM sodium chloride, 25 mM Bis TRIS, pH 7.5

(d) 0.075 M veronal acetate buffer pH 8.2 (Beckman B-2 for electrophoresis)

(e) 150 mM Phosphate Buffered Saline (PBS) pH 7.2 (Sigma Chemical Co., St . Louis MO)

(f) 50 mM solutions of: calcium alpha napthyl phosphate, diaminobenzidine, N,N-diethyl-p-phenylene diamine, N,N-dimethyl-p-phenylene diamine, 4-chloro-1 naphthol in diethylene glycol (DEG), alpha naphthol in diethylene glycol.

Example 1: Conjugation of Enzyme to Sephacryl Beads

This example describes preparation of beads conjugated to biotin-conjugated peroxidase.

Biotin-Horseradish peroxidase was conjugated to Sephacryl Beads according to the procedure of Dean et al. (1985) "Affinity Chromatography," IRL Press (Oxford and Washington DC) pp. 64-65. Briefly, sedimented Sephacryl 200 beads (Pharmacia) were equilibrated in 1 M phosphate buffer pH 7.4 containing 2.5% glutaraldehyde for 10 hours at 20°C, and thoroughly washed in distilled water. The beads were positive with Schiff reagent but the wash solutions became negative for Schiff reagent. The beads could be stored in active form at 4°C for several months.

10 mL of packed beads was resuspended in PBS, and biotin-conjugated Horseradish peroxidase (Vector Laboratories, Burlingame, CA) was added. The suspension was mixed in a rotary mixer for 2 hours at room temperature. The beads were washed 4x in PBS and the solution tested for peroxidase activity. Washing was repeated until the solution was negative for activity. The biotin-HRP-conjugated beads ["b-HRP-beads"] were stored at 4°C.

Example 2. Demonstration of peroxidase activity.

This example demonstrates that the beads described in Example 1 retain peroxidase activity.

10 mL of a 5% suspension of b-HRP-beads was incubated with 100 µL 3% H_2O_2, 100 µL of 50 mM N,N-diethyl-p-phenylene diamine, and 100 µL 50 mM alpha-naphthol in 5 mL of borax buffer (pH 9.2). The beads turned a bright blue color within about 5 minutes, and the solution became slightly blue in about 40 minutes. The concentration of the substrate components could be varied one at a time until the solution was consistently almost colorless and the beads were consistently blue.

Example 3. Dependence of peroxidase activity on alpha naphthol.

This example demonstrates the dependence of the production of the detectable reaction product on the presence of alpha-naphthol. In this example, alpha-naphthol is added as a reagent. In one embodiment of the invention, alpha-naphthol is produced as the product of a half-reaction (i.e., as "Reaction Product 1").

The experiment of Example 2 was repeated, using 50 µl of hydrogen peroxide and 50 µl of N,N-diethyl-p-phenylene diamine in 15 mL buffer. The concentration of alpha-naphthol was varied from 200 µl to 0 µl. Results were that from 200 and 100 µl, both solution and beads turned blue. At 50 µl, only beads turned blue. Below 50 µl, the beads became fainter blue and at 0 µl, no color developed.

To quantitate the production of blue product, the beads were transferred into a capillary (approximately 1.2 mm inside dimension by 75 mm length) and settled into one end by centrifugation. The capillaries were then placed into an adapter holder for a 96 well plate reader and read at 650 nm over both bead region and solution region according to the procedure described in US patent No. 5,674,699 "Two Phase Assay." Results of the measurements are provided in Table 5.

Table 5

Alpha-Naphthol Dilution Series

	Dilution	µl α-naphthol	OD 650 nm
1	1	200	0.498
2	0.5	100	0.237
3	0.25	50	0.152
4	0.125	25	0.067
5	0.0625	12.5	0.035
6	0.03125	6.25	0.02
7	0.015625	3.12	0.005
8	0.007813	1.56	0.007
9	0.003906	0.78	0.011

Example 4. Incubation of beads with alkaline phosphatase.

Alkaline phosphatase ("AP") (R&D Systems, Minneapolis, MN) and, separately, streptavidin alkaline phosphatase ("Strep-AP") (R&D Systems, Minneapolis, MN) were made up as 1 µg/ml stock solutions. Further serial 1:1 dilutions of each were made separately in BIS-TRIS buffer.

b-HRP-beads (20 µL) were incubated with 100 µL of each enzyme dilution for 2 h at 20°C. The beads were washed 3x in PBS and stained in 100 µL of solution I.

Solution I:

Borax buffer	15 ml
50 mM Calcium alpha naphthol phosphate	50 µL
50 mM N,N-diethyl-p-phenylene diamine	50 µL
3% hydrogen peroxide	50 µL

B-HRP-beads incubated with streptavidin-AP turned blue, while the beads incubated with AP remained colorless. The beads and solution were measured as in Example 3. The results are provided in Table 6.

Table 6

Staining Intensity Of B-HRP-Beads After Incubation In Various Dilutions Of Strep-AP

Strep-AP (μL)	Rel. Conc	OD 650 nm Beads	OD 650 nm Solution
0.46875	1	0.221	0.21
0.9375	2	0.648	0.211
1,875	4	0.815	0.205
3.75	8	0.835	0.19
7.5	16	0.871	0.224
15	32	1.012	0.212
30	64	1.094	0.239

This experiment demonstrates the requirement that both AP and HRP be present (i.e., associated with the beads), allowing both half-reactions to proceed in order to produce a detectable reaction product.

Example 5. Requirement for localized reaction.

The experiment of Example 4 was repeated, except that the beads were not washed after incubation with the enzyme solutions. In both strep-AP and AP incubations, both the beads and the solution turned blue. This indicates that oxidized developer may diffuse into the solution and couple with the free naphthol produced by the AP reaction (while free AP may also diffuse into the beads).

Example 6. Model histochemistry experiments.

Reagents:

(a) Primary antibody: mouse anti-cytokeratin antibody was obtained from Biogenix, San Ramon, CA.

(b) Secondary antibody: polyclonal rabbit anti-mouse antibody conjugated to AP ("AP-conjugated Ab") or to HRP was obtained from Vector Laboratories ("HRP-conjugated Ab")

(c) Bovine serum albumin (BSA) was obtained from Sigma Chemical Co., St. Louis, MO.

Cytokeratin-containing cells were obtained from scrapings of buccal mucosa of human volunteers. The scrapings were suspended in PBS, shaken to separate into single

cells or small clusters, and centrifuged. Concentrated cells were deposited on glass microscope slides in a manner similar to making blood smears.

The cells were fixed with acetone-ethanol (1:1) at -20°C for 10 minutes. The cells were then incubated for 30 minutes in 1% BSA in PBS (blocking solution). After washing with PBS the cells were incubated with the primary antibody, washed, and incubated with AP-conjugated Ab, HRP-conjugated Ab, or both AP-conjugated Ab and HRP-conjugated Ab. After an additional wash, color was developed with 40 mL of PBS buffer containing 200 μL each of hydrogen peroxide, alpha naphthyl phosphate and N,N-dimethyl-p-phenylenediamine for 5, 7, 10, 15 or 20 minutes. (The color develops gradually and may be stopped by washing at any desired time.)

When both AP-conjugated Ab and HRP-conjugated Ab secondary antibodies were present, the cytokeratin containing cells were blue in a pattern previously confirmed to be the cytokeratin pattern, *i.e.*, intracytoplasmic strands and meshworks as well as pale diffuse cytoplasmic staining. However, when only one of the enzyme secondary antibodies was used in the final incubation there was no color. This demonstrates that, in order to develop color with both HRP and AP half-reactions, both enzymes must be present in the same cells.

Example 7. Single secondary antibody.

The experiment of Example 6 was repeated, but the color was developed using a "complete" peroxidase reaction or a "complete" AP reaction. (In this example only, the term "complete" means that all necessary reagents are provided to produce color when only one enzyme is present, in contrast to the term "half-reaction" in which both enzymes must be present to generate the desired reaction product.) The "complete" peroxidase reaction used N,N dimethyl p phenylene diamine hydrogen peroxide and 4-chloro-1-naphthol. The "complete" AP reaction used alpha naphthyl phosphate and Fast Blue BB as diazonium salt (supplied as a kit from Vector Laboratories).

Staining of AP was positive when AP alone or when AP and HRP secondary antibody was used in the incubation. Similarly the staining was positive when for peroxidase when HRP alone or HRP and AP were in the second incubation. The AP incubation was negative in peroxidase staining, and likewise the HRP incubation was negative with AP staining.

This experiment confirms that the AP or HRP interactions in Example 6 were effective, even if the half reactions only produce blue produced when both enzymes are present.

Example 8. Detection of Fetal hemoglobin.

Fetal blood cells were enriched from human maternal blood as described in U.S. Pat. No. 5,432,054. Smears were made on microscope slides by conventional methods. Both maternal and fetal cells were present on the slide. Fetal cells were also obtained from fetuses at termination of pregnancy. In this case only fetal cells were placed on the slide. The cells were fixed in 80% ethanol at room temperature which is known to inactivate myeloperoxidase.

The cells were incubated in mouse anti fetal hemoglobin antibody (Cortex Biochem., San Leandro, CA, Cat. #CR815M) and washed. The secondary antibody (rabbit anti-mouse) conjugated with AP was applied as in Example 8. However no HRP antibody was used in this incubation. Instead, the pseudoperoxidase activity of hemoglobins supplies the peroxidase activity. Staining was carried out using hydrogen peroxide, alpha naphthyl phosphate and N,N dimethyl p phenylene dianine.

Slides with only fetal red blood cells had almost 100% positive-staining (i.e., blue) red blood cells on the slide. Slides with mixed fetal and maternal cells had only a small number of positive cells, as expected from the enrichment technique. This demonstrated that signal was detected only when both pseudoperoxidase activity from hemoglobin (fetal or adult) and AP activity (associated with fetal hemoglobin) were present in a cell. This provides lower background and greater confidence than staining with AP-labeled antibody alone.

Example 9. Electrophoresis of Hemoglobin.

Electrophoresis was carried out on blood. Lysed whole fetal blood alone, or lysed mixed fetal and maternal blood, was deposited on cellulose acetate electrophoresis strips equilibrated in Veronal buffer. Separation was for 30 minutes at 5 mAmps and 250 Volts. The post separation strip was fixed in 2% trichloroacetic acid in water, and washed 3 times in distilled water. The strip was incubated for 30 minutes in 3% BSA in Bis TRIS buffer, and washed twice in the same buffer.

The strip was the incubated for 1 hour with an anti-fetal hemoglobin antibody as in Example 8, washed and incubated for another hour with AP-labeled rabbit anti-mouse

IgG (Vector Laboratories) second antibody. Staining was with Solution I as described *supra* in Example 4.

Bands containing fetal hemoglobin were stained blue in both the fetal blood only and in the mixed fetal/maternal blood sample. The band corresponding to maternal hemoglobin on the electrophoretic strip did not stain blue, but was visibly red due to the presence of hemoglobin. This demonstrates that an antibody label may be applied to electrophoretic separations and that bands having both enzyme activities (e.g., pseudoperoxidase and antibody-linked hydrolase) may be specifically detected and distinguished from bands having only one activity (e.g., the pseudoperoxidase activity of maternal hemoglobin) which may co-migrate with fetal hemoglobin.

If the staining mixture used in this example is changed to substitute 4-chloro-1-naphthol in place of alpha naphthyl phosphate, then the maternal hemoglobin zone will also appear blue.

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All references cited herein are incorporated herein by reference in their entirety and for all purposes to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference in its entirety for all purposes.

Many modifications and variations of this invention can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. The specific embodiments described herein are offered by way of example only, and the invention is to be limited only by the terms of the appended claims, along with the full scope of equivalents to which such claims are entitled.

WHAT IS CLAIMED IS:

1. A method for determining whether a first analyte and second analyte are co-localized in a sample comprising:

a) associating a first enzyme with the first analyte if it is present in the sample;

b) associating a second enzyme with the second analyte if it is present in the sample;

c) providing a first substrate and a second substrate,

wherein the first enzyme acts on the first substrate to produce a first reaction product, the second enzyme acts on the second substrate to produce a second reaction product; and the first and second reaction products chemically combine to form a third reaction product;

d) detecting the third reaction product; and,

e) correlating the presence of the third reaction product with the presence of co-localized first analyte and second analyte in the sample.

2. The method of claim 1, wherein the first enzyme is a hydrolase.

3. The method of claim 2, wherein the hydrolase is a phosphatase, an esterase, a peptidase or an amidase.

4. The method of claim 1 wherein the second enzyme is an oxidase.

5. The method of claim 4 wherein the enzyme is a peroxidase or a pseudoperoxidase.

6. The method of claim 5, wherein the peroxidase is horseradish peroxidase.

7. The method of claim 1, wherein either the first or second enzyme is conjugated to an antibody.

8. The method of claim 7 wherein the antibody directly binds either the first or second analyte.

9. The method of claim 1, wherein the first substrate is a naphthol derivative.

10. The method of claim 9, wherein the napthol derivative is alpha-naphthol-phosphate.

11. The method of claim 1, wherein the second reaction product forms a detectable product in the absence of the first reaction product.

12. The method of claim 1 further comprising adding a compound that combines with the first reaction product to form a detectable colored product, wherein said colored product is distinguishable from the third reaction product.

13. The method of claim 12 wherein the compound is a diazonium salt.

14. The method of claim 1, wherein the sample is a tissue section.

15. The method of claim 14 wherein the first analyte and second analyte are co-localized in a cell.

16. A method for determining whether an analyte and a protein with hydrolytic activity are co-localized in a sample comprising:

a) associating an oxidase with the analyte if it is present in the sample;

b) providing a first substrate and a second substrate,

wherein the hydrolase acts on the first substrate to produce a first reaction product, the oxidase acts on the second substrate to produce a second reaction product; and the first and second reaction products chemically combine to form a third reaction product;

c) detecting the third reaction product; and,

d) correlating the presence of the third reaction product with presence of co-localized first analyte and hydrolase in the sample.

17. The method of claim 15 wherein the protein with hydrolytic activity is a phosphatase, esterase, galactosidase, lipase, glucuronidase, amidase, peptidase or sulphatase.

18. A method for determining whether an analyte and a protein with oxidase activity are both present in a sample comprising:

a) associating a hydrolase with the analyte if it is present in the sample;

b) providing a first substrate and a second substrate,

wherein the hydrolase acts on the first substrate to produce a first reaction product, the protein with oxidase activity acts on the second substrate to produce a second reaction product; and the first and second reaction products chemically combine to form a third reaction product;

c) detecting the third reaction product; and,

d) correlating the presence of the third reaction product with presence of co-localized first analyte and oxidase in the sample.

19. The method of claim 18 wherein the oxidase is a peroxidase or a pseudoperoxidase.

20. A method for determining whether a first enzyme and a second enzyme are both co-localized in a sample comprising:

a) providing a first substrate and a second substrate,

wherein the first enzyme acts on the first substrate to produce a first reaction product, the second enzyme acts on the second substrate to produce a second reaction product; and the first and second reaction products chemically combine to form a third reaction product;

b) detecting the third reaction product; and,

c) correlating the presence of the third reaction product with presence of co-localized first enzyme and second enzyme in the sample.

21. The method of claim 20, wherein the first enzyme is a hydrolase and the second enzyme is an oxidase.

22. At kit comprising a container, a compound that produces a color coupler upon hydrolysis, an oxidizable developer, and a hydrolase.

23. The kit of claim 22, wherein the compound that produces a color coupler upon hydrolysis and oxidizable developer are in a single vial.

INTERNATIONAL SEARCH REPORT

Int ational Application No
PCT/US 00/26841

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/28 C12Q1/42 G01N33/72 G01N33/58

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 04157 A (RAMBACH ALAIN) 9 February 1995 (1995-02-09) claims 1-4	1-3,11, 20,22,23
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P,X	WO 99 51767 A (BIO MERIEUX ;MONGET DANIEL (FR); ORENGA SYLVAIN (FR); ARMSTRONG LY) 14 October 1999 (1999-10-14) page 5, line 7 - line 27 page 19, line 17 -page 23, line 12; examples 4-6 claim 2	1-3,11, 20,22,23

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
15 March 2001	26/03/2001

Name and mailing address of the ISA	Authorized officer
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