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3,783,105

APPARATUS FOR ASSAYING ENZYME ACTIVITY

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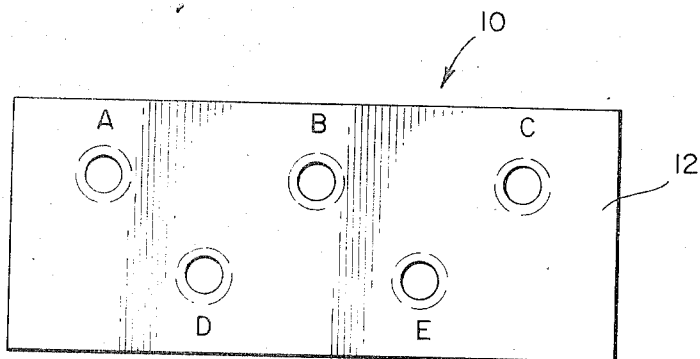


FIG. 1

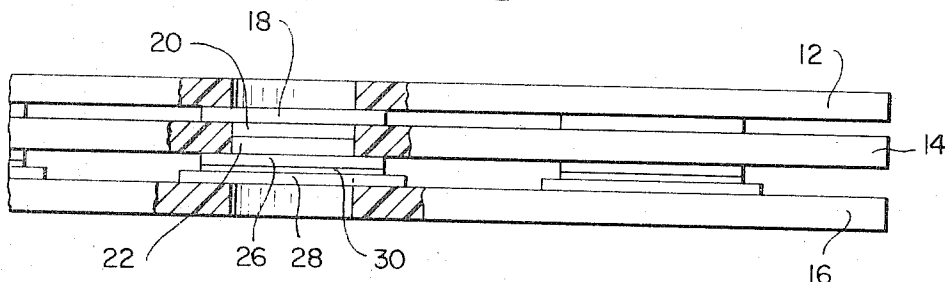


FIG. 2

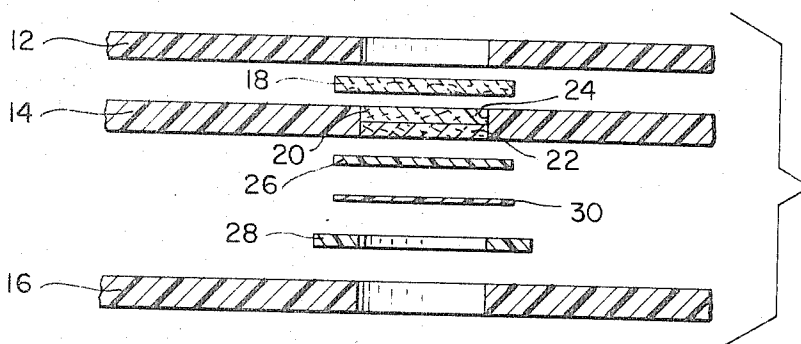


FIG. 3

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3,783,105

**APPARATUS FOR ASSAYING ENZYME ACTIVITY**  
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11 Claims

**ABSTRACT OF THE DISCLOSURE**

Improved method and apparatus for quantitating the activities of a wide selection of enzymes present in biological fluids, using a spot test technique. The test utilizes enzyme standards freeze-dried on transparent membranes assembled together with but separated from reagents, including substrates, dyes, and cofactors, which are freeze-dried in absorbent pads. The assembly may utilize micro-porous membranes as filters to assist in processing whole blood samples. As assembled, the spot test plates or slides are completely self-sufficient, require no added controls, instrumentation, or liquid volume measurement. The tests may be utilized under any environmental conditions such as those requiring rapid diagnostic support under emergency conditions, or routinely by medical practitioners in offices, hospitals or clinics. Test plates may assist in the diagnosis of a wide spectrum of pathological conditions relating to malfunctions of the liver, spleen, heart, neurological system, lungs, kidneys, muscle tissue, bones, etc.

The present invention relates to extensions and improvements of the concepts and principles disclosed in our co-pending application, Ser. No. 63,842, entitled Method and Apparatus for Quantitating Enzyme Activity, filed Aug. 14, 1970, now Pat. 3,663,374.

**BACKGROUND**

Rapid assays of the activities of enzymes in body fluids offer to the practicing physician a tool for confirmation of

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**Hepatobiliary diseases**

In application of blood or serum enzyme activity tests to the diagnosis of disorders of the liver and its duct systems, it is possible only in relatively few cases to discriminate between the possible sources of the enzymatic imbalance indicative of specific pathological conditions. However, the following enzymes are considered particularly useful in support of diagnosis of such diseases: alkaline phosphatase, lactic dehydrogenase, glutamic oxalacetic transaminase, isocitric dehydrogenase, sorbitol dehydrogenase, leucine aminopetidase, aldolase, and 5'-nucleotidase among others.

The following table shows some of the diseases which specifically give rise to considerably increased levels of these enzymes and the disorders indicated.

Enzymes	Paraen- chymal jaundice	Obstruc- tive jaundice	Others
Alkaline phosphatase...	No.....	Yes.....	Bone diseases.
Lactic dehydrogenases..	Yes <sup>1</sup> .....	No.....	Myocardial infarcts, <sup>2</sup> muscular dystrophy, <sup>3</sup> pulmonary infarcts,
Glutamic oxalacetic transaminase.	Yes.....	No.....	Myocardial infarcts, pancreatitis.
Glutamic-pyruvic transaminase.	Yes.....	No.....	Myocardial infarcts, pancreatitis.
Isocitric dehydrogenase.	Yes.....	No.....	Acute myocardial infarction.
Sorbitol dehydrogenase.	Yes.....	No.....	
Leucine amino peptidase. <sup>3</sup>	No.....	Yes.....	Cancer of the pancreas.
Aldolase.....	Yes.....	No.....	Muscular dystrophy.
5'-nucleotidase.....	No.....	Yes.....	

<sup>1</sup> Not heat stable.  
<sup>2</sup> Heat stable.  
<sup>3</sup> Serum and urine.

**Disease of the lung**

A number of experts have reported elevated enzyme levels in various pulmonary disorders. The following table indicates briefly a number of possibilities. Compared to cardiovascular and hepatobiliary diseases, elevation of enzyme activity is usually less dramatic in lung diseases. When sharply elevated it is frequently an indicator of complications.

**Diseases**

Enzymes	Pulmo- nary infarct or embolism	Pulmonary insufficiencies	Lung cancer	Others
Lactic dehydrogenases.....	Positive...	Positive for some disorders...	Usually positive.....	Myocardial infarcts, hepatobiliary diseases other carcinomas.
Glutamic-oxalacetic transaminase.....	do.....	do.....		Cardiovascular diseases, hepatobiliary dis- orders, etc.
Aldolase.....			Positive.....	Other carcinomas.
Phosphohexose isomerase.....			do.....	Do.
Malic dehydrogenase.....			Frequently elevated...	Do.
Isocitric dehydrogenase.....			do.....	Do.

diagnoses which has been generally unavailable in situa-  
 tions devoid of trained personnel and suitable instrumen-  
 tation. In addition, sufficient data on the correspondence  
 between various diseases and enzyme activity variations  
 are not available, partially because only time-consuming  
 or difficult enzyme assay methods are available. Our pre-  
 vious, above-identified patent application, discloses  
 methods for very rapid estimation of enzymes which are  
 indicative of cardiovascular disorders, particularly myo-  
 cardial infarctions. These methods may be modified for  
 screening enzymes indicative of a wide range of physio-  
 logical malfunctions even at subclinical levels. Thus, sim-  
 ple rapid enzyme assays employing the easily available  
 body fluids may be conveniently utilized in the practice  
 of preventative medicine as well as in assisting in diag-  
 nosis of pathological conditions. Exemplary examples are set  
 forth hereinbelow.

**Pancreatitis**

The laboratory diagnosis of pancreatitis may be asso-  
 ciated with (1) abnormalities in enzyme digestive capacity,  
 and (2) changes in blood and urine enzyme levels. Only  
 amylase and lipase have had extensive clinical trials. Di-  
 agnostic possibilities are indicated in the following table.

**Diseases**

Enzymes	Acute pancreatitis	Chronic pancreatitis	Others
Alpha amylase.	Positive with serum, higher levels in urine.	Usually positive.	Adrenocortical stress hepatocellular diseases, gastrointesti- nal disorders, (serum) drugs, hemolysis.
Lipase.....	Usually positive..	Negative.....	Drug use, gastroin- testinal disorders, cirrhosis, mumps.

Other enzymes of potential interest in diagnosis of pancreatic disorders are trypsin, lecithinase, leucine aminopeptidase, and deoxyribonuclease.

Enzymatic activity levels in biological fluids such as serum, cerebrospinal fluid, urine, etc., appear to have possible utility in assisting in diagnosis of a wide variety of disorders. In this area of biochemistry, the rapidly increasing volume of data indicates increasing need for techniques of appropriate utility. Other medical areas and enzymes of interest include:

Diseases	Enzymes
Neural and muscular.	Glutamic-oxalacetic transaminase, glutamic-pyruvic transaminase, creatine phosphokinase, lactic dehydrogenase, isocitric dehydrogenase, aldolase, cholinesterase, leucine aminopeptidase.
Skeletal muscle....	Aldolase, creatine phosphokinase, lactic dehydrogenase, transaminases, 5'-nucleotidase, ATP-ase, phosphohexoisomerase, adplahydroxy butyric dehydrogenase.
Bone.....	Alkaline phosphatase, acid phosphatase
Urinary, including prostate	Acid phosphatase, lactic dehydrogenase, alkaline phosphatase, glutamic-oxalacetic transaminase, beta-glucuronidase.
Gynecological....	6-phosphogluconic acid dehydrogenase, beta-glucuronidase.
Hematological....	Glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, pyruvate kinase, glyceraldehyd-3-phosphate dehydrogenase, Aldolase, phosphofruktokinase, phosphohexose isomerase, hexokinase, cholinesterase, methemoglobin reductase, glyoxalase, acid phosphatase, glutamic-acetic transaminase, pyrophosphatase.

### SUMMARY OF THE INVENTION

The method and apparatus utilize a simple spot test procedure in which the rate of color development on reference spots, containing pre-standardized increments of enzyme, is compared directly with that on a test spot where the enzyme in any of the several body fluids reacts. Direct comparison of the selected fluid enzyme activity with the enzyme activity on the reference spots eliminates need for temperature control and timing of reaction rates. The test measures enzyme activity in the biological fluids and requires neither instrumentation nor an operator. It can be employed for rapid screening in emergency situations where clinical test equipment and trained personnel may not be available; however, use in routine situations is also conceived within the scope of applications. Body fluids such as urine, blood, cerebrospinal fluid, serious cavity effusions, gastric juices, and vaginal fluid may serve as the source of enzymes for assay.

An illustrative embodiment of the apparatus and a working embodiment of a practical method will be explained with reference to the accompanying drawings in which:

FIG. 1 is a plan view of a test plate format embodying the concept of the invention;

FIG. 2 is a partial side elevational view showing in assembled relation the various components utilized in a stacked array in the test plate; and

FIG. 3 is a fragmentary exploded view disclosing an appropriate assembly of the components in a stacked test area in a test plate.

In one form of the test, which is illustrative only, a plate format is used. This test may be used to measure the activity of lactic dehydrogenase, pyruvic kinase, hexokinase, sorbitol dehydrogenase, amylase, or many other enzymes—all indicators of simple or complex physiological problems, some of which have been indicated above. All reagents, along with the enzyme standards appropriate to the assay, are freeze-dried in pads or discs of absorbent material which are held in assemblies similar to those indicated in the illustrative figures.

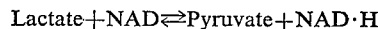
The reagent systems utilized for the enzyme assays are similar to those routinely employed in standard assays; however, readout is coupled to dye systems which produce developable colors. A typical dye of application is nitro blue tetrazolium which may be coupled to a number of reduction reactions by use of the electron carrier, N-methyl phenazonium methosulfate. Concentrations of reagents in the absorbent pads are adjusted to provide opti-

mum reaction conditions when the freeze-dried components are reconstituted by wetting the discs. The following examples illustrate the utility of the method.

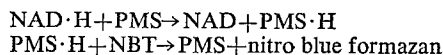
### EXAMPLE 1

#### Lactic dehydrogenase

Lactic (lactate) dehydrogenase catalyzes the reaction:



where NAD=nicotinamide-adenine dinucleotide and NAD·H is its reduced form. The indicator color is developed by coupling the reduction of nitro blue tetrazolium (NBT) to NAD·H by use of N-methyl phenazonium methosulfate (PMS) as follows:



The following reagents are described in appropriate literature: Henry, R. J. Chiamori, N., Golub, O. J., and Berkman, S., *Amer. J. Clin. Path.*, 34, 381-398 (1960); Honier, G. M., Yott, B., and Lim, J. G., *Amer. J. Clin. Path.*, 51, 287-292 (1969). Concentrations were adjusted on the basis of experience.

Reagents	Concentrations (mg./ml.)
Nicotinamide adenine dinucleotide (NAD).....	0.8.....
Sodium lactate.....	4.0.....
N-methyl phenazonium methosulfate.....	0.04.....
Nitro blue tetrazoleum.....	0.8.....
Lactate dehydrogenase.....	As required.
Dextran (Dextran, Clinical—200,000-300,000, Nutritional Biochemicals Corp.).....	3.0.....

All materials were made up in 0.05 M phosphate buffer, pH=7.5. The NAD and sodium lactate components (Reagent A) were adsorbed on the glass fibre pads, the PMS, NBT, LDH and Dextran (Reagent B) were freeze-dried on the transparent Nuclepore membrane, where color development was most readily followed.

A test plate generally designated 10 for practicing the invention is shown in detail in FIG. 3. It includes a plurality of test spots on areas A, B, C, D, and E. It consists of three slides, 12, 14, and 16, measuring 1¼ x 3¼ inches of opaque, white, high-impact polystyrene. Each slide measures 0.040 inch in depth. Each slide contains five coincident holes, measuring 0.161 inch in diameter. A top glass fibre pad, 18 (Whatman GF/A glass fibre paper is a typical material) is 0.250 inch in diameter. Glass fibre pads, 20 and 22, are 0.161 inch in diameter. These two fill hole 24 through slide 14. Reagent A is absorbed on pads 18, 20, and 22. Pads 18, 20, and 22 are retained between the upper slide, 12, and a porous filter material, 26, such as Acropor AN-200 (Gelman Instrument Company) or cellulose acetate such as Sephaphore III (Gelman Instrument Company, Cat. No. 51003). The bottom slide, 16, supports an ¼-inch (O.D.) spacer 28 containing a 0.161-inch hole made of 0.015-inch high-impact polystyrene. Across the spacer opening, a transparent film 30 such as Nuclepore (General Electric Company, 8-micron average pore diameter) is sealed. In preparation of the test plates 5 microliters of Reagent B mixture are freeze-dried on the inner surface of the transparent membrane 30.

Assembly of test plates requires a technique which will maintain liquid-tight seals between slide 12 and glass fibre pad, 18, and between slide 14 and membrane 26. Accordingly, a number of sealing methods can be utilized to achieve this purpose. Pressure heat seals on the edges, plastic edge binders, eyelets keyed through all plates, and thermal spot seals have been used to maintain the liquid seal integrity.

For use with whole blood, glass fibre pads are consecutively: (1) washed with glacial acetic acid, (2) washed with glass-distilled water, (3) dried, (4) saturated with 30 microliters of a solution of bovine serum albumin containing 10 mg./ml. and, (5) dried under vacuum at room temperature in a desiccator.

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The glass fibre discs which serve as reagent reservoirs, remove white and red blood cells to prevent clogging of the membrane filter. In practice, the reagents are applied as indicated and slides 14 and 16 are freeze-dried separately. The third slide, 12, is added as a retainer and the slides are sealed together and packaged in a low humidity atmosphere (<5% relative humidity). Spots A, B, and C contain LDH standards representing normal, elevated, and very high enzyme levels respectively. Spot D is a blank from which sodium lactate has been eliminated. Spot E is for assay of LDH and contains all reagents. A reordering of spots can be appropriate.

In conducting a test, the slide is positioned with the dull, glass fibre surfaces uppermost. Water is applied to Spots A, B, and C; whole blood to Spots D and E. Fluid uptake is controlled by the glass fibre pads; about 30 microliters is absorbed. Extra fluid remains unabsorbed. After application of the samples and water, the slide is inverted and development of blue color is the measure of reaction. After a few minutes, the intensity of color on Spot E is compared to the color development on Spots A, B, and C. Matching gives a direct measure of LDH activity.

## EXAMPLE 2

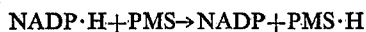
## Glucose-6-phosphate dehydrogenase (G-6-PDH)

Glucose - 6 - phosphate dehydrogenase is a glycolytic enzyme and impairment of its function is frequently associated with the chemotherapy of malaria and a number of metabolic diseases. Deficiency in G-6-PDH function has world-wide distribution with regional prevalences and a genetic transmission.

Methods of measurement together with reagent concentrations have been described by Glock, G. E., and McLean, P., *Biochem. J.*, 55, 400 (1953), and Thompson, R. H., and Todd, J. R., *Nature*, 201, 718 (1964).

In the plate or slide format described above, G-6-PDH may be estimated by application of the following reactions:

Glucose-6-phosphate (G-6-P) + nicotinamide - adenine-dinucleotide phosphate (NADP) → 6-phosphogluconate (6-PG) + reduced nicotinamide adenine - dinucleotide phosphate (NADP·H)



The following concentrations have been utilized in the glass fibre layers:

Components	Concentrations (mg./ml.)
NADP	0.8
PMS	0.04
G-6-P	4.0
Magnesium acetate: 4H <sub>2</sub> O	0.8
Nitro blue tetrazoleum	0.8

The glass fibre layers were impregnated with 30 microliters. Preparations are made in 0.05 M phosphate buffer adjusted to pH=7.4.

The transparent layer on the bottom slide was coated with:

Component	Concentrations (mg./ml.)
Glucose-6-phosphate dehydrogenase	As necessary
Dextran	3.0

These were also dissolved in phosphate buffer.

The procedure described in Example 1 was carried out to complete preparation of the test devices. Use is practiced in an identical fashion.

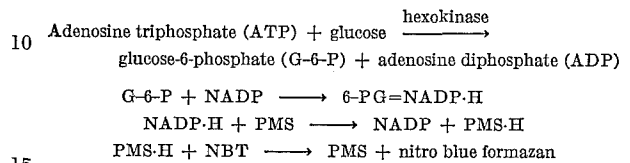
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## EXAMPLE 3

## Hexokinase

Hexokinase is another glycolytic enzyme which may be assayed by techniques similar to those explained above.

The reactions involved are:



Components freeze-dried in the glass fibre layers are:

Components:	Concentrations (mg./ml.)
NADP	0.8
G-6-P	4.0
Magnesium acetate: 4H <sub>2</sub> O	0.8
PMS	0.04
NBT	0.8

These components were applied to the glass fibre layers in 30 microliters of a solution prepared in 0.05 phosphate buffer, adjusted to a pH=7.4.

The transparent layer contained:

Components:	Concentrations (mg./ml.)
Hexokinase	As necessary.
G-6-PDH	1 I.U.
Dextran	3.0.

These were also prepared in phosphate buffer and 5 microliters of solution was dried on each site.

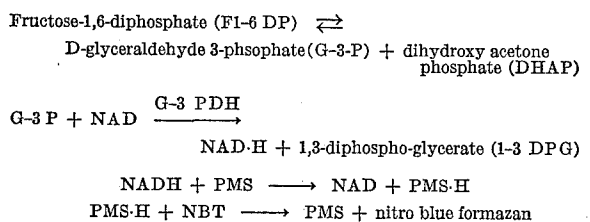
Techniques described in Example 1 were utilized to prepare and use the test slides.

## EXAMPLE 4

## Aldolase

Aldolase is a widely distributed enzyme which is found in many human tissues. It is a normal component of blood serum. Elevated aldolase activity may be used as a confirmatory test for viral hepatitis, progress muscular dystrophy, and some cancers. It is usually elevated after irradiation treatments.

Its assay is conducted by use of the following reaction scheme:



Components freeze-dried in the glass fibre layers are:

Component:	Concentrations (mg./ml.)
NAD	0.8
F1-6 DP	4.0
PMS	0.04
NBT	0.8
Disodium arsenate	4.0

This solution is prepared in 0.05 tris buffer, pH=7.4. A volume of 30 microliters is used to impregnate the glass fibre pads.

The transparent lower layer is coated with 5 microliters of:

Component:	Concentration (mg./ml.)	
G-3 PDH	2 I.U.	
Aldolase	For standards, as required.	5
Cysteine hydrochloride	2.0.	
Dextran	3.0.	

The solution is also prepared in 0.5 M tris buffer.

Completion of the preparation and use of these plates is described in Example No. 1. 10

#### EXAMPLE 5

##### Glyceraldehyde-3-phosphate dehydrogenase 15

Glyceraldehyde-3-phosphate dehydrogenase may be assayed by use of the same chemical system described in Example 4. However, aldolase is included in the preparatory reagents while glyceraldehyde-3-phosphate dehydrogenase is eliminated. The components included in preparation of the upper, glass fibre layers are: 20

Components:	Concentration (mg./ml.)	
NAD	0.8	
F1-6 DP	4.0	25
PMS	0.04	
NBT	0.8	
Disodium arsenate	4.0	

This solution is prepared in tris buffer as in Example 4 and used to impregnate the glass fibre layers. 30

The coating on the transparent surface is comprised of:

Components:	Concentration (mg./ml.)	
Aldolase	3 I.U.	
G-3-PDH	For standards, as required.	35
Cysteine hydrochloride	2.0.	
Dextran	3.0.	

Most kinase and dehydrogenase enzymes may be assayed by procedures similar to those indicated above. Included among such are: glycerol dehydrogenase, pyruvic kinase, malate dehydrogenase, isocitric dehydrogenase, malic dehydrogenase, alpha-hydroxy butyrate dehydrogenase, glutamic dehydrogenase, sorbital dehydrogenase, 6-phosphogluconic acid dehydrogenase. With appropriate reaction modifications, assay of alkaline and acid phosphatases and amylase may also be carried out in the indicated format. 40

Although the foregoing example of test plates have been evolved with nitro blue tetrazoleum as the indicator of enzyme activity, any chromogen may be used which demonstrates an appropriate color change on reduction. Another tetrazoleum dye which is directly applicable is MTT [3-(4,5 dimethylthiazol-2)-2,5-diphenyltetrazoleum bromide] which is available from G. T. Gurr, Ltd. Dyes which decolorize may also be used, whether singly or in combinations. Such techniques may be utilized to give many color combinations in the test plates. For example, decolorization of 2,6-dichlorophenol-indophenol (a deep blue dye) may be used in conjunction with a permanent yellow dye such as p-amino azobenzene (at basic or neutral pH's) to form an indicator which passes from blue-gray to green and eventually becomes yellow. Such color combinations may greatly improve the ease of comparison of the standards and the test spots. This indicator technique with mixed dyes may be used with a wide variety of enzyme reactions. 50

Manifestly minor changes and variations can be effected in the embodiment shown and described without departing from the spirit and scope of the invention as defined in and limited solely by the appended claims. 70

We claim:

1. In a system for visually quantitating enzyme activity in biological and the like fluids:

(A) a support;

(B) means constituting a plurality of separate restricted test zone areas in a rigid array mounted on said support;

(C) at least one of said areas including as a stacked array a plurality of superposed test reagent impregnated members in a rigidly confined column and adapted for placement thereon of a fluid test media, said stacked array including in descending sequence:

- (i) a top porous glass fiber disc;
- (ii) an intermediate porous glass fiber disc;
- (iii) a lower porous glass fiber disc;
- (iv) a porous filter membrane; and
- (v) a transparent film membrane.

(D) said glass fiber discs and said porous filter membrane constituting filters to remove contaminants including white and red blood cells;

(E) said glass fiber discs constituting dried reagent storage reservoirs for a first test reagent for elution therefrom by clear filtrate passing through the said discs;

(F) said transparent film membrane having a second, freeze dried, reagent on the upper surface thereof;

(G) said glass fiber discs constituting fluid control units and functioning to draw liquid therethrough;

(H) said support including first, second and third superimposed slides having a plurality of sets of mating holes therethrough, a said stacked array being mounted at each of said set of mating holes and confined between said slides.

2. In a system as claimed in claim 1, said top glass fiber disc being contained between said first and second slides and being of a size greater than that of said holes, said intermediate and lower glass fiber discs being closely contained and mounted within the hole in said second slide, said porous filter membrane and said transparent film membrane being of greater size than said holes and mounted between said second and third slides.

3. In a system as claimed in claim 2, further including a spacer having a hole mating with said holes of said slides, said spacer interposed between said transparent film membrane and said third slide.

4. In a system as claimed in claim 3, said slides as assembled in an array being sealed together and including liquid tight seals between said first slide and said top porous glass fiber disc, and between said third slide and said transparent film membrane.

5. In a system as claimed in claim 2, wherein some of said stacked arrays are standardized with the enzyme to be assayed, each of the arrays having the enzyme freeze-dried on said film membrane and separate from said fiber discs.

6. In a system as claimed in claim 5, wherein said glass fiber discs contain reagents including substrates, dyes, and co-factors which are freeze-dried therein.

7. In a system as claimed in claim 6, said reagents including for a color readout test result a dye material which indicates activity of the assayed enzyme.

8. In a system as claimed in claim 7, wherein the dye consists of a nitro blue tetrazolium and N-methyl phenazonium methosulfate as an electron carrier to produce a reduction reaction.

9. In a system as claimed in claim 4, wherein five test zone areas are provided, three prearranged said areas containing lactate dehydrogenase standards representing normal, elevated, and very high enzyme levels respectively, a fourth said area constituting a blank and the remaining said area being for lactate dehydrogenase assay of a test specimen and containing all reagents for said assay, said lactate dehydrogenase standards areas being adapted for application thereto of water, and said fourth and remaining area being adapted for application thereto of the test specimen whereby, after such application the assembly is inverted and color develops in the test areas indicative of the measure of reaction and the intensity of color developed in said remaining area, upon matching comparison with color development in the standards containing 75

areas provides a direct measure of lactate dehydrogenase activity in the test specimen.

10. In a system as claimed in claim 7, wherein said dye system consists of MTT.

11. In a system as claimed in claim 1, wherein reagents constituting said first and second reagents consist of substantially the following:

Reagents:	Concentrations
Nicotinamide adenine dinucleotide (NAD) --	0.8
Sodium lactate -----	4.0
N-methyl phenazonium methosulfate (PMS) -	0.04
Nitro blue tetrazolium (NBT) -----	0.08
Lactate dehydrogenase (LDH) -----	As required
Dextran (Dextran, Clinical-200,000-300,000	
Nutritional Biochemicals Corp.) -----	3.0

and wherein, all materials being made up in 0.05 phosphate buffer, pH=7.5, the NAD and sodium lactate com-

ponents being adsorbed on said glass fibre discs, the PMS, NBT, LDH and Dextran being freeze-dried on the upper surface of the transparent membrane, where color development is most readily followed.

References Cited

UNITED STATES PATENTS

3,526,480	9/1970	Findl et al. -----	195-103.5 R
3,367,841	2/1968	Buissiere et al. --	195-103.5 R
3,663,374	5/1972	Moyer et al. ----	195-103.5 R

ALVIN F. TANENHOLTZ, Primary Examiner

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U.S. Cl. X.R.

23-253 TP; 195-103.5 R, 100