

REAGENT SYSTEMS FOR DETECTING THE PRESENCE OF A REDUCED COFACTOR IN A SAMPLE AND METHODS FOR USING THE SAME

INTRODUCTION

5 Field of the Invention

The field of this invention is analyte detection, particularly reagent systems for use in analyte detection.

Background of the Invention

10 Analyte detection in physiological fluids, e.g. blood or blood derived products, is of ever increasing importance to today's society. Analyte detection assays find use in a variety of applications, including clinical laboratory testing, home testing, etc., where the results of such testing play a prominent role in diagnosis and management in a variety of disease conditions. Analytes of interest include alcohol, formaldehyde, glucose, glutamic acid,
15 glycerol, beta-hydroxybutyrate, L-lactate, leucine, malic acid, pyruvic acid, steroids, etc. In response to this growing importance of analyte detection, a variety of analyte detection protocols and devices for both clinical and home use have been developed. Many of the protocols and devices that have been developed to date employ a signal producing system to identify the presence of the analyte of interest in a physiological sample, such as blood.

20 One type of signal producing system that finds use in the detection of a variety of different analytes is one in which a dehydrogenase oxidizes the analyte of interest and concomitantly reduces an enzyme cofactor, such as NAD(P)⁺. The reduced form of the cofactor, e.g. NAD(P)H, is then detected through subsequent reaction with a cofactor oxidizing agent, e.g. phenazine methosulfate or a diaphorase, that transfers an electron to a
25 redox indicator, such as a tetrazolium salt, to produce a detectable product.

While a variety of such signal producing systems have been developed to date for use in the detection of a wide variety of different analytes, there continues to be a need for the further development of such systems. For example, a signal producing system which provided for an enhanced reaction rate and a lower cost would be of great interest.

30 Relevant Literature

U.S. Patents of interest include: 4,629,697; 5,126,247 and 5,902,731. See also Raap et al., Histochem. J. (1983) 15:881-893.

SUMMARY OF THE INVENTION

Signal producing systems, reagent compositions, test strips and kits of the same, as well as methods for their use in the detection of an analyte in a sample, are provided. The subject signal producing systems are characterized by having at least a first and second
5 electron transfer agent and a redox indicator, where in many preferred embodiments the systems include a proteinaceous and non-proteinaceous electron transfer agent, e.g. a phenazine compound and a diaphorase. In many preferred embodiments, the subject systems and kits further include both an enzyme cofactor and an enzyme having an analyte oxidizing activity, e.g. an analyte dehydrogenase. The subject systems, reagent compositions, test
10 strips and kits find use in the detection of a wide variety of analytes in a sample, such as a physiological sample, e.g. blood or a fraction thereof.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 provides a graphical representation of the observed rate of reaction in a test
15 strip according to the subject invention vs. the theoretical expected rate of reaction for a test strip that includes both PMS and a diaphorase, clearly demonstrating that the use of both a non-proteinaceous and proteinaceous electron transfer agent, e.g. PMS and a diaphorase, provides for an unexpected increase in the rate of reaction.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Signal producing systems, reagent compositions, test strips and kits of the same, as well as methods for their use in the detection of an analyte in a sample, are provided. The subject signal producing systems are characterized by having at least a first and second
25 electron transfer agent and a redox indicator, where in many preferred embodiments the systems include a proteinaceous and non-proteinaceous electron transfer agent, e.g. a phenazine compound and a diaphorase. In many preferred embodiments, the subject systems and kits further include both an enzyme cofactor and an enzyme having an analyte oxidizing activity, e.g. an analyte dehydrogenase. The subject systems, reagent compositions, test strips and kits find use in the detection of a wide variety of analytes in a sample, such as a
30 physiological sample, e.g. blood or a fraction thereof.

Before the subject invention is described further, it is to be understood that the invention is not limited to the particular embodiments of the invention described below, as

variations of the particular embodiments may be made and still fall within the scope of the appended claims. It is also to be understood that the terminology employed is for the purpose of describing particular embodiments, and is not intended to be limiting. Instead, the scope of the present invention will be established by the appended claims.

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In this specification and the appended claims, singular references include the plural, unless the context clearly dictates otherwise. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs.

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SIGNAL PRODUCING SYSTEMS

As summarized above, the subject invention provides a signal producing system that is capable of detecting the presence of a reduced enzyme cofactor in a sample. By signal producing system is meant a collection of two or more compounds or molecules which are capable of acting in concert, when combined, to produce a detectable signal that is indicative of the presence of, and often amount of, a particular analyte in a given sample. The term signal producing system is used broadly to encompass both a mixture of all of the reagent constituents of the signal producing system as well as a system in which one or more of the reagent constituents are separated from the remainder of the reagent constituents, e.g. as is present in a kit.

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A feature of the subject signal producing systems is the presence of two distinct electron transfer agents. By electron transfer agent is meant a compound or molecule that can transfer an electron, in the form of a hydride ion, from a reduced enzyme cofactor to a redox indicator. In the subject signal producing systems, the first of the distinct electron transfer agents is a low molecular weight molecule, while the second electron transfer agent is a high molecular weight molecule. In this specification, low molecular weight means a molecular weight that does not exceed about 2000 daltons, usually about 1000 daltons and in many embodiments about 500 daltons. High molecular weight means a molecular weight of at least about 5000 daltons and in many embodiments 10,000 or 20,000 daltons or higher. The molecular weight of the high molecular weight electron transfer agent often will not exceed about 100,000 daltons. In many embodiments, the low molecular weight electron transfer agent is a non-proteinaceous compound while the high molecular weight electron

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transfer agent is a proteinaceous compound. By proteinaceous is meant a polypeptide or polymeric mimetic thereof.

A variety of low molecular weight non-proteinaceous electron transfer agents are of interest. These agents include: flavins such as riboflavin (RBF), alloxazine (ALL) and lumichrome (LC); phenazines such as phenazine, phenazine methosulfate (PMS), phenazine ethosulfate, methoxyphenazine methosulfate and safranine; methyl-1, 4-naphthol (menadione), phenothiazines such as PT and its radical cation, PT⁺, thionin (TH), azure A (AA), azure B (AB), azure C (AC), methylene blue (MB), methylene green (MG) and toluidine blue O (TOL); phenoxazines such as phenoxazine (POA), basic blue 3 (BB3), and brilliant cresyl blue ALD (BCBA), benzo- α -phenazoxonium chloride (Medola's blue); Indophenols such as 2,6-dichlorophenol indophenol (DCIP); and Indamines such as Bindschedler's green and phenylene blue; and the like.: Of particular interest in many embodiments are phenazine compounds, e.g. PMS, phenazine ethosulfate, methoxyphenazine methosulfate and safranine, where PMS is the low molecular weight, non-proteinaceous electron transfer agent in many embodiments.

In many embodiments, the high molecular weight proteinaceous electron transfer agent is an enzyme that is capable of oxidizing a reduced cofactor, e.g. NAD(P)H, and concomitantly reducing a redox indicator. In many embodiments, this electron transfer enzyme is a diaphorase, such as lipoic dehydrogenase, ferredoxin-NADP reductase, lipoamide dehydrogenase, NADPH dehydrogenase, etc. A variety of diaphorases are available and may be employed, where representative commercially available diaphorases that may be present in the subject signal producing systems include bacillus diaphorase, clostridium diaphorase, vibrio diaphorase, porcine diaphorase, and the like.

In the subject signal producing systems, the ratio of the first to the second electron transfer agent is chosen to provide for an accelerated reaction rate as compared to a control, e.g. a comparable signal producing system with a single electron transfer agent, e.g. only PMS or a diaphorase. Typically, the ratio of the first to the second electron transfer agent in the subject systems ranges from about 0.001 to 10, usually from about 0.01 to 1.0 and more usually from about 0.05 to 0.5 (nmole/U), respectively.

In addition to the above described first and second electron transfer agents, the subject signal producing systems also include a redox indicator. By redox indicator is meant a compound that is capable of being reduced by the electron transfer agents to produce a detectable, e.g. chromogenic, product. Where the redox indicator produces a chromogenic

product, i.e. the redox indicator is a chromogen, suitable chromogens are any compound capable of changing color upon reduction by one or more electrons, where suitable chromogens are generally ones that accept electrons from the electron transfer agents, described above.

5 A variety of different redox indicator compounds are of interest. Compounds of interest include: oxazines, thiazines, and tetrazolium salts. Of particular interest in many embodiments are tetrazolium salts which are capable of accepting the captured hydride from the electron transfer agents to form a colored formazan product. In many embodiments, these salts have the advantageous feature of being faint yellow in the oxidized form, but turn
10 bright visible colors upon electron reduction and conversion to formazan dyes. Tetrazolium compounds or salts that are of particular interest include: 2-(2' benzothiazolyl)-5-styryl-3-(4'-phthalhydrazidyl) tetrazolium (BSPT); 2-benzothiazolyl-(2)-3,5-diphenyl tetrazolium (BTDP); 2,3-di(4-nitrophenyl) tetrazolium (DNP); 2,5-diphenyl-3-(4-styrylphenyl) tetrazolium (DPSP); distyryl nitroblue tetrazolium (DS-NBT); 3,3'-[3,3'-dimethoxy-(1,1'-
15 biphenyl)-4,4'-diyl]-bis[2-(4-nitrophenyl)-5-phenyl(2H tetrazolium (NBT); 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium (MTT); 2-phenyl-3-(4-carboxyphenyl)-5-methyl tetrazolium (PCPM); tetrazolium blue (TB); thiocarbamyl nitroblue tetrazolium (TCNBT); tetranitroblue tetrazolium (TNBT); tetrazolium violet (TV); 2-benzothiazothiazolyl-3-(4-carboxy-2-methoxyphenyl)-5-[4-(2-sulfoethylcarbamoyl)phenyl]-
20 2H-tetrazolium (WST-4); 2,2'-dibenzothiazolyl-5,5'-bis[4-di(2-sulfoethyl)carbamoylphenyl]-3,3'-(3,3'-dimethoxy-4,4'-biphenylene)ditetrazolium, disodium salt (WST-5); 2-(p-nitrophenyl)-3-(p-iodophenyl)-5-phenyltetrazolium chloride (INT); and the like. WST-5 is preferred in many embodiments because it readily dissolves in an aqueous medium, which is most compatible with biological samples. Furthermore, the
25 resulting formazan compound exhibits strong spectral absorption at the purple-blue region, thus reducing the need for correcting the background signal from hemoglobin. Other useful tetrazolium salts are disclosed in U.S. Pat. Nos. 4,490,465; 4,491,631; 4,598,042; 4,351,899; 4,271,265; 4,247,633; 4,223,090; 4,215,917; 4,142,938; 4,024,021; 3,867,259; 3,867,257; 3,791,931; and 4,254,222; the disclosures of which are herein incorporated by reference.

30 The above described signal producing systems are capable of detecting the presence of a reduced enzyme cofactor in a sample, particularly an aqueous sample and more particularly a physiological sample, e.g. whole blood or a fraction or derivative thereof. A variety of different reduced enzyme cofactors may be detected using the subject signal

producing systems, where representative reduced enzyme cofactors include the reduced forms of the following cofactors: beta-nicotinamide adenine dinucleotide (beta-NAD), beta-nicotinamide adenine dinucleotide phosphate (beta-NADP), thionicotinamide adenine dinucleotide, thionicotinamide adenine dinucleotide phosphate, nicotinamide 1,N6-ethenoadenine dinucleotide, nicotinamide 1,N6-ethenoadenine dinucleotide phosphate, and pyrrolo-quinoline quinone (PQQ). The subject signal producing systems are particularly suited for use in the detection of NADH or NAD(P)H.

In many applications in which the subject signal producing systems find use, the reduced enzyme cofactor is one that is produced following the oxidation of an analyte of interest in a sample. In many embodiments, therefore, the subject signal producing systems also include the enzyme cofactor and an analyte oxidizing enzyme that is capable of oxidizing the analyte of interest and concomitantly reducing the enzyme cofactor. Enzyme cofactors of interest include those described above, i.e. beta-nicotinamide adenine dinucleotide (beta-NAD), beta-nicotinamide adenine dinucleotide phosphate (beta-NADP), thionicotinamide adenine dinucleotide, thionicotinamide adenine dinucleotide phosphate, nicotinamide 1,N6-ethenoadenine dinucleotide, nicotinamide 1,N6-ethenoadenine dinucleotide phosphate, and pyrrolo-quinoline quinone (PQQ). Enzyme cofactors of particular interest that may be included in the subject signal producing systems include: NADH or NAD(P)H.

The analyte oxidizing enzyme present in the signal producing system necessarily depends on the nature of the analyte to be detected with the system. Representative analyte oxidizing enzymes of interest include: alcohol dehydrogenase for alcohol, formaldehyde dehydrogenase for formaldehyde, glucose dehydrogenase for glucose, glucose-6-phosphate dehydrogenase for glucose-6-phosphate, glutamate dehydrogenase for glutamic acid, glycerol dehydrogenase for glycerol, beta-hydroxybutyrate dehydrogenase for beta-hydroxybutyrate, hydroxysteroid dehydrogenase for steroid, L-lactate dehydrogenase for L-lactate, leucine dehydrogenase for leucine, malate dehydrogenase for malic acid, and pyruvate dehydrogenase for pyruvic acid. As can be seen from the above representative list, the analyte oxidizing enzyme is typically a dehydrogenase.

REAGENT COMPOSITIONS

Also provided by the subject invention are reagent compositions for use in detecting at least a reduced enzyme cofactor, and in many embodiments and analyte, in a sample. The

reagent compositions may be fluid, e.g. aqueous, or dry compositions, where in many embodiments the reagent compositions are dry compositions. At a minimum, the subject reagent compositions are ones that include the first and second electron transfer agent and the redox indicator, where these components are described above. Such reagent compositions are suitable for use in the detection of reduced enzyme cofactors, e.g. NAD(P)H, in a sample. In many embodiments, however, the reagent compositions further include an enzyme cofactor and an analyte oxidizing enzyme, where these components are described above.

REAGENT TEST STRIPS

Also provided by the subject invention are reagent test strips for use in detecting the presence of an analyte in a sample. In particular, the invention provides dry strips for assaying for a particular analyte in whole blood, e.g. in beta-hydroxybutyrate, glucose, etc. In the broadest sense, the reagent test strip includes a solid support and a dry reagent composition present thereon, where the dry reagent composition is made up of all of the reagent compounds necessary to produce a detectable signal in the presence of the analyte of interest. In most embodiments of the subject invention, the dry reagent composition present on the subject test strip is one that includes the following members: an analyte oxidizing enzyme, an enzyme cofactor, first and second electron transfer agents and a redox indicator, where each of these constituent members are described in greater detail *supra*.

In many embodiments, the subject test strips include a membrane test pad that is affixed to a solid support. The support may be a plastic – e.g., polystyrene, nylon, or polyester - or metallic sheet or any other suitable material known in the art. In many embodiments, the test pad preferably comprises a bibulous, such as filter paper or polymer membrane. Associated with the test pad, e.g. coated onto the test pad, incorporated into the test pad, etc., is the reagent composition. The strip may also be configured in more complex arrangements, e.g. where the test pad is present between the support and a surface layer, where one or more reagents employed in sample processing may be present on the surface layer. In addition, flow paths or channels may be present on the test strip, as is known in the art. Of interest in many embodiments are the test strip configurations disclosed in U.S. Patent No. 5,902,731, the disclosure of which is herein incorporated by reference.

The subject test strips may be fabricated employing any convenient protocol. One convenient protocol is to contact at least the test pad portion of the strip with an aqueous

composition that includes all of the members of the reagent composition that is to be associated with the test pad in the final reagent test strip. Conveniently, the test pad may be immersed in the aqueous composition, maintained therein for a sufficient period of time and then dried, whereby the test pad of the reagent test strip which has associated therewith the reagent composition is produced. As stated above, the aqueous composition will include the various members of the reagent composition to be associated with the test pad of the reagent test strip, where the various members are present in amounts sufficient to provide for the desired amounts in the reagent composition that is produced on the test pad. As such, the concentration of non-proteinaceous electron transfer agent present in this aqueous composition typically ranges from about 1 μM to 1000 μM , usually from about 10 μM to 500 μM ; while the concentration of proteinaceous electron transfer agent in the aqueous composition ranges from about 50U to 3000 U, usually from about 100 U to 1000 U. The concentration of redox reagent present in the aqueous composition ranges from about 3 mM to 36 mM, usually from about 6 mM to 24mM. When present, the enzyme cofactor ranges in concentration from about 1.5 mM to 28 mM, usually from about 3.5mM to 14 mM. Similarly, the analyte oxidizing agent enzyme ranges in concentration from about 100 U to 2000 U, and usually from about 200 U to 1000 U when present. Other components that may be present in this aqueous composition employed to prepare the reagent test strip include sodium chloride, magnesium chloride, Tris, PSSA, Tetronic 1307, Crotein-SPA, sucrose, oxamic acid, sodium salt, and the like. See the experimental section, *infra*, for a more detailed description of a representative method for preparing the subject reagent test strips.

METHODS OF ANALYTE DETECTION

The above described signal producing systems, reagent compositions and test strips find use in methods of detecting the presence of, and often the amount of, an analyte in a sample. A variety of different analytes may be detected using the subject methods, where representative analytes include those described above, e.g. alcohol, formaldehyde, glucose, glutamic acid, glycerol, beta-hydroxybutyrate, L-lactate, leucine, malic acid, pyruvic acid, steroids, etc. While in principle, the subject methods may be used to determine the presence, and often concentration, of an analyte in a variety of different physiological samples, such as urine, tears, saliva, and the like, they are particularly suited for use in determining the concentration of an analyte in blood or blood fractions, e.g. blood derived samples, and more particularly in whole blood.

An important feature of the subject methods is that use of the subject signal producing systems that include both first and second electron transfer agents provides for an accelerated reaction time as compared to a control system, e.g. a system that includes a single electron transfer agent. Generally, the reaction rate is accelerated or enhanced by a factor of 1 to 3 depending on the ratio of PMS and diaphorase. Furthermore, the reaction rate is greater than the theoretical or predicted rate which would be expected based on the summation of the rates provided by the individual electron transfer agents by a factor of 1 to 3. Where the reaction rate is measured in terms of K/S per sec (see the experimental section *infra*) the K/S per sec for a reaction in which the subject signal producing systems are employed typically ranges from about 0.01 to 10, usually from about 0.05 to 5 and more usually from about 0.1 to 2.

In the subject methods, the sample and the signal producing system are combined into a reaction mixture, the reaction is allowed to proceed for a sufficient period of time to generate a signal indicative of the presence of (and often amount of) analyte in the sample, and the resultant signal is detected and related to the presence of (and often amount of) analyte in the sample. In the broadest sense, the reaction mixture may be produced in any convenient environment, such as a cuvette or other fluid containment means. However, in many embodiments, the above steps take place on a reagent test strip as described *supra*. As such, the subject methods are now discussed further in terms of methods in which a reagent test strip is employed.

In practicing the subject methods, the first step is to apply a quantity of the physiological sample to the test strip, where the test strip is described *supra*. The amount of physiological sample, e.g. blood, that is applied to the test strip may vary, but generally ranges from about 2 μ L to 40 μ L, usually from about 5 μ L to 20 μ L. Because of the nature of the subject test strip, the blood sample size that is applied to the test strip may be relatively small, ranging in size from about 2 μ L to 40 μ L, usually from about 5 μ L to 20 μ L. Where blood is the physiological sample, blood samples of a variety of different hematocrits may be assayed with the subject methods, where the hematocrit may range from about 20% to 65%, usually from about 25% to 60%.

Following application of the sample to the test strip, the sample is allowed to react with the members of the signal producing system to produce a detectable product that is present in an amount proportional to the initial amount of the analyte of interest present in the sample. The amount of detectable product, i.e. signal produced by the signal producing

system, is then determined and related to the amount of analyte in the initial sample. In certain embodiments, automated instruments that perform the above mentioned detection and relation steps are employed. The above described reaction, detection and relating steps, as well as instruments for performing the same, are further described in U.S. Patent Nos. 4,734,360; 4,900,666; 4,935,346; 5,059,394; 5,304,468; 5,306,623; 5,418,142; 5,426,032; 5,515,170; 5,526,120; 5,563,042; 5,620,863; 5,753,429; 5,573,452; 5,780,304; 5,789,255; 5,843,691; 5,846,486; 5,902,731; 5,968,836 and 5,972,294; the disclosures of which are herein incorporated by reference. In the relation step, the derived analyte concentration takes into account the constant contribution of competing reactions to the observed signal, e.g. by calibrating the instrument accordingly.

KITS

Also provided by the subject invention are kits for use in practicing the subject methods. The kits of the subject invention at least include a signal producing system as described above, where the signal producing system components may be combined into a single reagent composition or separated, e.g. present in separate containers. In certain embodiments, the signal producing system will be present in the kits in the form of a reagent test strip, as described supra. The subject kits may further include a means for obtaining a physiological sample. For example, where the physiological sample is blood, the subject kits may further include a means for obtaining a blood sample, such as a lance for sticking a finger, a lance actuation means, and the like. In addition, the subject kits may include a control solution or standard, e.g. an analyte control solution that contains a standardized concentration of analyte. In certain embodiments, the kits also include an automated instrument, as described above, for detecting the amount of product produced on the strip following sample application and relating the detected product to the amount of analyte in the sample. Finally, the kits include instructions for using the subject kit components in the determination of an analyte concentration in a physiological sample. These instructions may be present on one or more of the packaging, a label insert, containers present in the kits, and the like.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL**A. Preparation of Ketone Test Strip**

An 0.8 μ m nylon membrane obtained from Cuno (Meriden, CT) was dipped into the reagent of Table 1, until saturated.

Table 1

Component	Quantity
Water	100 ml
Tris(hydroxymethyl)aminomethane	1.2 gm
sodium chloride (MW 56.44, Sigma St. Louis, MO)	560 mg
Magnesium Chloride (MW 203, Sigma, St. Louis, MO)	2.5 gm
PSSA, polystyrenesulfonic acid, sodium salt (MW 70,000)	3 gm
Croton-SPA (Croda Inc., Parsippany, NJ)	3 gm
Oxamic acid, sodium salt	250 mg
Tetronic 1307 (BASF Corporation, Mount Olive, NJ)	2 gm
Sucrose (MW 342.30, Aldrich Chemicals, Milwaukee WI)	5 gm
NAD (MW 663.4, N7004, Sigma, St. Louis, MO)	450 mg
D-3-hydroxybutyrate dehydrogenase	50,000 U
WST-5 (MW 1331.37, Dojindo, Japan)	1.8 gm
Diaphorase	0-15000 U
Phenazine Methosulfate (PMS)	0-3 mg

The excess reagent was scraped off gently with a glass rod. The resulting membrane was hung to dry in a 56 °C oven for 10 minutes. Porex (0.6 mm thick) was soaked in the 5% nitrite solution and then hung to dry in a 100 °C oven for ten hours. Finally, the membrane was laminated between a polyester stock (0.4 mm Melenex® polyester from ICI America, Wilmington DE) and the nitrite impregnated Porex.

B. Assays

Using the following protocol, 10 μ L of aqueous samples comprising 40mg/dL (D) β -hydroxybutyrate were tested on strips as described above, where the strips varied in terms of the amount of PMS and/or Diaphorase present on the strip. A 10 ml aqueous sample was applied onto a freshly prepared test strip. The strip was inserted into a reflectometer and data acquisition was commenced. The reflectance of the reading strip was monitored at 660 nm at one-second intervals for two minutes. Next, the data were uploaded from the reflectometer's memory buffer to a personal computer via a modified serial cable. The reaction rate was calculated based on the initial rate of change in K/S at the range where the reaction profile was linear. The results summarized in Table 2 were averages of five replicates. In each individual assay, the reaction rate (in terms of K/S per sec) was observed.

(K/S is a measure of reflectance, discussed and defined in USP 4,935,346, col. 14, the disclosure of which is herein incorporated by reference.) Table 2 provides the results.

Table 2

DAD and PMS Formulation Separately					DAD and PMS Formulated Together		
DAD U/ml	Rate (K/S/sec)	PMS (μ m)	Rate (K/S/sec)	Separate† (K/S/sec)	DAD U/ml	PMS (μ m)	Together (K/S/sec)
150	0.0166	10	0.013	0.0296	150	10	0.0385
300	0.0392	20	0.0225	0.0617	300	20	0.1054
600	0.0943	40	0.0484	0.1427	600	40	0.3095
900	0.1666	60	0.078	0.2446	900	60	0.5861
1200	0.1787	80	0.1085	0.2872	1200	80	0.9107
1500	0.2866	100	0.13	0.4166	1500	100	Too fast*

† Theoretical rate, i.e. the predicted rate based on the sum of the reaction rate catalyzed by diaphorase and PMS individually.

* The reaction is too fast and difficult to calculate the rate precisely.

C. Observed Rate v. Theoretical Rate

Table 3 below provides a comparison of the observed rate and the expected or theoretical rate for the above described assays.

Table 3

Theoretical Rate (K/S per sec)	Observed Rate (K/S per sec)
0.0296	0.0385
0.0617	0.1054
0.1427	0.3095
0.2446	0.5861
0.2872	0.9107

Fig. 1 provides a graph of the observed rate vs. the theoretical rate.

As can be seen by the graph of Figure 1, the reaction rate of the signal producing system of the test strip is accelerated by the presence of both PMS and Diaphorase, where the magnitude of the observed acceleration is unexpectedly greater than the predicted amount of acceleration based on the sum of the reaction rates of systems having PMS or Diaphorase individually.

It is evident from the above results and discussion that the subject invention provides for a significant and unexpected enhancement in the rate of reaction observed in an analyte detection protocol based on the oxidation of an analyte and the concomitant reduction of a redox indicator. In addition, the subject invention provides for a more economical manner of analyte detection, as compared to certain prior art methods, e.g. ones that rely solely on

diaphorase as the electron transfer agent. As such, the subject invention represents a significant contribution to the art.

5 All publications and patents cited in this specification are herein incorporated by reference as if each individual publication or patent were specifically and individually indicated to be incorporated by reference. The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention.

10 Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

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WHAT IS CLAIMED IS:

1. A signal producing system for detecting the presence of a reduced cofactor in a sample, said system comprising:
a dry reagent composition, said dry reagent composition comprising:
(a) first and second electron transfer agents capable of oxidizing a reduced cofactor;
and
(b) a redox indicator.
2. The signal producing system according to Claim 1, wherein said first electron transfer agent is a low molecular weight electron transfer agent.
3. The signal producing system according to Claim 2, wherein said low molecular weight electron transfer agent is a phenazine compound.
4. The signal producing system according to Claims 1, 2 or 3, wherein said second electron transfer agent is a high molecular weight electron transfer agent.
5. The signal producing system according to Claim 4, wherein said high molecular weight electron transfer agent is a proteinaceous compound.
6. The signal producing system according to Claim 5, wherein said proteinaceous compound is an enzyme.
7. The signal producing system according to any of the preceding claims, wherein said redox indicator is a tetrazolium compound.
8. The signal producing system according to any of the preceding claims, wherein said dry reagent composition is present on a test strip.
9. In a method for detecting the presence of an analyte in a sample, the improvement comprising:
employing a signal producing system comprising a dry reagent composition according to any of Claims 1 to 8, 11 or 12.
10. A kit for use in detecting the presence of an analyte in a sample, said kit comprising:
a signal producing system according to any of Claims 1 to 8, 11 or 12.
11. The signal producing system according to any of Claims 1-7, wherein said first and said second electron transfer agents are present in amounts that provide for a reaction rate of said signal producing system that is greater than the theoretically expected reaction rate for said signal producing system.
12. The signal producing system according to Claim 11, wherein said first electron transfer agent and said second electron transfer agent are present at a respective nmol/U ratio of about 0.001 to 10