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USE OF 4' OF 5'-HYDROXY AND 4'- OR 5'-ALKOXY-SUBSTITUTED ELECTRON
TRANSFER AGENTS IN COMPOSITIONS, ELEMENTS, TEST KITS
AND ANALYTICAL METHODS

This invention relates to compositions,

- 5 analytical elements, test kits and methods which
utilize certain 4'-hydroxy- and alkoxy-substituted
anilides as electron transfer agents. In particular,
such compounds are useful to improve sensitivity in
analytical and diagnostic methods.
- 10 It is well known to perform a quantitative or
qualitative analysis of an aqueous liquid by contacting
that liquid with a combination of reagents capable of
yielding a detectable product in proportion to the
concentration of the analyte in the liquid. One type
- 15 of useful assay utilizes enzymatic reactions wherein
the analyte, upon contact with the appropriate
reagents, reacts with oxygen in the presence of a
suitable enzyme to produce hydrogen peroxide in
proportion to concentration of the analyte. A
- 20 detectable product is then produced by the reaction of
hydrogen peroxide in proportion to the concentration of
the analyte in the test liquid. Peroxidase is
generally used in such assays.

- In other assays, a peroxidase is reacted in
- 25 the presence of hydrogen peroxide which has been added
to the system to measure the amount of a particular
analyte. Analytes such as glucose, triglycerides, uric
acid, cholesterol and creatine kinase can be so
detected as well as specific binding ligands in
- 30 specific binding assays wherein the peroxidase is used
as a detectable label. Such determinations can be
carried out in solution, dry analytical assays or
diagnostic test devices. The signals produced in such
assays can be a colorimetric, chemiluminescent or
- 35 fluorescent signal using well known signal generating
reagents.



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It has been previously demonstrated that certain phenols and anilines can be used to enhance the production of a colorimetric signal using peroxidase as a reagent in signal generation.

5 There are also several major types of
luminescent or luminometric assays which produce an
emission of light as a result of the presence of the
analyte of interest. These assays are also known as
chemiluminescent assays and are described, for example,
10 in US-A-4,729,950 and publications noted therein.
Various aromatic amines and phenols, such as p-
iodophenol, are considered useful for enhancing the
production of light in such assays (see also US-A-
4,598,044).

15 One preferred enhancer of colorimetric assays
has been 4'-hydroxyacetanilide, which is described in
US-A-4,828,983.

 Although 4'-hydroxyacetanilide shows
remarkable behavior in many analytical systems, there
20 is a need for further improvement. For example, high
concentrations of 4'-hydroxyacetanilide do not produce
correspondingly high signal generation. In addition,
the stability of the compound needs to be increased.

 These problems have been solved with a
25 composition for providing a colorimetric or
chemiluminescent signal having a pH of from 5 to 10,
and comprising:

 a) a colorimetric or chemiluminescent
signal generating reagent which provides a signal in
30 response to the catalytic activity of peroxidase, the
signal generating reagent being

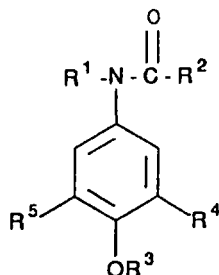
 a 2,3-dihydro-1,4-phthalazinedione
derivative,

 a tetrazolium salt,
35 a diazonium salt, or

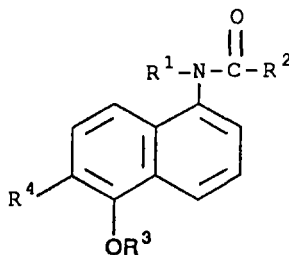
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an imidazole or triarylmethane leuco
dye, and

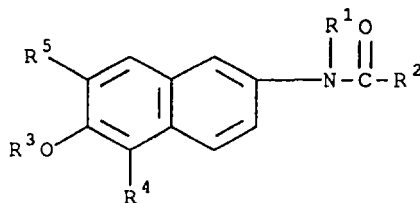
- b) an electron transfer agent,
the composition characterized wherein
5 the electron transfer agent has structure (I):



structure (II):

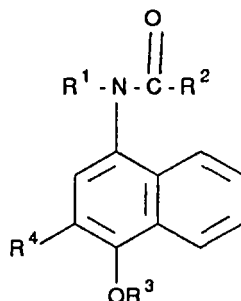


structure (III):



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or structure (IV):



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wherein R^1 is hydrogen, alkyl of 1 to 4 carbon atoms, hydroxyalkyl of 1 to 4 carbon atoms, alkoxyalkyl of 1 to 4 carbon atoms, aminoalkyl of 1 to 4 carbon atoms, halogenated alkyl of 1 to 4 carbon atoms,

5 R^2 is hydrogen, alkyl of 1 to 4 carbon atoms, hydroxyalkyl of 1 to 4 carbon atoms, alkoxyalkyl of 1 to 4 carbon atoms, aminoalkyl of 1 to 4 carbon atoms, halogenated alkyl of 1 to 4 carbon atoms, or alkenyl of 2 to 5 carbon atoms,

10 R^3 is hydrogen or alkyl of 1 to 4 carbon atoms, and

R^4 and R^5 are independently hydrogen or an electron withdrawing group having a Hammett sigma value of at least 0.01,

15 provided that in structure (I), at least one of R^4 and R^5 is an electron withdrawing group having a Hammett sigma value of at least 0.01.

Further, an aqueous composition which is buffered to a pH of from 5 to 10 comprises a peroxidase or peroxidase-labeled specific binding molecule, and is characterized as having an electron transfer agent as defined above.

This invention also provides a diagnostic test kit for the determination of an analyte as the result of the catalytic activity of peroxidase, the kit comprising, in individual packaging:

i) the composition described above, and
ii) peroxidase or a peroxidase-labeled specific binding molecule.

30 A test device for the detection of peroxidase or an analyte catalytically related to peroxidase comprises an absorbent carrier material, and contains a colorimetric or chemiluminescent signal generating reagent,

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the test device characterized as further comprising an electron transfer agent having structure (I), (II), (III) or (IV) as described above.

Another embodiment of this invention is a method for producing a detectable signal in response to peroxidase comprising:

- A. reacting a peroxidase in the presence of
 - a) the signal generating reagent described above,
 - 10 b) an electron transfer agent having structure (I), (II), (III) or (IV) as described above, and
 - c) an oxidant,
to produce a detectable colorimetric or
15 chemiluminescent signal, and
 - B. determining the resulting colorimetric or chemiluminescent signal as a measure of peroxidase.
- A specific binding assay for the determination of a specific binding ligand comprises:
- 20 A. complexing a specific binding ligand with a receptor specific for the ligand to form a specific binding complex,
 - B. labeling the specific binding complex with a peroxidase which is either conjugated with the
25 receptor, or is conjugated with a specific binding molecule which is specifically reactive with either the specific binding ligand or the receptor,
 - C. after separating uncomplexed materials from the peroxidase-labeled complex, contacting the
30 peroxidase-labeled complex with:
 - a) the colorimetric or chemiluminescent signal generating reagent described above,
 - b) an electron transfer agent having structure (I), (II), (III) or (IV) as described above,
35 and

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c) an oxidant,
to produce a detectable colorimetric or
chemiluminescent signal, and

D. determining the resulting signal as a measure
5 of the specific binding ligand.

The present invention provides all of the
known advantages associated with the use of various
colorimetric or chemiluminescent assays utilizing
peroxidase in the reaction scheme. However, by using
10 the noted electron transfer agents defined by
structures (I), (II), (III) and (IV), the assays are
more sensitive.

Moreover, the present invention demonstrates
stable kinetics, especially for the chemiluminescent
15 assays described herein, wherein the emitted light
signal remains at a constant intensity for a longer
period of time.

FIGURE 1 is a graphical representation of
chemiluminescent signal obtained using a composition of
20 this invention and a Control composition, as described
in Example 4 below.

FIGURE 2 is a graphical representation of
chemiluminescent signal obtained using a composition of
this invention and a Control composition at various
25 concentrations of horseradish peroxidase, as described
in Example 4 below.

FIGURE 3 is a graphical representative of
chemiluminescent signal obtained in assays versus the
concentration of enzyme label, as described in Examples
30 5-7 below.

FIGURE 4 is a graphical representation of
chemiluminescent signal obtained in assay for thyroid
stimulating hormone (TSH), as described in Example 8
below.

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FIGURE 5 is a graphical plot of dye signal versus *Chlamydia trachomatis* antigen concentration after 1 minute of incubation time as described in Example 9 below.

5 FIGURE 6 is a graphical plot of dye signal versus *Chlamydia trachomatis* antigen concentration after 2 minutes of incubation time as described in Example 9 below.

10 FIGURE 7 is a graphical plot of dye signal versus *Chlamydia trachomatis* antigen concentration after 3 minutes of incubation time as described in Example 9 below.

15 FIGURE 8 is a graphical plot of the change in dye signal versus concentration of electron transfer agent for the assays described in Example 10 below.

20 The present invention can be practiced to advantage in any analytical method designed to generate a colorimetric or chemiluminescent signal in response to the presence of a peroxidase. Such assays can involve the detection of an organic or inorganic peroxide (such as hydrogen peroxide) or peroxidase (in its free form), or the detection of a non-immunological analyte other than peroxidase or hydrogen peroxide. In particular, the invention is useful in the practice of specific binding assays which generate a colorimetric, chemiluminescent or fluorescent signal.

25 The assay can be qualitative or quantitative or both, and be used to detect a biological or chemical substance (that is, an analyte) in aqueous liquids, including human or animal biological fluids, waste fluids, foods, environmental effluent, chemical processing liquids and other specimens readily apparent to one skilled in the art.

30 Hydrogen peroxide (or another peroxide) can be determined with this invention. In addition, the

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invention can be used to determine analytes which are capable of producing hydrogen peroxide, that is the analyte participates in one or more reactions which produce hydrogen peroxide in the presence of suitable
5 signal generating reagents and a peroxidase. Such analytes are considered herein as "analytes catalytically related to peroxidase".

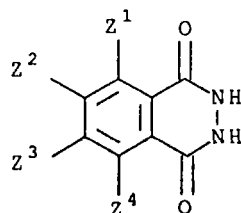
In a preferred embodiment, the invention is useful for the determination of a specific binding
10 ligand, or its corresponding receptor (that is, a substance which specifically binds with the ligand). Such ligands include, but are not limited to, antibodies and other proteins (including lipoproteins, blood proteins, enzymes and glycoproteins), haptens,
15 drugs, hormones, steroids, toxins, viruses, bacteria, vitamins, saccharides (including polysaccharides), lipids, nucleic acids, nonproteinaceous blood components, or any component thereof as readily understood by one skilled in the art.

20 A preferred chemiluminescent signal generating reagent is a 2,3-dihydro-1,4-phthalazinedione derivative (identified herein as "DPD"). Any free or conjugated 2,3-dihydro-1,4-phthalazinedione derivative that can be converted to an
25 excited state in a chemiluminescent reaction and then returns to a non-excited state with the emission of light, is useful in the practice of this invention. A considerable number of such compounds are known in the art, including those described in US-A-4,598,044 and
30 Chemiluminescence in Organic Chemistry, Gundermann and McCapra, Springer-Verlag, Berlin, 1987, pages 204-207. Such compounds are generally known as "luminol type hydrazides" and include phthalic hydrazides, naphthalene-1,2-dicarboxylic acid hydrazides,
35 anthracene-2,3-dicarboxylic acid hydrazides,

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phenanthrene-1,2-dicarboxylic acid hydrazides, fluorene-1,2-dicarboxylic acid hydrazides, benzo[g,h,i]perylene-1,2-dicarboxylic acid hydrazides, coronene-1,2-dicarboxylic acid hydrazides, and others readily
 5 apparent to one skilled in the art.

In particular, the DPD is defined by the structure (V):



wherein Z^1 , Z^2 , Z^3 and Z^4 are independently hydrogen,
 10 alkyl of 1 to 6 carbon atoms (such as methyl, ethyl, isopropyl, t-butyl, sec-pentyl and hexyl), alkenyl of 2 to 6 carbon atoms [such as ethenyl, 1-propenyl, isobutenyl, 2-(N,N-diisopropylamino)vinyl, 2-(N,N-diisobutylamino)vinyl, 2-(N,N-diisopentylamino)vinyl
 15 and 2-hexenyl], hydroxy, alkoxy of 1 to 6 carbon atoms (such as methoxy, ethoxy, isopropoxy, t-butoxy and hexoxy), carboxy, amino [including amino substituted with alkyl or alkanoyl, such as methylamino, ethylamino, amido (for example, acetamido and
 20 hexanamido), dimethylamino, diethylamino and diisobutylamino], conjugated aminoalkenyl (for example, as defined below) or aminoaryl [including substituted aminoaryl, such as p-(N,N-dimethylamino)phenyl, p-(N,N-diethylamino)phenyl and 5-amino-2,3-dihydro-1,4-
 25 phthalazinedion-8-yl (also known as luminy)]].

At least one of Z^1 and Z^2 is amino (including substituted amino, as defined above), conjugated aminoalkenyl (including substituted aminoalkenyl as described above) or aminoaryl [such as p-(N,N-
 30 dimethylamino)phenyl, p-(N,N-diethylamino)phenyl and 5-amino-2,3-dihydro-1,4-phthalazinedion-8-yl]. As used

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herein, "conjugated aminoalkenyl" refers to a monovalent group capable of electron resonance from the amino group through the alkenyl group to the aromatic ring of the phthalazinedione where it is substituted, and includes for example, a dialkylaminovinyl group [such as 2-(N,N-diisopropylamino)vinyl, 2-(N,N-diisobutylamino)vinyl and 2-(N,N-diisopentylamino)vinyl], and dialkylaminobutadienyl groups, such as 4-(N,N-diethylamino)-1,3-butadien-1-yl.

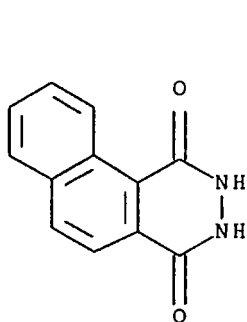
Alternatively, any adjacent two, adjacent three or all of Z^1 , Z^2 , Z^3 and Z^4 (that is, combinations of two or three adjacent groups, or all four groups) can be taken together to form a fused ring system containing one or more aromatic rings. Such fused rings can be substituted with one or more hydroxy, amino (substituted or unsubstituted as described above) or alkoxy of 1 to 4 carbon atoms (such as methoxy, ethoxy and isopropoxy). Preferably, such fused rings are substituted with one or more primary, secondary or tertiary amines, hydroxy or alkoxy as described above.

Representative useful DPD compounds include, but are not limited to, luminol, isoluminol, N-(4-aminobutyl)-N-ethylisoluminol hemisuccinimide, N-(6-aminoethyl)-N-ethylisoluminol, N-ethylisoluminol and 7-dimethylaminonaphthalene-1,2-dicarboxylic acid hydrazide. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) and isoluminol (6-amino-2,3-dihydro-1,4-phthalazinedione) are preferred, and luminol is most preferred.

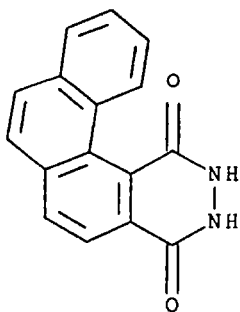
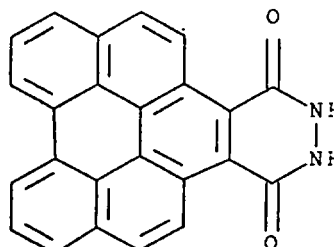
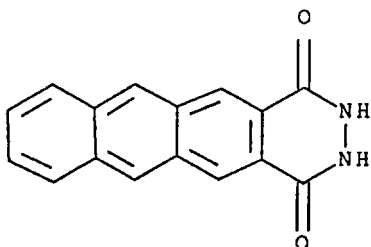
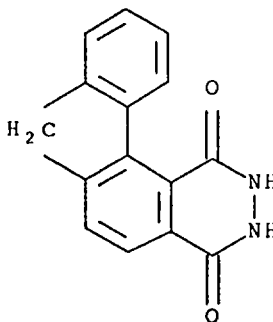
Other useful classes of DPD compounds are described in the Gundermann and McCapra publication noted above, and include substituted or unsubstituted phthalic acid hydrazides, anthracene-2,3-dicarboxylic acid hydrazides, phenanthrene dicarboxylic acid

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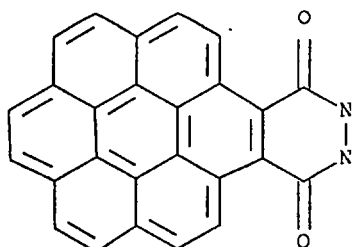
hydrazides, fluorene-1,2-dicarboxylic acid hydrazides, benzo[g,h,i]perylene-1,2-dicarboxylic acid hydrazides and coronene-1,2-dicarboxylic acid hydrazides, such as those illustrated by the following structures:



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and



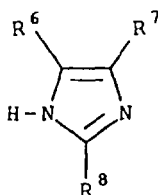
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Other useful signal generating reagents include imidazole or triarylmethane leuco dyes, such as those described in US-A-4,089,747 and references noted therein, EP-A-0 122 641, Japanese Patent Publication
15 No. 58(1983)-045557 and US-A-4,670,385.

The triarylimidazole leuco dyes described in the Bruschi patent are preferred for generating a

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colorimetric signal in the practice of this invention.
Such leuco dyes generally have the structure (VI):



wherein R^6 , R^7 and R^8 are independently an organic
5 group such that at least one of them is an *o*- or *p*-
hydroxysubstituted aryl group of up to 18 carbon atoms
in the aromatic ring, and the other two groups being
aryl groups chosen such that the imidazole oxidation
potential is within the range of from -70 to +100 mV as
10 measured by cyclic voltammetry against a standard
calomel electrode using a carbon based electrode.
Oxidation potential measurements can be made according
to conventional electrochemical techniques (see for
example, Sawyer et al, Experimental Electrochemistry
15 for Chemists, John Wiley & Sons, New York, 1974).

As used in the definition of the leuco dyes,
"aryl" is meant to include aromatic hydrocarbon groups
such as phenyl, naphthyl or anthryl, tolyl, xylyl and
other substituted aromatic groups. The number of
20 carbon atoms refers to the total nuclear carbon atoms
as well as those in the substituents. At least one of
the R^6 , R^7 and R^8 groups has an *ortho* or *para* electron
donating substituent such as an alkoxy ($-OR'$) wherein
 R' is alkyl of 1 to 8 carbon atoms (such as methyl,
25 ethyl, isopropyl, *t*-butyl, hexyl, chloromethyl or
methoxymethyl), or a dialkylamino wherein alkyl is as
just defined.

Particularly useful triarylimidazole leuco
dyes are:

30 2-(4-hydroxy-3,5-dimethoxyphenyl)-4,5-bis(4-
methoxyphenyl)imidazole,

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2-(3,5-dibromo-4-hydroxyphenyl)-4,5-diphenylimidazole,

3-(3-bromo-5-methoxy-4-hydroxyphenyl)-4,5-bis(4-methoxyphenyl)imidazole,

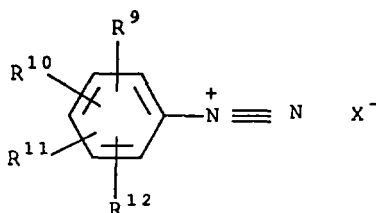
5 4,5-bis(4-dimethylaminophenyl)-2-(4-hydroxyphenyl)imidazole,

4,5-bis(4-dimethylaminophenyl)-2-(4-hydroxy-3-methoxyphenyl)imidazole, and

10 2-(4-hydroxyphenyl)-4,5-bis(4-methoxyphenyl)imidazole.

The diazonium and tetrazolium salts useful in the practice of this invention must be those capable of reacting in the presence of peroxidase and an oxidant to produce a chromophore. A diazonium
15 salt is generally an organic salt of a compound having a diazonium radical.

Particularly useful diazonium salts include, but are not limited to, those having the following structure (VII):



20 wherein R⁹, R¹⁰, R¹¹ and R¹² are independently hydrogen, halo (such as chloro, bromo or iodo), alkyl of 1 to 12 carbon atoms (such as methyl, ethyl, isopropyl, t-butyl, sec-butyl, n-butyl, pentyl,
25 octyl, isononyl and dodecyl), nitro, cyano, alkoxy of 1 to 6 carbon atoms (such as methoxy, ethoxy, pentoxy, isopropoxy, t-butoxy and hexoxy), aryloxy of 6 to 10 carbon atoms in the aromatic ring, including aryloxy substituted with alkoxy and alkyl as defined
30 above (such as phenoxy, naphthoxy, tolyloxy and p-nitrophenoxy), aryl of 6 to 10 carbon atoms (such as

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phenyl, naphthyl, *p*-methylphenyl, *m*-ethoxyphenyl, *p*-cyanophenyl and *o*-methoxyphenyl), acyl of 1 to 12 carbon atoms (such as acetyl, propionyl, benzoyl and butyryl), and substituted or unsubstituted carbamoyl or sulfamoyl (such as carbamoyl, sulfamoyl, *N,N*-dimethylcarbamoyl and *N,N*-diethylsulfamoyl). Preferably, R^9 , R^{10} , R^{11} and R^{12} are independently hydrogen, methyl, chloro, *N,N*-diethylsulfamoyl, nitro or methoxy.

10 In addition, any two of these radicals at adjacent positions on the ring can be taken together to represent a 6- to 10-membered fused aromatic ring system, including both heterocyclic or carbocyclic fused aromatic rings. Such rings can include carbon, 15 nitrogen, oxygen or sulfur atoms in the ring structure. Preferably, 6- to 10-membered carbocyclic ring systems are formed in this manner.

 In the salt noted above, X^- represents any suitable anion which does not inhibit or act as a 20 substrate for peroxidases, such as halide (for example, chloride, bromide and fluoride), tetrafluoroborate, nitrate, perchlorate, *p*-toluenesulfonate and others readily apparent to one skilled in the art.

25 Representative useful diazonium salts include, but are not limited to, *N,N'*-diethyl-4-methoxymetanilamide diazonium salt (known as Fast Red-ITR salt), 4-chloro-2-methylbenzediazonium salt (known as Fast Red TR salt), diazotized 2-methoxy-5- 30 chloroaniline (known as Fast Red RC salt) and diazotized 5-nitro-2-amino-1-methoxybenzene (known as Fast Red B).

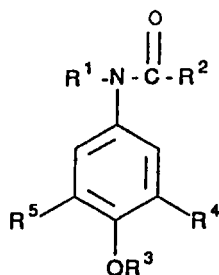
 Tetrazolium salts are organic salts in which the organic portion contains one or two 35 tetrazole rings, generally with aryl substituents at

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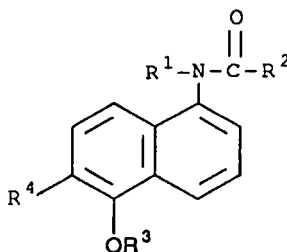
various positions. Tetrazolium salts having two tetrazole rings can be formed to provide a biphenyl nucleus with a tetrazole ring in each of the 4- and 4'- positions of the biphenyl nucleus.

5 Many useful diazonium and tetrazolium compounds are described for example, in US-A-3,905,872, US-A-4,772,553, US-A-4,892,817 and A-4,892,833 and Japanese Publication 63/088,000 (published April 19, 1988).

10 A second critical component of the signal-providing composition of this invention is an electron transfer agent having any of structures (I):

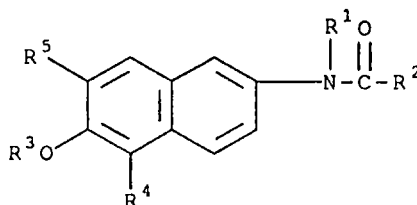


(II):



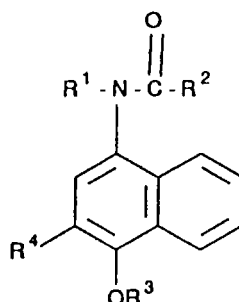
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(III):



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or (IV):



wherein R^1 is hydrogen or alkyl of 1 to 4 carbon
 5 atoms (such as methyl, ethyl, isopropyl,
 hydroxymethyl, aminomethyl and methoxymethyl).
 Preferably, R^1 is hydrogen.

In structure (I), (II), (III) and (IV), R^2
 is hydrogen, alkyl of 1 to 4 carbon atoms (such as
 10 methyl, ethyl, isopropyl, t-butyl and isobutyl),
 alkoxyalkyl of 1 to 4 carbon atoms (such as
 methoxymethyl and methoxyethyl), hydroxyalkyl of 1 to
 4 carbon atoms (such as hydroxymethyl, 1-
 hydroxyethyl, 2-hydroxyethyl and 2,3-
 15 dihydroxypropyl), aminoalkyl of 1 to 4 carbon atoms
 (such as aminomethyl, 2-aminoethyl, 3-aminopropyl,
 2,4-diaminobutyl, methylaminomethyl, 2,2-
 dimethylaminoethyl and 4-aminobutyl), haloalkyl of 1
 to 4 carbon atoms (such as chloromethyl, bromomethyl,
 20 2-chloroethyl, 1,1-dichloromethyl, 1,1,1-
 trichloromethyl, 2,2,2-trichloroethyl and 3-
 chloropropyl), or alkenyl of 2 to 5 carbon atoms
 (such as ethenyl, 1-propenyl, isopropenyl and 2-
 butenyl). Preferably, R^2 is hydrogen, methyl or
 25 ethenyl.

R^3 is hydrogen or alkyl of 1 to 4 carbon
 atoms (such as methyl, ethyl, isopropyl, t-butyl, n-
 butyl and isobutyl). Preferably, R^3 is hydrogen or
 methyl.

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R^4 and R^5 are independently hydrogen or an electron withdrawing group having a Hammett sigma value of at least 0.01, and preferably at least 0.3.

Hammett sigma values are calculated in accordance

5 with standard procedures described, for example, in Steric Effects in Organic Chemistry, John Wiley & Sons, Inc., 1956, pp. 570-574 and Progress in

Physical Organic Chemistry, Vol. 2, Interscience Publishers, 1964, pp. 333-339. Representative

10 electron withdrawing groups having positive Hammett sigma values include cyano, carboxy, nitro, halo (fluoro, bromo, chloro or iodo), trihalomethyl (for example, trifluoromethyl or trichloromethyl), carbonyl, carbamoyl, sulfonyl, sulfamoyl, esters, and
15 others readily apparent to one skilled in the art.

Preferred electron withdrawing groups are halo (such as chloro or bromo) and cyano. Chloro and cyano are more preferred electron withdrawing groups, and chloro is most preferred for either of R^4 and R^5 .

20 In the foregoing structure (I), at least one of R^4 and R^5 must be an electron withdrawing group as defined above. In foregoing structures (II), (III) and (IV), at least one of R^4 and R^5 can be an electron withdrawing group, but this is not
25 required. Most preferably, R^4 is an electron withdrawing group.

Representative electron transfer agents having structure (I) include:

3'-chloro-4'-hydroxyacetanilide,
—30 3',5'-dichloro-4'-hydroxyacetanilide,
3'-fluoro-4'-hydroxyacetanilide,
3',5'-difluoro-4'-hydroxyacetanilide,
3'-bromo-4'-hydroxyacetanilide,
3',5'-dibromo-4'-hydroxyacetanilide,
35 3'-cyano-4'-hydroxyacetanilide,

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3',5'-dicyano-4'-hydroxyacetanilide,
N-methyl-N- (3-chloro-4-
hydroxyphenyl) acetamide,
N- (3-chloro-4-hydroxyphenyl) methacrylamide,
5 N- (3-chloro-4-methoxyphenyl) acetamide,
N- (3-chloro-4-hydroxyphenyl) -2-
chloroacetamide,
N- (3-chloro-4-hydroxyphenyl) -2,2-
dichloroacetamide,
10 N- (3-chloro-4-hydroxyphenyl) -2,2,2-
trichloroacetamide,
N- (3-chloro-4-hydroxyphenyl) -2-
hydroxyacetamide,
N- (3-chloro-4-hydroxyphenyl) -2-
15 methoxyacetamide, and
N- (3-chloro-4-hydroxyphenyl) -2-
aminoacetamide.

Representative electron transfer agents of
structure (II) include:

20 N- (5-hydroxy-1-naphthyl) acetamide,
N- (5-hydroxy-6-fluoro-1-naphthyl) acetamide,
N- (5-hydroxy-6-chloro-1-naphthyl) acetamide,
N- (5-hydroxy-6-cyano-1-naphthyl) acetamide,
N-methyl-N- (5-hydroxy-1-naphthyl) acetamide,
25 N-methyl-N- (5-hydroxy-6-chloro-1-
naphthyl) acetamide,
N- (5-methoxy-1-naphthyl) acetamide,
N- (5-methoxy-6-chloro-1-naphthyl) acetamide,
N- (5-hydroxy-1-naphthyl) -2-chloroacetamide,
30 N- (5-hydroxy-1-naphthyl) -2,2-
dichloroacetamide,
N- (5-hydroxy-6-chloro-1-naphthyl) -2,2-
dichloroacetamide,
N- (5-hydroxy-1-naphthyl) -2,2,2-
35 trichloroacetamide,

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N-(5-hydroxy-6-chloro-1-naphthyl)-2,2,2-trichloroacetamide,

N-(5-hydroxy-1-naphthyl)-2-hydroxyacetamide,

5 N-(5-hydroxy-6-chloro-1-naphthyl)-2-hydroxyacetamide,

N-(5-hydroxy-1-naphthyl)-2-methoxyacetamide,

10 N-(5-hydroxy-6-chloro-1-naphthyl)-2-methoxyacetamide,

N-(5-hydroxy-1-naphthyl)-2-aminoacetamide,
and

N-(5-hydroxy-6-chloro-1-naphthyl)-2-aminoacetamide.

15 Representative electron transfer agents of structure (III) include:

N-(6-hydroxy-2-naphthyl)acetamide,

N-(6-hydroxy-5-fluoro-2-naphthyl)acetamide,

N-(6-hydroxy-7-fluoro-2-naphthyl)acetamide,

20 N-(6-hydroxy-5,7-difluoro-2-naphthyl)acetamide,

N-(6-hydroxy-5-chloro-2-naphthyl)acetamide,

N-(6-hydroxy-5,7-dichloro-2-naphthyl)acetamide,

25 N-(6-hydroxy-7-bromo-2-naphthyl)acetamide,

N-(6-hydroxy-5,7-dibromo-2-naphthyl)acetamide,

N-(6-hydroxy-5-cyano-2-naphthyl)acetamide,

30 N-(6-hydroxy-5,7-dicyano-2-naphthyl)acetamide,

N-methyl-N-(6-hydroxy-5-chloro-2-naphthyl)acetamide,

N-methyl-N-(6-hydroxy-7-chloro-2-naphthyl)acetamide,

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N-methyl-N-(6-hydroxy-5,7-dichloro-2-naphthyl)acetamide,
N-(6-methoxy-2-naphthyl)acetamide,
N-(6-methoxy-5-chloro-2-naphthyl)acetamide,
5 N-(6-methoxy-5,7-dichloro-2-naphthyl)acetamide,
N-(6-hydroxy-2-naphthyl)-2-chloroacetamide,
N-(6-hydroxy-7-chloro-2-naphthyl)-2-chloroacetamide,
10 N-(6-hydroxy-2-naphthyl)-2,2-dichloroacetamide,
N-(6-hydroxy-7-chloro-2-naphthyl)-2,2,2-trichloroacetamide,
N-(6-hydroxy-2-naphthyl)-2-hydroxyacetamide,
15 N-(6-hydroxy-5-chloro-2-naphthyl)-2-methoxyacetamide,
N-(6-hydroxy-2-naphthyl)-2-aminoacetamide,
and
20 N-(6-hydroxy-5,7-dichloro-2-naphthyl)-1-aminoacetamide.
Representative compounds of structure (IV) include:
N-(4-hydroxy-1-naphthyl)acetamide,
25 N-(4-hydroxy-5-chloro-1-naphthyl)acetamide,
N-methyl-N-(4-hydroxy-3-chloro-1-naphthyl)acetamide,
N-(4-methoxy-1-naphthyl)acetamide,
N-(4-hydroxy-1-naphthyl)-2-chloroacetamide,
30 N-(4-hydroxy-3-chloro-1-naphthyl)-2-chloroacetamide,
N-(4-hydroxy-1-naphthyl)-2,2-dichloroacetamide,
N-(4-hydroxy-5-chloro-1-naphthyl)-2-methoxyacetamide,
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N-(4-hydroxy-5-chloro-1-naphthyl)-2-aminoacetamide,

N-(4-hydroxy-3-chloro-1-naphthyl)-2-aminoacetamide, and

5 N-(4-hydroxy-3-fluoro-1-naphthyl)acetamide.

The most preferred electron transfer agent is 3'-chloro-4'-hydroxyacetanilide.

10 These electron transfer agents can be prepared generally from known starting materials as follows:

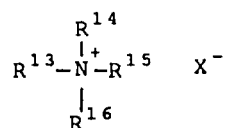
In general, the halogenated compounds of Structures (I)-(IV) are prepared by halogenation of the known precursor anilide (for example, 4'-hydroxy- or alkoxyacetanilide, or an anilide of naphthalene) with a
15 known halogenating agent such as sulfuryl chloride, sulfuryl bromide, or the free halogen in the presence of acid. Where the desired precursor is not available, an appropriately substituted phenol or naphthol can be nitrated by mild nitration using known techniques (for
20 example, with nitric acid in a solvent such as glacial acetic acid) followed by hydrogenation, typically over platinum or palladium to produce the amine (see *J. Am. Chem. Soc.* 49, 1093, 1927). The amine is then acylated, for example, by condensation with the desired
25 acylating agent such as an anhydride (for example, acetic anhydride) or an acid chloride such as acrylic acid chloride, to produce the anilide. Suitable acylation procedures are also described by Challis et al, The Chemistry of Amides, pp. 731-857, Intersciences
30 Publishing, New York, 1970. If the selected starting materials do not already provide the requisite electron withdrawing groups, the resulting anilide can be conveniently halogenated as described above. Alternatively, the amine precursor to the anilide can
35 be acylated with an acylating agent that provides the

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group at R² (for example, trichloroacetic acid chloride or maleic anhydride), or the aromatic ring of the anilide can be alkylated, acylated or nitrated at the R⁴ or R⁵ (or both) positions using known techniques to
 5 provide the requisite electron withdrawing groups from R⁴ and R⁵.

An optional but preferred component of the signal-providing composition of this invention is a low molecular weight cationic surfactant to provide
 10 micelles, or a cationic polymer to provide a hydrophobic environment for increased sensitivity, storage stability and kinetic stability.

Particularly useful cationic surfactants and polymers can be represented by the structure (VIII):



15 wherein R¹³ is substituted or unsubstituted alkyl of at least 7 carbon atoms, and preferably from 10 to 20 carbon atoms (such as n-octyl, isononyl, isodecyl, dodecyl, tetradecyl, hexadecyl, octadecyl, eicosyl,
 20 2,7,8-trimethyldecyl, 4-ethyl-6-methyldodecyl, benzyl and phenethyl), substituted or unsubstituted aryl of 6 to 14 carbon atoms in the aromatic nucleus (such as phenyl, naphthyl or anthryl) which can be substituted with one or more hydrophobic groups such as linear or
 25 branched alkyl of 1 to 10 carbon atoms (such as methyl, ethyl, isopropyl, t-butyl, hexyl, octyl, isooctyl, nonyl or isononyl), halo and others known to one skilled in the art. Such substituted aryl groups include, but are not limited to xylyl, tolyl,
 30 isononylphenyl, dimethylphenyl and trichlorophenyl. R¹³ can also be substituted or unsubstituted alkenyl of 8 to 20 carbon atoms (such as 1-octenyl, 1-decenyl and 2-dodecenyl), or a polymeric moiety (described below).

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Preferably, R^{13} is alkyl or alkenyl of 14 to 16 carbon atoms, with groups such as 2,4-dimethyl-6-ethyldecyl, tetradecyl and hexadecyl being more preferred.

5 In structure (VIII), R^{14} can be alkyl or alkenyl as defined for R^{13} , substituted or unsubstituted alkyl of 1 to 7 carbon atoms (such as methyl, ethyl, isopropyl, *t*-butyl, methoxymethyl, benzyl and hexyl), substituted or unsubstituted alkenyl
10 of 2 to 7 carbon atoms (such as a ethenyl, allyl, isopropenyl and *n*-butenyl), or carbocyclic aryl of 6 to 10 carbon atoms in the ring system (such as phenyl, tolyl, xylyl, naphthyl and *p*-methoxyphenyl). One of R^{14} , R^{15} and R^{16} can be one of the nonpolymeric groups
15 defined above for R^{10} .

R^{15} and R^{16} are independently substituted or unsubstituted alkyl of 1 to 7 carbon atoms (such as methyl, ethyl, isopropyl, *t*-butyl, methoxymethyl, benzyl and hexyl), substituted or unsubstituted alkenyl
20 of 2 to 7 carbon atoms (such as a ethenyl, isopropenyl and allyl), or carbocyclic aryl of 6 to 10 carbon atoms in the ring system (such as phenyl, tolyl, xylyl, naphthyl and *p*-methoxyphenyl).

Alternatively, any two or three of R^{14} , R^{15}
25 and R^{16} can be taken together to represent sufficient carbon atoms and an oxygen, nitrogen or sulfur atom to complete, with the quaternary ammonium atom, a 5- to 6-membered heterocyclyl cationic group. Examples of such groups include, but are not limited to pyridinium,
30 piperidinium, pyrrolidinium, morpholinium, quinolinium, pyrimidinium, acridinium, benzothiazolium, benzoxazolinium and imidazolium.

Preferably, R^{14} , R^{15} and R^{16} are independently methyl or ethyl.

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Y⁻ is a suitable monovalent acid anion which is not a substrate or inhibitor for peroxidases, including but not limited to, perchlorate, halide (such as fluoride, chloride and
5 bromide), tetrafluoroborate, triflate, methyl sulfate, hexafluorophosphate, nitrate, p-toluenesulfonate and others readily apparent to one skilled in the art. Halide anions are preferred.

Examples of useful nonpolymeric cationic
10 surfactants are hexadecyltrimethylammonium chloride, dodecyltrimethylammonium chloride, cetyltrimethylammonium bromide (also known as hexadecyltrimethylammonium bromide), cocotrimethylammonium chloride,
15 tallowtrimethylammonium chloride, soyatrimethylammonium chloride, myristyltrimethylammonium bromide, stearyltrimethylammonium chloride, cetyldimethylammonium bromide,
20 didodecyldimethylammonium bromide, cetylpyridinium chloride and myristyldimethylbenzylammonium chloride.

Where R¹³ in structure (VIII) is a polymeric moiety, the backbone of such polymers can be conventional polyesters, polyamides,
25 polyethyleneimines, polycarbonates, cellulosic materials, and vinyl addition homo- and copolymers comprised of recurring units of a monomer having the desired positive charge. These materials can be prepared from conventional materials using
30 conventional procedures. The polymer can have the charges incorporated therein from starting materials, or from chemical reaction after preparation.

Particularly useful cationic polymers are vinyl addition homo- or copolymers prepared from
35 ethylenically unsaturated polymerizable monomers

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having the requisite positively charged groups, and one or more comonomers which provide hydrophobic regions characteristic of surfactants, crosslinked regions or other suitable properties.

5 Representative cationic monomers include, but are not limited to, N-cyclohexyl-N,N-dimethyl-N-(m- & p-vinylbenzyl)ammonium chloride, N-benzyl-N,N-dimethyl-N-(m- & p-vinylbenzyl)ammonium chloride, 3-(2-hydroxypropyl)-1-vinylimidazolium chloride and 1-
10 methyl-4-vinylpyridinium chloride. Useful comonomers include, but are not limited to, styrene and its derivatives (such as vinyltoluene and p-t-butylstyrene), acrylic and methacrylic acid esters (such as methyl acrylate, methyl methacrylate, butyl
15 acrylate and butyl methacrylate), crosslinkable monomers [such as divinylbenzene, ethylene diacrylate, ethylene dimethacrylate and N,N'-methylenebis(acrylamide)]. Other useful polymers are described, for example, as mordants in US-A-4,069,017
20 and US-A-4,024,839. Such materials generally have quaternary ammonium or quaternary phosphonium groups pendant from the polymer backbone, and preferably at least from 40 to 100 weight percent of the ethylenically unsaturated polymerizable monomer
25 derived recurring units have such groups. The remaining recurring units can be derived from a wide variety of ethylenically unsaturated polymerizable monomers as noted in the patents identified above.

 Representative cationic polymers include,
30 but are not limited to, poly(N,N,N-trimethyl-N-vinylbenzylammonium chloride), poly[styrene-co-benzyl-N,N-dimethyl-N-(m- & p-vinylbenzyl)ammonium chloride-co-divinylbenzene], poly(N,N,N-trioctyl-N-vinylbenzylphosphonium chloride), poly[styrene-co-N-
35 vinylbenzyl-N,N,N-trihexylammonium chloride],

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poly(styrene-co-N,N,N-trimethyl-N-vinylbenzylammonium chloride), poly[N-cyclohexyl-N,N-dimethyl-N-(m- & p-vinylbenzyl)ammonium chloride], poly[styrene-co-1-vinylimidazole-co-3-(2-hydroxyethyl)-1-

5 vinylimidazolium chloride] and others readily apparent to one skilled in the art. A preferred cationic polymer is poly[N-cyclohexyl-N,N-dimethyl-N-(m- & p-vinylbenzyl)ammonium chloride].

The signal-providing composition of this
10 invention is generally buffered to a pH of from 5 to 10 using one or more suitable buffers well known in the art. For example, buffers such as tris(hydroxymethyl)aminomethane, borate, phosphate, bis-tris-propane and tricine can be used.
15 Tris(hydroxymethyl)aminomethane and phosphate are preferred. Where the signal generating reagent is a DPD and the signal to be produced is light, the pH is generally from 7 to 9.5, whereas where a colorimetric signal is to be generated using a leuco dye,
20 tetrazolium salt or diazonium salt, the pH is generally from 5 to 9.

The amount of buffer would be readily apparent to a skilled worker since it is well known how much of any buffer is needed to maintain a
25 desired pH. The amount of signal generating reagent is generally at least 0.01 mmolar, with an amount in the range of from 0.1 to 10 mmolar being preferred. The electron transfer agent having structure (I), (II), (III) or (IV) is generally present in an amount
30 of at least 0.01 mmolar, with an amount in the range of from 0.05 to 10 mmolar being preferred.

When used, the nonpolymeric cationic surfactant is present in an amount which is from 0.05 to 0.25% above its critical micelle
35 concentration. This can be readily determined for a

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given surfactant since the "critical micelle concentration" for many surfactants is well known, or can be readily determined using procedures described, for example, in Surfactant Science and Technology,
5 Meyers, VCH Publishers, New York, Chapter 3, 1988. If a cationic polymer is included in the composition, it is present in an amount of from 0.01 to 2% (by weight).

This invention also provides a peroxidase-
10 containing aqueous composition which contains a peroxidase in free form, or as a label conjugated with a specific binding molecule (such as an antibody, avidin or biotin), and the electron transfer agent described herein. Such a composition
15 can also be buffered as described for the signal-providing composition of this invention.

Besides the compositions described above, the present invention also provides a kit of individually packaged reagents, equipment and
20 instructions useful for carrying out a variety of analytical methods (described below). The packaging of kit components is well known in the art.

In one embodiment, a kit comprises, individually packaged: the signal-providing
25 composition described above, and a peroxidase or peroxidase-labeled specific binding molecule. By "specific binding molecule" is meant any biological or chemical compound which will specifically bind with a receptor therefor which will not bind with
30 other materials. Particularly useful labeled specific binding species are peroxidase-labeled proteins or oligonucleotides. Such proteins include antibodies and avidin. Suitable receptors for given specific binding species are well known. Other

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components useful in an assay (such as an oxidant, described below) can be included in the test kit.

Another kit can include the peroxidase-containing compositions as defined above, and a
5 separately packaged signal generating reagent.

As used herein, "peroxidase" is meant to be any peroxidative substance (enzymatic or otherwise) which catalyzes the oxidation of a substrate, such as luminol or a leuco dye, to produce the appropriate
10 signal. Microbial, fungal and plant peroxidases are preferred with horseradish peroxidase being most preferred. The amount of peroxidase can vary widely due to the amount of other components used in the reaction. A useful amount would be readily apparent
15 to one skilled in the art, but a minimum amount would generally be at least 1×10^{-7} I.U./ml (or equivalent amount for nonenzymatic peroxidative substances). I.U. represents the International Unit for enzyme activity and is defined as the amount of enzyme
20 activity required to catalyze the conversion of 1 micromole of substrate to product per minute under standard pH and temperature conditions.

An oxidant is needed to produce the desired signal in the presence of peroxidase and the electron
25 transfer agent of structure (I), (II), (III) or (IV). Various useful oxidants are known, but perborate ion and hydrogen peroxide are preferred with the latter being most preferred. The amount of oxidant used in a given assay is readily apparent to one skilled in
30 the art, but a minimum amount of 0.1 mmolar is generally used.

Another embodiment of a test kit can include, in individual packaging, a signal generating reagent as described herein, and an electron transfer
35 agent having structure (I), (II), (III) or (IV), and

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any additional optional components as would be readily understood by one skilled in the art. In some methods of this invention, peroxidase is used in "free form" (non-conjugated) for clinical analysis.

5 In specific binding methods, however, the peroxidase is used as a conjugate with a specific binding ligand or receptor therefor, or with a specific binding molecule which is reactive with either the ligand or receptor. The ligand and receptor are complexed in
10 such assays and thereby labeled with the peroxidase for eventual detection of the complex or uncomplexed materials. The preparation of such conjugates can be achieved using a variety of known techniques.

Various specific binding assay formats are
15 useful in the practice of this invention, and include nucleic acid hybridization assays, immunochemical assays (such as enzyme immunoassays, sandwich assays, competitive binding assays, direct binding assays) and others well known in the art. Such assays are
20 generally described, for example in US-A-4,598,044, US-A-4,745,077, US-A-5,077,198, US-A-5,085,986, Anal.Biochem. 169, pages 1-25 (1988), and WO 88/01302 (published February 25, 1988). The method of the invention can be preceded by an amplification
25 process, such as polymerase chain reaction (commonly known as PCR) as described for example in US-A-4,965,188 and ligase chain reaction which is generally described by Weiss, Science, 254, pages 1292-3, 1991 to increase the amount of targeted
30 nucleic acid which can then be detected using the composition of this invention.

Thus, the method of this invention can be used to detect any of a wide variety of chemical and biological materials, such as amino acids, peptides,
35 polypeptides, proteins (including enzymes, avidin,

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antibodies and antigenic proteins), carbohydrates (including monosaccharides, polysaccharides and lipopolysaccharides), hormones (such as human chorionic gonadotropin, thyroid stimulating hormone, 5 leutinizing hormone, thyroxin, follicle stimulating hormone, parathyroid hormone and growth hormone), metabolites (such as glucose, lactate and pyruvate), oligonucleotides, nucleic acids, vitamins (such as B₁₂ and biotin), intact cells from various organisms 10 (including microorganisms) and drugs (narcotics, therapeutic and those abused).

Particularly useful specific binding methods of this invention are those known in the art as sandwich assays whereby the ligand of interest is 15 complexed with at least a first and second receptor either simultaneously or in a desired sequence. One of the receptors is insolubilized on a suitable support (such as microtiter plate, polymeric, magnetic or glass particles, film, membrane, filter 20 paper and others known in the art) by adsorption, covalent or other known attachment procedures, or is capable of being insolubilized through further complexation or reaction. For example, the receptor can be labeled with a specific binding moiety (for 25 example, biotin) which is reactive with its corresponding receptor moiety (for example, avidin) which is insolubilized on the support.

In the sandwich assays, the second receptor for the ligand of interest can be labeled with 30 peroxidase, or is capable of being so labeled through additional specific binding reactions (such as labeling the ligand through an avidin-biotin complex). Detection of the label is accomplished using the composition described above.

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In more preferred embodiments, the ligand of interest is an antigenic material with which antibodies are reactive, or a nucleic acid with which complementary nucleic acids (such as
5 oligonucleotides) can be hybridized.

The assays described above can be carried out in solution or in a dry format. Solution assays generally refer to methods carried out in solution in a suitable container, and in the case of
10 heterogeneous specific binding assays, suitable separation techniques and equipment are then used to separate unbound materials from the bound materials. In dry assays, chemical or specific binding reactions can be carried out in a dry element, test strip or
15 fibrous sheet and the presence of the analyte is detected by adding the stabilized chemiluminescent composition of this invention. For example, a specific binding reaction can be carried out using a peroxidase-labeled specific binding material which is
20 used to generate a chemiluminescent signal. Details regarding such elements are well known in the art, including for example, US-A-3,992,158, US-A-4,258,001, US-A-4,292,272, US-A-4,430,436 and US-A-4,670,381.

25 In the following examples, all percentages are by weight, unless otherwise noted.

The following preparations describe representative procedures for making the electron transfer compounds useful in the practice of this
30 invention.

Preparation A:

Into a 1 liter round bottom flask (fitted with a magnetic stirrer), glacial acetic acid (700 ml) was mixed with 4'-hydroxyacetanilide (50 g) until
35 the solid was completely dissolved. The resulting

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solution was cooled to between 10-15°C, and to it was added SO₂Cl₂ (47 g) and the resulting solution was stirred for 1 hour. Water (200 ml) was then added, and 3'-chloro-4'-hydroxyacetanilide crystallized as a
5 white precipitate (60 g, 98% yield).

Preparation B:

Into a 500 ml round bottom flask (fitted with a magnetic stirrer), water (200 ml) was mixed with 4-amino-2,6-dichlorophenol hydrochloride (20 g)
10 until the solid was completely dissolved. The resulting solution was warmed to 70°C, and to it was added acetic anhydride (11 g) and the resulting solution was stirred for 1 hour. Upon cooling, crystals of 3',5'-dichloro-4'-hydroxyacetanilide
15 formed (19.6 g, 95% yield).

Preparation C:

Into a 500 ml round bottom flask (fitted with a magnetic stirrer), a 50:50 mixture of water:ethanol was combined with 5-amino-1-naphthol
20 (20 g, 125 mmole) until the solid was completely dissolved. The solution was warmed to boiling and to it was added acetic anhydride (14 g) and the resulting solution was stirred for 1 hour. Upon cooling, crystals of N-(5-hydroxy-1-naphthyl)acetamide
25 (21 g, 84% yield) formed.

Preparation D:

Into a high pressure reaction vessel (fitted for shaking), tetrahydrofuran (200 ml) was combined with 6-bromo-2-naphthol. The reaction
30 vessel was then charged with gaseous ammonia (800 psi, 56.2 kg/cm²) and warmed to 80°C for 3 days. The resulting solution was removed by reduced pressure and some of the solid precipitate 6-amino-2-naphthol (10 g) was placed (without further purification) into
35 a 500 ml round bottom flask). The solid was

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completely dissolved into a 50:50 mixture of water:ethanol. The resulting solution was warmed to boiling and acetic anhydride (7 g) was added. After 1 hour of continuous stirring, the solution was
5 cooled and crystals of N-(6-hydroxy-2-naphthyl)acetamide formed (8 g, 63% yield).

Preparation E:

Into a 500 ml round bottom flask (fitted with a magnetic stirrer), a 50:50 mixture of
10 water:ethanol was combined with 4-amino-1-naphthol (20 g, 125 mmole) until the solid was completely dissolved. The solution was warmed to boiling, and to it was added acetic anhydride (14 g) and the resulting solution was stirred for 1 hour. Upon
15 cooling, crystals of N-(4-hydroxy-1-naphthyl)acetamide (20 g, 79% yield) formed.

Examples 1-2 Aqueous Chemiluminescent Compositions

An aqueous composition for providing a
20 chemiluminescent signal was prepared as follows:

A buffer solution was prepared, containing diethylenetriaminepentaacetic acid (100 μ molar) in tris(hydroxymethyl)aminomethane buffer (0.1 molar, pH 8).

25 Solution 1 was prepared by mixing luminol, sodium salt (8 mg/10 ml, 4 mmolar) and 3'-chloro-4'-hydroxyacetanilide (0.75 mg/10 ml, 0.4 mmolar) in the buffer solution. Solution 2 contained 9.07 μ l of a 30% stock solution of hydrogen peroxide in the buffer
30 solution (10 ml).

A composition of this invention was prepared by adding horseradish peroxidase (1 μ l of 2 pmolar solution) to the buffer solution (199 μ l) and Solution 1 (100 μ l). To this mixture was added Solution 2 (100
35 μ l) to provide a chemiluminescent composition of this

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invention. The final concentrations were as follows: luminol (1 mmolar), hydrogen peroxide (2 mmolar), 3'-chloro-4'-hydroxyacetanilide (100 μ molar) and horseradish peroxidase (5 femtomolar).

5 A second composition of this invention (Example 2) was like Example 1 except hexadecyltrimethylammonium bromide (0.1%) was also added.

10 Example 3 Aqueous Colorimetric Composition

 An aqueous composition of this invention was prepared containing 2-(4-hydroxy-3-methoxyphenyl)-4,5-bis(4-methoxyphenyl)imidazole leuco dye (0.008%), poly(vinyl pyrrolidone) (1%), sodium phosphate (10
15 mmolar, pH 6.8), diethylenetriaminepentaacetic acid (10 μ molar), hydrogen peroxide (10 mmolar) and 3'-chloro-4'-hydroxyacetanilide (5 mmolar).

 Example 4 Assay for Horseradish Peroxidase

20 The composition of Example 1 (400 μ l) was placed in a commercially available Turner TD 20e Luminometer to evaluate the chemiluminescent signal in response to the presence of horseradish peroxidase. The data was collected as 10 second intervals of the
25 Turner light signal, expressed as relative light units.

 A Control composition was similarly prepared and tested, except the 3'-chloro-4'-hydroxyacetanilide was replaced with 4'-hydroxyacetanilide.

 The resulting chemiluminescence signals from
30 both compositions are plotted on the "Y" axis in FIG. 1, as measured at 25°C over a period of twenty minutes ("X" axis). The plot for the Control is labeled as "1" and the plot for the invention is labeled as "2". It is clear that higher signal is provided by the use of
35 the composition of this invention.

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Similar chemiluminescent signals were generated at various concentrations (100 to 5000 attomoles) of horseradish peroxidase. The results are shown in FIG. 2 where the Control plot is labeled as "3" while the invention plot is labeled as "4". The results, as shown in FIG. 2 (signal vs. enzyme concentration), make it evident that 3'-chloro-4'-hydroxyacetanilide provided an improvement at each concentration.

10

Examples 5-7 Use of Several Electron Transfer Agents to Detect Hydrogen Peroxide

A solution (300-"x" μ l) of hydrogen peroxide (2 mmolar), luminol (1 mmolar) and each electron transfer agent and diethylenetriaminepentaacetic acid (100 μ molar) in tris(hydroxymethyl)aminomethane hydrochloride (0.05 molar, pH 8) was prepared and added to individual test wells of a microtiter plate. Example 5 contained 3'-bromo-4'-hydroxyacetanilide (0.15 mmolar), Example 6 contained 3'-chloro-4'-hydroxyacetanilide (0.15 mmolar), and Example 7 contained 3',5'-dichloro-4'-hydroxyacetanilide (2 mmolar).

At the beginning of the assay (time "zero"), aliquots ("x" μ l) of commercially available Sigma XII horseradish peroxidase (0 to 100,000 attamoles) in buffer were added to each test well of the microtiter plate so that each final cup volume was 300 μ l. After five minutes, the chemiluminescent signal was determined using a TITERTEK LUMINOSKAN™ reader using a 10 second integration time.

A Control assay was similarly carried out using 4'-hydroxyacetanilide as the electron transfer agent (0.15 mmolar).

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The results are illustrated in FIG. 3 which is a plot of log (chemiluminescence signal-5 minutes) versus log (horseradish peroxidase concentration, attomoles). The plot identified as "1" was for the Control assay, the plot identified as "2" was for Example 5, the plot identified as "3" was for Example 6, and the plot identified as "4" was for Example 7.

Examples 5 and 6 provided a higher chemiluminescent signal over the five minute assay period as opposed to the Control assay. Example 7 showed a lower chemiluminescent signal than the Control assay, but it demonstrated less variation in chemiluminescence over time than the Control assay.

Example 8 Solution Immunoassay for Thyroid Stimulating Hormone (TSH)

This example demonstrates the practice of this invention for the detection of TSH in a biological specimen. The assay was carried out using a commercially available AMERLITE™ Fast TSH immunoassay kit, plate washer/incubator and chemiluminescence reader. The chemiluminescent signal was generated using a commercial AMERLITE™ solution of 4-iodophenol, sodium perborate and luminol in borate buffer (pH 8.5) for the Control assay, or using a solution of 3'-chloro-4'-hydroxyacetanilide (0.15 mmolar), luminol (1 mmolar), hydrogen peroxide (2 mmolar), diethylenetriaminepentaacetic acid (0.1 mmolar) and cetyltrimethylammonium bromide (0.1%) in tris(hydroxymethyl)aminomethane hydrochloride buffer (0.05 molar, pH 8) for the assay of this invention.

The protocol described in the commercial AMERLITE™ Fast TSH kit noted above was followed:

- 1) A specimen containing 0-200 μ I.U./ml of TSH was added to a test well of the kit test device

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containing anti-TSH monoclonal antibodies adsorbed to the walls thereof.

2) Anti- β -TSH-horseradish peroxidase conjugate of the commercial kit was added to the test well to form a ternary complex.

3) Incubation was carried out at 37°C for about 30 minutes.

4) Unbound materials were washed from the test well using the commercial kit wash solution.

5) The solution (described above) for generating the chemiluminescent signal was then added, and the resulting signal was evaluated after 5 minutes.

The resulting signals for both the Control and invention were acceptable for calibration, but the invention assay provided improved sensitivity at the lower TSH levels (that is, from 0-5 μ I.U./ml). This distinction is important for clinical differentiation between a hyperthyroid and euthyroid status of a patient. These results are illustrated in FIG. 4 which contains graphical plots of log(light units) versus log(TSH concentration, μ I.U./ml). The plot identified as "1" is the Control assay, and the plot identified as "2" is the assay of this invention.

Example 9 Immunoassay for *Chlamydia trachomatis*

This example shows the practice of the present invention for the determination of *C.trachomatis* in a biological specimen and compares it to a Control assay for the same analyte using 4'-hydroxyacetanilide as the electron transfer agent.

The following materials and compositions were used in the assays of this example.

The microporous membrane used in the assays was an uncharged and uncoated nylon 66 membrane commercially available as LOPRODYNETM membrane from

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Pall Corporation. The membrane was mounted in each of the three test wells of a commercially available SURECELLTM test device (Eastman Kodak Company).

Specimens for testing were obtained from female
5 patients using endocervical swabs. Each specimen was tested using a separate test device.

A conventional flexible extraction tube was used to extract antigen from *C. trachomatis* organisms in the specimens, the tube having at separate locations
10 on the inside thereof, dried coatings: (1) tris(hydroxymethyl)aminomethane buffer (20 μ l of a 1.65 molar solution, pH 11) with thimerosal preservative (0.01%), and (2) 2-(N-morpholino)ethane sulfonic acid (10 mmolar, 50 μ l solution, pH 6), sodium azide (1.54
15 molar), ethylenediaminetetraacetic acid (5.4 mmolar), 5,5-dimethyl-1,3-cyclohexanedione (21.4 mmolar), dithiothreitol (0.188 molar) and poly(acrylamide) (6.35%).

Composition 1 contained AMIDECKTM protease (4
20 mg/ml, 170 units/mg, Genencor, International) in 2-(N-morpholino)ethane sulfonic acid buffer (10 mmolar, pH 6), sodium chloride (50 mmolar), calcium chloride (5 mmolar), 1,2-propanediol (10% w/v) and thimerosal (0.01%).

25 An antigen extraction composition contained ethanolamine hydrochloride (0.47 molar), sodium chloride (0.27 molar), thimerosal (30 mmolar), ethylenediaminetetraacetic acid (50 mmolar), EMCOLTM CC-36 cationic surfactant (0.45%, Witco Chemical) and
30 sodium hydroxide (0.66 normal, to provide pH 13.5).

Composition 2 contained hydrogen peroxide (12% in water), diethylenetriaminepentaacetic acid (10 μ molar) and thimerosal (0.01%).

A wash solution contained 3-cyclohexylamino-
35 2-hydroxy-1-propanesulfonic acid buffer (0.05 molar, pH

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10), EMCOL™ CC-9 cationic surfactant (0.75%) and thimerosal (0.01%).

Composition 3 contained creatine kinase-MB F(ab')₂ antibody fragment conjugated to horseradish peroxidase (5 µg/ml), casein (0.01%), LONZAINETM C amphoteric surfactant (0.01%, Lonza Corp.) and thimerosal (0.01%) in phosphate buffered saline solution (pH 7.2). This composition was used in a test well of each test device as a negative control. The labeled antibody fragment was prepared using known procedures (EPA-0 456 309) including a final chromatography step using a standard DEAE column and (1) tris(hydroxymethyl)aminomethane (20 mmolar, pH 8), and (2) tris(hydroxymethyl)aminomethane (20 mmolar, pH 8) containing sodium chloride (0.75 molar) as eluting buffers.

A horseradish peroxidase labeled F(ab')₂ anti-chlamydial labeled antibody fragment was prepared using known procedures (EPA-0 456 309) (4 µg/ml of solution, final concentration) was mixed in solution with casein (0.05%), LONZAINETM amphoteric surfactant (0.01%, Lonza Corp.), and thimerosal (0.01%) in phosphate buffered saline solution (pH 7.2).

The dye-providing composition used for the assay of this invention was that of Example 3. In the Control assay, a similar dye-providing composition was prepared and used, that composition containing 4'-hydroxyacetanilide as the electron transfer agent.

Assay Protocol:

In assaying each patient specimen, Composition 1 (7 drops) was added to the extraction tube and a swab containing each patient specimen was placed into the tube, rotated 10 seconds, followed by incubation of the tube for 1 minute at room temperature. The antigen extraction composition was

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then added to the tube, and the swab was rotated again for 10 seconds, and incubation was again carried out for 1 minute at room temperature. Composition 2 was then added, and the swab was rotated again for 10
5 seconds, followed by a third incubation for two minutes at room temperature. The swab was wrung out by squeezing the tube, and removed therefrom. The extraction procedure required about 4 to 5 minutes.

The resulting solution containing extracted
10 lipopolysaccharide antigen from *C. trachomatis* was removed from the tube using a pipette, prefiltered, and transferred to each test well of each test device (4 drops per test well). Fluid was allowed to drain through the microporous membranes in the test wells.
15 Each test well was then washed with the wash solution (4 drops per test well).

Composition 3 (2 drops) was then added to one of the test wells of each test device (considered a negative control test well). The composition
20 containing enzyme-labeled anti-chlamydial antibody fragment (2 drops) was added to each of the two remaining test wells of each test device. One of those two test wells (considered a positive control test well) contained dried *C. trachomatis* lipopolysaccharide
25 antigen on the membrane.

After incubation for two minutes at room temperature, the wash step was repeated twice. Each dye-providing composition was added to individual test devices and after one, two or three minutes incubation
30 at room temperature, the test sample well in each test device was evaluated for dye signal on the membrane.

The resulting dye signals are illustrated in FIGS. 5-7. In each FIGURE, dye signal is plotted versus antigen concentration (pg). The dye signal is
35 determined from a color chart having values of dye

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density ranging from 0 (no density) to 10 (highest density). The results are shown for 1, 2 and 3 minute incubations in FIGURES 5, 6 and 7, respectively. In each FIGURE, the plot for the invention is labeled as "1" and the Control assay plot is labeled as "2".

It is apparent that the assay of the present invention using 3'-chloro-4'-hydroxyacetanilide as the electron transfer agent was more sensitive to the presence of various concentrations of antigen over the three different incubation time periods.

Example 10 Comparative Example

The present example is a solution assay for peroxidase, and compares the present invention using 3'-chloro-4'-hydroxyacetanilide as an electron transfer agent with two Control assays (A and B) using 3'-methyl-4'-hydroxyacetanilide and 2'-methyl-4'-hydroxyacetanilide, respectively, as electron transfer agents.

The assay for peroxidase was carried out as a colorimetric rate assay in cuvettes (3 ml) using a Beckman DU-68 spectrophotometer. A stock dye solution containing 2-(4-hydroxy-3-methoxyphenyl)-4,5-bis(4-methoxyphenyl)imidazole leuco dye (0.008%) was prepared as described in Example 3, except no electron transfer agent was included. Specific amounts of electron transfer agent were then added to aliquots (25 ml) of the dye solution. Admixtures of the dye solution and the electron transfer agent solutions (10 mmolar) were then prepared to obtain 5 and 1 mmolar concentrations of the electron transfer agent in the resulting admixtures.

To start the assay, 3 ml of the test concentration of electron transfer agent (1, 5 or 10 mmolar) were added to a spectrophotometer cuvette. At

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time zero, horseradish peroxidase stock solution (10
μl, 3 nmolar in 50 mmolar phosphate buffer, pH 7
containing 0.01% ethylmercurithiosalicylic acid and 10
mmolar 4'-hydroxyacetanilide) was vortex-mixed into the
5 cuvette solution (3 ml), and the resulting change in
absorbance at 515 nm was measured. Initial rates of
absorbance change from 0 to 50 seconds were calculated
as dA/dT (milliabsorbance units/minute).

FIG. 8 shows the data provided by these
10 assays (plotted as rate of change in absorbance of dye
signal versus concentration of electron transfer agent,
μmolar). In the FIGURE, the invention plot is labeled
as "1", and Controls A and B are labeled "2" and "3",
respectively. It is apparent that the present
15 invention has greater sensitivity compared to the
Control assays over a range of electron transfer agent
concentrations.

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CLAIMS:

1. An aqueous composition for providing a colorimetric or chemiluminescent signal having a pH of from 5 to 10, and comprising:

5 a) a colorimetric or chemiluminescent signal generating reagent which provides a signal in response to the catalytic activity of peroxidase, the signal generating reagent being selected from the group consisting of:

10 a 2,3-dihydro-1,4-phthalazinedione derivative,

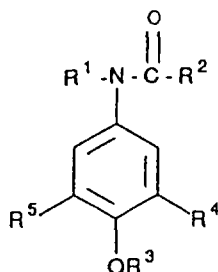
a tetrazolium salt,

a diazonium salt, and

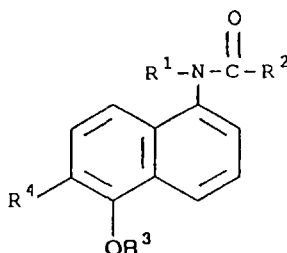
15 dye, and

b) an electron transfer agent,

the composition characterized wherein the electron transfer agent has structure (I):



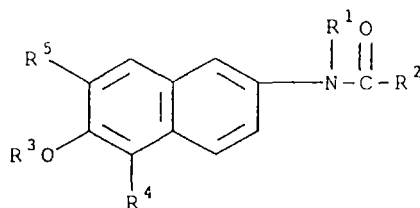
20 structure (II):



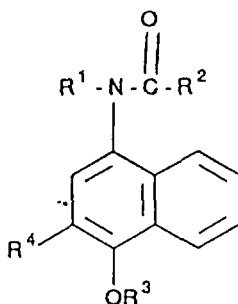
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structure (III):



or structure (IV):



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wherein R^1 is hydrogen, alkyl of 1 to 4 carbon atoms, hydroxyalkyl of 1 to 4 carbon atoms, alkoxyalkyl of 1 to 4 carbon atoms, aminoalkyl of 1 to 4 carbon atoms, halogenated alkyl of 1 to 4 carbon atoms,

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R^2 is hydrogen, alkyl of 1 to 4 carbon atoms, alkoxyalkyl of 1 to 4 carbon atoms, hydroxyalkyl of 1 to 4 carbon atoms, aminoalkyl of 1 to 4 carbon atoms, haloalkyl of 1 to 4 carbon atoms, or alkenyl of 2 to 5 carbon atoms,

15

R^3 is hydrogen or alkyl of 1 to 4 carbon atoms, and

R^4 and R^5 are independently hydrogen or an electron withdrawing group having a Hammett sigma value of at least 0.01,

20

provided that in structure (I), at least one of R^4 and R^5 is an electron withdrawing group having a Hammett sigma value of at least 0.01.

2. The composition as claimed in claim 1 wherein R^1 is hydrogen, R^2 is hydrogen or methyl, R^3 is

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hydrogen or methyl, and R^4 and R^5 are independently hydrogen, halo or cyano.

3. The composition as claimed in claim 2 wherein R^2 is methyl and both of R^4 and R^5 are chloro.

5 4. The composition as claimed in any of claims 1 to 3 further comprising a low molecular weight cationic surfactant present at from 0.05 to 0.25% above its critical micelle concentration, or from 0.01 to 2% of a cationic polymer.

10 5. The composition as claimed in any of claims 1 to 4 further comprising peroxidase or a peroxidase-labeled specific binding molecule.

15 6. The composition as claimed in any of claims 1 to 5 having a pH of from 5 to 9 and wherein the signal generating reagent is a triarylimidazole leuco dye to provide a colorimetric signal.

20 7. The composition as claimed in any of claims 1 to 6 further comprising an oxidant for the reaction of peroxidase with the signal generating reagent.

 8. A diagnostic test kit for the determination of an analyte as the result of the catalytic activity of peroxidase, the kit comprising, in individual packaging:

25 i) the aqueous composition as claimed in any of claims 1 to 7, and

 ii) peroxidase or a peroxidase-labeled specific binding species.

30 9. A diagnostic test kit for the determination of an analyte as the result of the catalytic activity of peroxidase, the kit comprising, in individual packaging:

 a) a signal generating reagent which provides a colorimetric or chemiluminescent signal in

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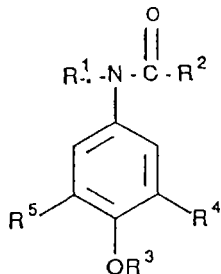
response to the catalytic activity of peroxidase, the
signal generating reagent being

a 2,3-dihydro-1,4-phthalazinedione
derivative,

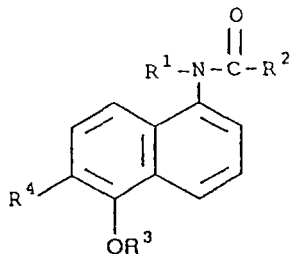
5 a tetrazolium salt,
a diazonium salt, or
an imidazole or triarylmethane leuco

dye, and

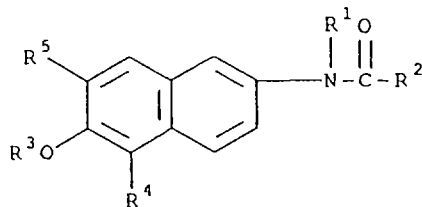
10 b) an electron transfer agent,
the kit characterized wherein the
electron transfer agent has structure (I):



structure (II):

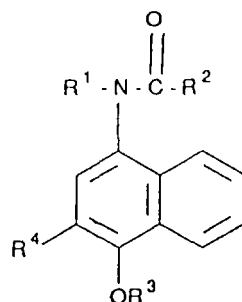


15 structure (III):



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or structure (IV):



wherein R¹ is hydrogen, alkyl of 1 to 4 carbon atoms, hydroxyalkyl of 1 to 4 carbon atoms, alkoxyalkyl of 1 to 4 carbon atoms, aminoalkyl of 1 to 4 carbon atoms, halogenated alkyl of 1 to 4 carbon atoms,

R² is hydrogen, alkyl of 1 to 4 carbon atoms, alkoxyalkyl of 1 to 4 carbon atoms, hydroxyalkyl of 1 to 4 carbon atoms, aminoalkyl of 1 to 4 carbon atoms, haloalkyl of 1 to 4 carbon atoms, or alkenyl of 2 to 5 carbon atoms,

R³ is hydrogen or alkyl of 1 to 4 carbon atoms, and

R⁴ and R⁵ are independently hydrogen or an electron withdrawing group having a Hammett sigma value of at least 0.01,

provided that in structure (I), at least one of R⁴ and R⁵ is an electron withdrawing group having a Hammett sigma value of at least 0.01.

10. A test device for the detection of peroxidase or an analyte catalytically related to peroxidase, the test device comprising an absorbent carrier material, and containing:

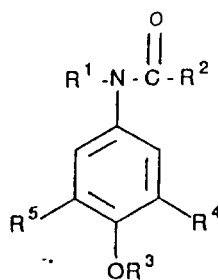
a) a colorimetric or chemiluminescent signal generating reagent which provides a signal in response to the catalytic activity of peroxidase, the signal generating reagent being

a 2,3-dihydro-1,4-phthalazinedione derivative,

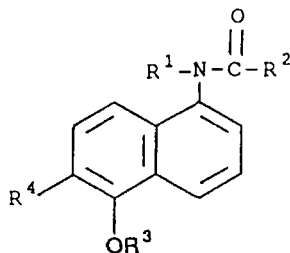
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a tetrazolium salt,
 a diazonium salt, or
 an imidazole or triarylmethane leuco
 dye, and

- 5 b) an electron transfer agent,
 the test device characterized wherein
 the electron transfer agent has structure (I):

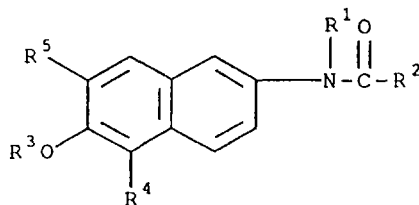


structure (II):

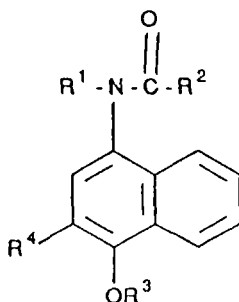


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structure (III):



or structure (IV):



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wherein R^1 is hydrogen, alkyl of 1 to 4 carbon atoms, hydroxyalkyl of 1 to 4 carbon atoms, alkoxyalkyl of 1 to 4 carbon atoms, aminoalkyl of 1 to 4 carbon atoms, halogenated alkyl of 1 to 4 carbon atoms,

5 R^2 is hydrogen, alkyl of 1 to 4 carbon atoms, alkoxyalkyl of 1 to 4 carbon atoms, hydroxyalkyl of 1 to 4 carbon atoms, aminoalkyl of 1 to 4 carbon atoms, haloalkyl of 1 to 4 carbon atoms, or alkenyl of 2 to 5 carbon atoms,

10 R^3 is hydrogen or alkyl of 1 to 4 carbon atoms, and

R^4 and R^5 are independently hydrogen or an electron withdrawing group having a Hammett sigma value of at least 0.01,

15 provided that at least one of R^4 and R^5 is an electron withdrawing group having a Hammett sigma value of at least 0.01.

11. An aqueous composition buffered to a pH of from 5 to 10, and comprising:

20 peroxidase or a peroxidase-labeled specific binding molecule, and

an electron transfer agent,

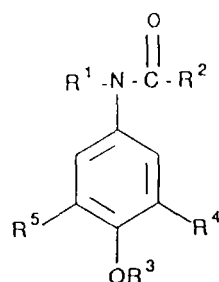
the composition characterized wherein the electron transfer agent has structure (I):

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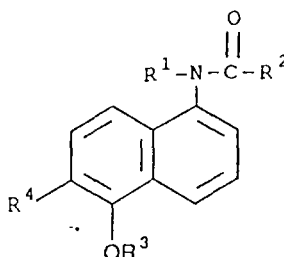
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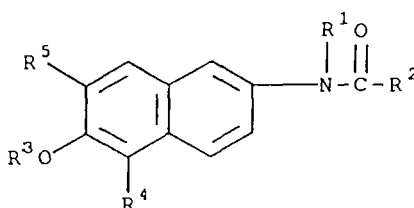
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structure (II):

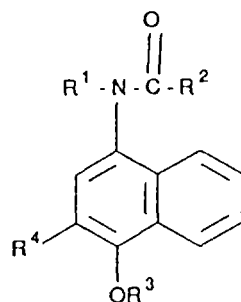


structure (III):



5

or structure (IV):



wherein R^1 is hydrogen, alkyl of 1 to 4 carbon atoms,
hydroxyalkyl of 1 to 4 carbon atoms, alkoxyalkyl of 1
10 to 4 carbon atoms, aminoalkyl of 1 to 4 carbon atoms,
halogenated alkyl of 1 to 4 carbon atoms,

R^2 is hydrogen, alkyl of 1 to 4 carbon atoms,
alkoxyalkyl of 1 to 4 carbon atoms, hydroxyalkyl of 1
to 4 carbon atoms, aminoalkyl of 1 to 4 carbon atoms,

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haloalkyl of 1 to 4 carbon atoms, or alkenyl of 2 to 5 carbon atoms,

R^3 is hydrogen or alkyl of 1 to 4 carbon atoms, and

5 R^4 and R^5 are independently hydrogen or an electron withdrawing group having a Hammett sigma value of at least 0.01,

provided that in structure (I), at least one
10 R^4 and R^5 is an electron withdrawing group having a Hammett sigma value of at least 0.01.

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