(54) Title: ENZYMATICALLY AMPLIFIED PIEZOELECTRIC SPECIFIC BINDING ASSAY

(57) Abstract

A quartz crystal microbalance assay in which the binding of analyte to a surface on or near a quartz crystal microbalance (QCM) is detected by a conjugate which comprises an enzyme capable of catalyzing the conversion of a substrate to a product capable of accummulating on or reacting with a surface of the QCM leading to a mass change and, hence, a change in resonant frequency.

1

TITLE

ENZYMATICALLY AMPLIFIED PIEZOELECTRIC SPECIFIC BINDING ASSAY

Field of the Invention

The present invention relates to an enzymatically amplified piezoelectric specific binding assay in which analyte suspected of being present in a liquid sample is bound either on or in the proximity of a quartz crystal microbalance by means of a capture reagent, and the bound analyte is reacted with an anti-analyte reagent/enzyme conjugate. The conjugate is reacted with a substrate specific for the enzyme to form a product that is capable of either reacting with and/or accumulating on the surface of the quartz crystal microbalance. The mass change on the surface of the quartz crystal microbalance resulting from these reactions results in a change in the resonant frequency of the quartz crystal microbalance, which can be used to determine the analyte concentration in the sample.

Background of the Invention

The use of quartz crystal microbalances (also known as piezoelectric oscillators) in immunoassays has been described previously. These devices consist of single crystal wafers sandwiched between two electrodes. The electrodes are provided with means for connecting these devices to an external oscillator circuit that drives the quartz crystal at its resonant frequency. This frequency is dependent on the mass of the crystal, as well as the mass of any layers confined to the electrode areas of the crystal. Thus, the frequency is altered by changes in mass on the surface of the electrodes or in any layers on those electrodes. In general, the

2

change in resonant frequency of these devices can be correlated to the amount of mass change; if the quartz crystal microbalance and any attached layers obey rigid-layer behavior, the mass change can be determined from the frequency change by the Sauerbrey relationship

$$\Delta f = -\frac{2f_0^2 \Delta m}{A\sqrt{\rho_q \mu_q}}$$

where Δf is the measured frequency shift, f_0 the parent frequency of the quartz crystal, Δm the mass change, A the piezoelectrically active area, ρ_q the density of quartz (2.648 g cm^{-3}) and u_q the shear modulus (2.947 x 10^{11} dynes cm^{-2} for AT-cut quartz).

Shons et al. describe a piezoelectric quartz crystal microbalance which has been modified for the determination of antibody activity in solution. A quartz crystal, precoated with antigen, is exposed to antisera of varying concentration and specificity. Antisera specific for the antigen coating will form an additional protein layer on the crystal. The thickness of the antibody layer, measured by the frequency shift of the dry crystal, is proportional to the concentration of specific antibody in solution. [J. Biomed. Mater. Res., Vol. 6, pp. 565-570 (1972)]

U. S. Patent No. 4,235,983, issued to Rice on December 2, 1980, discloses a method for the determination of a particular subclass of antibody. The method utilizes a piezoelectric oscillator having bound to its surface an antigen specific for the antibody to be determined. The antigen-coated

oscillator is exposed to a solution containing an
unknown amount of the antibody. After the antibody
in the solution is attached to the antigen on the
oscillator, the oscillator is exposed to a so-called
sandwiching substance which selectively binds to a
specific subclass of the antibody being determined.
The frequency of the oscillator is measured in the
dry state before and after exposure to the
sandwiching substance. The change in frequency is
related to the amount of the subclass of antibody
bound to the oscillator, and the amount of the
subclass of antibody in the solution can be
determined by reference to a standard curve.

Roederer et al. disclose an in-situ
immunoassay using piezoelectric quartz crystals,
specifically, surface acoustic wave devices. Goat
anti-human IgG was immobilized on the quartz crystal
surface with a coupling agent. The piezoelectric
crystals were then placed in an electric oscillator
circuit and tested for detection of the antigen human
IgG. Detection was based upon the fact that surface
mass changes by adsorption are reflected as shifts in
the resonant frequencies of the crystals. The
authors concluded that the method suffers from both
poor sensitivity and poor detection limits. The
authors also concluded that the antigen to be
detected must be of high molecular weight; low
molecular weight analytes cannot be directly detected
by this methodology. [Analytical Chemistry, Vol. 55,
(1983)]

Ngeh-Ngwainbi et al. describe the use of
piezoelectric quartz crystals coated with antibodies
against parathion which are used for the assay of
parathion in the gas phase. When the coated antibody
binds with parathion by a direct reaction in the gas

4

phase, the resulting mass change on the crystal
generates a frequency shift proportional to the
concentration of the pesticide. [J. Mat. Chem. Soc.,
Vol. 108, pp. 5444-5447 (1986)]

European patent application 0 215 669,
published March 25, 1987, discloses an analytical
device and method for the in-situ analysis of
biochemicals, microbes and cells. Again, the method
is predicated on a resonant frequency change caused
by a weight change on the surface of a piezoelectric
crystal on which are immobilized receptor materials
specific for the analyte to be detected.

Grabbe et al. describe a quartz crystal
resonator, used in conjunction with cyclic
voltammetry, to study the binding of human IgG and
anti-IgG at a silver electrode. [G. Electroanal.
Chem. Vol. 223, pp. 67-78 (1987)]

As discussed by Roederer et al.,
piezoelectric crystal-based immunoassays in which
mass change is attributable only to the immunological
reaction between an antigen and an antibody can,
under certain circumstances, suffer from poor
sensitivity and poor detection limit. Consequently
there is a need in the art for a piezoelectric
crystal-based specific binding assay in which the
reaction between a binding agent and its ligand can
be amplified to provide a more sensitive and reliable
assay.

Summary of the Invention

This need is met by the present invention
which, in one aspect, is a process for measuring an
analyte utilizing a conjugate comprising an enzyme
and either an anti-analyte reagent or the analyte.
The conjugate is capable of reacting with (or
competing with) an analyte indirectly bound to a

quartz crystal microbalance by a capture reagent that
is directly bound to a surface of the quartz crystal
microbalance. The quartz crystal microbalance may
have at least one of its surfaces modified by any
combination of chemically reactive, priming, coating
or anti-analyte layers prior to exposure to the
analyte. Such a modified quartz crystal microbalance
is referred to herein as a biologically modified
quartz crystal microbalance, or BMQCM. Once the
conjugate is bound to the BMQCM-bound analyte, a
substrate specific for the enzyme is added to the
system. The enzyme catalyzes a reaction in which the
substrate is converted to a product which either (1)
accumulates on the surface of the BMQCM; (2) reacts
with and is subsequently incorporated into the BMQCM,
either electrostatically, covalently or by simple
adsorption; or (3) reacts with the BMQCM, but results
in incorporation of a species other than the
enzymatic reaction product. The resulting mass
changes produce corresponding changes in the resonant
frequency of the quartz crystal, as measured by an
external oscillator circuit and frequency meter.

In another aspect, the present invention is
a process for measuring an analyte utilizing a
reaction chamber in which a quartz crystal
microbalance is placed opposite and in close
proximity to a surface having capture reagent
adsorbed thereon. Upon exposure to the sample,
analyte is bound by the capture reagent. The
resulting bound complex is then reacted with a
conjugate comprising an enzyme and either an
anti-analyte reagent or the analyte. A substrate is
then introduced. The enzyme catalyzes a reaction in
which the substrate is convered into a product which
accumulates on the surface of the quartz crystal

6

microbalance, thereby changing its mass and resonant frequency. The accumulation of product on the microbalance can be mediated by a reactive layer on the microbalance. The reactive layer can be chosen to mediate mass accumulation by, for example, physical adsorption, ion complexation or covalent attachment of the catalysis product. Alternatively, the reactive layer can be chosen so that the catalysis product causes a change in the reactive layer that results in simple adsorption, ion exchange or covalent attachment of another reagent in the reaction medium.

Brief Description of the Drawing

The drawing consists of eight figures. Figures 1 through 7 depict various modes of carrying out the present invention. Figure 8 depicts suitable circuitry for measuring the resonant frequency of the BMQCM.

Detailed Description of the Invention

The invention may be understood by reference to the Drawing wherein like reference numerals are used to indicate like elements.

Referring now to Figure 1, there is seen a biologically modified quartz crystal microbalance (BMQCM) indicated generally by the reference numeral 10. The BMQCM comprises a quartz crystal wafer 12 sandwiched between two electrodes 14, 16. Adsorbed to one surface 18 of the electrode 16 is capture reagent 20.

Upon exposure of the BMQCM having capture reagent bound thereto to a solution (not shown) containing analyte 22, the analyte 22 will be bound by the adsorbed capture reagent 20, thus forming a bound complex. After a suitable incubation period, unbound analyte is washed away.

7

The QCM is then contacted with a conjugate 24 comprising anti-analyte reagent and an enzyme, designated generally by E. After a suitable incubation period, unbound conjugate is washed away.

The QCM, having the conjugate bound thereto, is then contacted with a solution containing a substrate, designated generally by S, which is specific for the enzyme E. The enzyme will then catalyze a reaction in which the substrate is converted to a product P. The enzyme and substrate system is chosen such that the product P is insoluble and precipitable on the BMQCM surface. The product P will accumulate on the surface 18, thereby leading to a change in mass and hence a change in resonant frequency, as measured by an external circuit 26.

Suitable anti-analyte reagents and capture reagents include those reagents which are capable of participating in complexation reaction with the analyte. Preferred reagents include antibodies, lectins, chelating agents, binding proteins, DNA and RNA polynucleic acid probes, and cell receptors. The choice of reagent will depend on the analyte to be measured. The anti-analyte reagent and capture reagent may be the same or different chemically.

Suitable analytes include proteins, hormones, enzymes, antibodies, drugs, carbohydrates, nucleic acids, etc.

Examples of enzyme/substrate systems which are capable of producing an insoluble product which is capable of accumulating on the surface of the BMQCM include alkaline phosphatase and 5-bromo-4-chloro-3-indolylphosphate (BCIP). The enzymatically catalyzed hydrolysis of BCIP produces an insoluble dimer which precipitates on the surface of the BMQCM. Other analogous substrates having the phosphate

8

moiety replaced with such hydrolytically cleavable
functionalities as galactose, glucose, fatty acids,
fatty acid esters and amino acids can be used with
their complementary enzymes.

Other enzyme/substrate systems include
peroxidase enzymes, for example horseradish
peroxidase (HRP) or myeloperoxidase, and one of the
following: benzidene, benzidene dihydrochloride,
diaminobenzidene, o-tolidene, o-dianisidine and
tetramethylbenzidene, carbazoles, particularly
3-amino-9-ethylcarbazole, all of which have been
reported to form precipitates upon reaction with
peroxidases. Also, oxidases such as alphahydroxy
acid oxidase, aldehyde oxidase, glucose oxidase,
L-amino acid oxidase and xanthine oxidase can be used
with oxidizable substrate systems such as a phenazine
methosulfate-nitriblue tetrazolium mixture.

Referring now to Figure 2, there is seen an
alternative embodiment of the BMQCM shown in Figure
1. Specifically, the surface 18 has been modified by
coating it with a layer 28. The layer 28 can serve
as a "priming" layer, which enhances attachment of
the capture reagent 20. The layer 28 can also serve
to enhance mass accumulation on the BMQCM by (1)
specific reaction between product P and the layer 28,
(2) ion exchange between P and the layer 28 or (3)
simple absorption of P into the layer 28.

Illustrative surfaces 28 are polymer films
and silane reagents that serve to enhance the binding
of the capture reagent during equilibration by
either hydrophobic interactions or covalent
interactions. An example of a polymer film is
polystyrene, which, itself, can be applied by
conventional methods, such as spin coating. Higher
surface area coatings for greater capture reagent

9

coverages can be achieved by fabrication of irregular
and three dimensionally shaped surfaces, such as by
aerosol application which deposits minute droplets of
polymer. Suitable silanes include the general class
of alkyl trichlorosilanes, which covalently bind to
the metal and glass surfaces of the quartz crystal
microbalance by M-O-Si and Si-O-Si linkages,
respectively. The general class of aminosilanes,
when attached to the QCM surface via M-O-Si or
Si-O-Si linkages, can be used to bind the capture
reagent by covalent linkages between the nitrogen
atom of the aminosilane and an appropriate functional
group on the capture reagent. Surfaces can also be
treated with reactive films, for example, redox
polymer films such as polyvinylferrocene, PV-Fc,
which serve as hydrophobic layers to enhance binding
of the capture reagent, as well as reactive layers
that react with the enzymatic reaction product, P,
leading to an increased mass and changed resonant
frequency.

Examples of enzyme/substrate systems which
result in the production of a product for which the
BMQCM surface can have a specific affinity include
horseradish peroxidase and hydrogen peroxide/iodide
mixtures. In this system, the substrate is
catalytically converted by the enzyme to I_2/I_3^- which
oxidizes a PV-Fc film on the surface of the BMQCM.
After oxidation, I_3^- is specifically bound by the
PV-Fc$^+$ sites in the film.

Referring now to Figure 3, there is seen
yet another embodiment of the BMQCM. Specifically, a
layer 28 is chosen to react with the product P to
induce a chemical change in the layer 28 making it
specifically reactive with a chemical species,

10

designated generally by A, which is different from P
and is present in the reaction system.

Suitable layers 28 for the embodiments
shown in Figures 2 and 3 could comprise organic thin
films, redox polymers and conducting polymers which
are capable of incorporating anions upon oxidation.
Illustrative organic redox polymers and thin films
are polyvinylferrocene, polypyrrole, polythiophene,
polyacetylene and phthalocyanines. Suitable anions
for the embodiment shown in Figure 3 include halides,
polyhalides, ferro/ferricyanide and nitrate.
Illustrative enzyme/substrate systems are
peroxidase/H_2O_2/I^- and peroxidase/ferrocyanide.

Referring now to Figure 4, there is seen an
alternative embodiment of the method according to the
present invention. In this embodiment, the capture
reagent 20 is attached to a support surface 30 which
is different from the QCM surface 18 or layer 28.
However, the support surface 30 must be in close
proximity to the layer 28. In this embodiment,
analyte 22 is bound by capture reagent 20. The bound
analyte is then reacted with conjugate 24. The
conjugate 24 is then reacted with substrate S to
produce product P which diffuses to layer 28 where it
accumulates to produce a mass change and, hence, a
resonant frequency change.

Figure 5 depicts substantially the same
embodiment, except that the interaction of the
product P with the layer 28 induces a chemical change
in the layer 28, making it reactive with a species A
present in the reaction system. Suitable layers 28,
enzyme/substrate systems and species A have been
discussed above in connection with Figure 3.

11

Figure 6 depicts a simple device for
carrying out the assays described in Figures 4 and 5.
The QCM is mounted at the bottom of a chamber 32 so
that its reactive layer 28 is exposed to the interior
of the chamber. The side walls of the chamber are
formed by an o-ring 34. At the top of the chamber is
a nylon support mesh 36 which provides support
surface 30. Adsorbed on surface 30 is capture
reagent 20. In use, the nylon mesh 36 is incubated
in any convenient vessel (not shown) with the sample
solution. After a suitable incubation, the mesh 36
is washed and reacted with conjugate 24. After a
suitable incubation, the mesh 36 is washed and placed
on the top of chamber 32. The substrate S can then
be introduced into the chamber 32 by a suitable inlet
tube 38.

For the embodiments described in Figures 4
5 and 6, suitable support surfaces include porous and
nonporous polymeric thin films, cellulosic membranes
and nitrocellulose membranes. The reactive films 28
may include organic thin films, polymers, redox
polymers and conducting polymers which are capable of
adsorbing the product P or reacting with P, followed
by incorporation of P or a different species A.
The polymers or organic thin films may include
polyvinylferrocene, polypyrrole, polythiophene,
polyacetylene and phthalocyanines. Illustrative
anions are halides, polyhalides, ferro/ferricyanide
and nitrate. An illustrative enzyme/substrate system
is peroxidase/H_2O_2/I^-. In this enzyme/substrate
system, peroxidase catalyzes the conversion of I^- to
I_2/I_3^-, which, in turn, oxidizes a polyvinylferrocene
film. Subsequent incorporation of I_3^- in the film to
maintain charge balance results in a mass increase
and a measurable change in frequency of the QCM.

12

In the description relating to Figures 1 through 5, above, the sample and reagents are described as being added sequentially. It should be understood, however, that the reagents may be added simultaneously.

Figure 7 depicts another embodiment of the invention in which the analyte 22 and a conjugate of analyte and enzyme 24 compete for a limited number of capture reagent 20 sites on the surface of the QCM. The sample can be added before the conjugate 24 or together with the conjugate 24. After a suitable incubation period, the unbound conjugate 24 is washed away, and substrate added. The remainder of the assay is performed as described above. In this case, the response due to accumulation of mass on the BMQCM is inversely proportioned to the analyte concentration. It should be understood that this embodiment may also be performed using a capture reagent surface which is in proximity to the surface of the QCM, analogous to the embodiments shown in Figures 4 and 5.

Figure 8 depicts circuitry which may be used to measure the resonant frequency of the BMQCM. Such circuitry is conventional and well-known in the art.

In general, attachment of the capture reagent 20 is accomplished by incubation of the QCM in a phosphate buffered saline (PBS) buffer solution of the capture reagent, in concentrations ranging from 50 to 100 ug/ml, depending on the capture reagent and the type of surface on the quartz crystal microbalance. Another suitable method involves immobilization of the capture reagent by chemical crosslinking, for example with glutaraldehyde. After

13

equilibration, the BMQCM is washed with PBS buffer
solution to remove any excess capture reagent that is
not strongly attached to the desired surface.

    After formation of the BMQCM assembly, the
BMQCM is exposed to solutions of the analyte 22 at
room temperature for a period of time predetermined
to be optimal for the capture reagent/analyte system
under study. After this exposure, the surface is
washed with TRIS test buffer to remove non-
specifically adsorbed material. The conjugate 24 is
then added in concentrations ranging from 0.05 to 20
ug/mL in TRIS test buffer to the BMQCM/analyte
assembly at room temperature. Conjugate can be
prepared, for example, according to the method of
Imagawa [J. Biochem., 92, 1413 (1982)] or Avrameas et
al. [Scand. J. Immunol., Vol. 8, Suppl. 7, 7-23
(1978)] and maintained at 4°C as a stock solution.
Conjugates of enzymes and synthetic polynucleic acids
can be prepared according to the method of Ruth et
al. [DNA, 4, 93 (1985)]. This is then followed by
washing with TRIS test buffer solution to remove
nonspecifically adsorbed conjugate 24. The total
amount of conjugate 24 should be added at a
concentration to exceed the amount of specifically
adsorbed analyte 22. Alternatively, the analyte and
conjugate may be added simultaneously.

    In a so-called competitive mode, the
analyte and an analyte/enzyme conjugate may be
reacted with the BMQCM. The analyte and conjugate
may be added sequentially or simultaneously.

    The frequency of the quartz crystal
microbalance is measured in 50 MM TRIS wash buffer
solution, and then a standard solution of the
substrate is directly added. The rate of frequency
change, as well as the total frequency change after a

14

time considered to be the optimum measurement
interval, are measured in solution and, since
conjugate is present only when analyte is bound to
the surface, are indicative of the amount of analyte
exposed to the BMQCM. The signal measured is
amplified by the large turnover numbers of the
enzymatic reaction, which produces concentrations of
product far exceeding that of the analyte.

The present invention can be embodied in
diagnostic kits comprising crystals treated with the
desired capture reagent and any modifying layers 28,
and an oscillator circuit with direct readout of the
resonant frequency of the quartz crystal
microbalance. In typical use, the analyte solution,
for example patient serum, would be added to a
compartment containing the BMQCM, followed by a wash
with buffer solution, followed by addition of the
appropriate enzyme/anti-analyte reagent conjugate,
followed by a wash. The substrate would then be
added and the frequency change directly measured.
The preferred mode of operation would include the use
of a reference crystal which is exposed to the
identical solutions, but which has not been modified
so as to have capture reagent on or in proximity to
its surface. In this manner, the difference in
frequency between the sample and reference crystals
can be measured and errors due to changes in
viscosity, temperature and non-specific binding
minimized. Operation with only the sample crystal,
however, is also feasible, because (1) the viscosity
and temperature changes during addition of the
substrate and during measurement are not large, (2)
interference from nonspecific adsorption is minimized

15

by the washing step, and (3) the measurement step poses no risk of mass changes from processes other than those induced by P.

The invention is further illustrated by the following nonlimiting examples.

Example 1

Assay of adenosine-5'-phosphosulfate (APS) reductase using alkaline phosphatase/5-bromo-4-chloro-3-indolylphophate (BCIP) on unmodified quartz crystals.

The procedure was performed according to the mode illustrated in Figure 1, with anti-APS reductase antibody as the capture reagent 20, APS reductase as the analyte 22, anti-APS reductase antibody with alkaline phosphatase enzyme as the conjugate 24, and 5-bromo-4-chloro-3-indolylphophate (BCIP) as the substrate, S. The first step was adsorption of the anti-APS antibody on the gold/quartz surfaces of a quartz crystal. This was performed by equilibration of the gold/quartz crystal with 2 mL of 100 ug/mL anti-APS reductase antibody in PBS buffer solution for 2 hours. The crystal was then washed once with PBS buffer containing 0.1% bovine serum albumin (BSA) and then 3 times with PBS buffer to remove excess anti-APS reductase antibody and block any nonspecific binding sites. The crystal was then exposed to varied concentrations of APS reductase ranging from 0 to 400 ng in 1.5 mL of TRIS test buffer solution for 20 minutes; the dosage concentrations were varied to determine the response characteristics of the device. After being washed with TRIS test buffer to remove

16

nonspecifically adsorbed APS reductase, the crystal
was exposed for twenty minutes to 1.5 mL TRIS test
buffer solution containing 30 mL conjugate comprised
of anti-APS reductase antibody and alkaline
phosphatase enzyme. The crystal was then washed
again 2 times with test buffer and once with 50 MM
TRIS wash solution.

The detection step was performed by
immersing the crystal in 0.5 mL of TRIS wash buffer
in a cell holder, followed by addition of 0.5 mL of a
standard solution of BCIP reagent solution (SIGMA).
A positive response for antigen was measured by a
decrease in frequency, corresponding to the
precipitation of the oxidized dimer of BCIP, an
indigo dye analog. Precipitation results from the
enzymatically catalyzed hydrolysis of the phosphate
functionality of BCIP, which, in turn, only occurs if
the alkaline phosphatase conjugate is present, which,
in turn, is only possible when the APS reductase is
present. Table 1 indicates the frequency response of
the BMQCM to different dosage levels of APS
reductase. It is clear that the frequency change and
the rate of change increase with larger dosage rates,
as expected. The relative responses agreed with
those determined by the spectroscopically measured
optical density of the blue BCIP indigo dimer
deposited on the surface 18 of the quartz crystal
microbalance. Notably, these responses were observed
for APS reductase levels in which the direct binding
of APS reductase could not be observed. That is, the
frequency change resulting from addition of APS
reductase to the BMQCM was not detectable.

17

Table 1

APS reductase concentration (ng/mL)	Δ frequency/sec (Hz/sec)	Δ frequency in 30 min.
200	0.24	406
75	0.17	250
25	0.10	170
7.5	0.002	6.3

Example 2

Assay of APS using alkaline phosphatase/5-bromo-4-chloro-3-indolylphophate (BCIP) on quartz crystals with polyvinylferrocene modifying layers.

The procedure was performed according to the mode illustrated in Figure 2, with a polyvinyl-ferrocene (PV-Fc) layer 28 on the quartz crystal, an anti-APS reductase antibody as the capture reagent 20, APS reductase as the analyte 22, anti-APS reductase antibody with alkaline phosphatase enzyme as the conjugate 24, and 5-bromo-4-chloro-3-indolyl-phophate (BCIP) as the substrate, S. The PV-Fc layer serves as a hydrophobic layer, enhancing adsorption of the anti-APS reductase antibody.

The first step was adsorption of the anti-APS reductase antibody onto a PV-Fc layer, applied by spin coating from a 10% o-chlorotoluene solution onto the gold/quartz surface 18 of a quartz crystal. This was performed by equilibration of the PV-Fc modified gold/quartz crystal with 1.5 mL of

18

100 ug/mL anti-APS reductase antibody in PBS buffer
solution for 2 hours. The crystal was then washed 3
times with PBS buffer to remove excess anti-APS
reductase antibody. The crystal was then exposed to
0 to 80 mL of APS reductase (0.5 mg/mL) in 2 mL TRIS
test buffer solution for 30 minutes; the dosage rate
was varied to determine the response characteristics
of the device. After being washed with TRIS test
buffer to remove nonspecifically adsorbed APS
reductase, the crystal was exposed for thirty minutes
to 1.5 mL TRIS test buffer solution containing 30 mL
of conjugate comprised of anti-APS reductase antibody
and alkaline phosphatase enzyme. The crystal was
then washed again with TRIS test and TRIS wash
buffers as described in Example 1.

     The detection step was performed by
immersing the crystal in 0.5 mL of TRIS buffer in a
cell holder, followed by addition of 0.5 mL of a
standard detection solution containing 50% dilution
of BCIP substrate reagent (SIGMA) in 50 MM TRIS
buffer (SIGMA). A positive response for antigen was
measured by the decrease in frequency, corresponding
to the precipitation of the oxidized dimer of BCIP,
an indigo dye analog. Precipitation results from the
enzymatically catalyzed hydrolysis of the phosphate
functionality of BCIP, which, in turn, only occurs if
the alkaline phosphatase conjugate is present, which,
in turn, is only possible when the APS reductase is
present. Table 2 indicates the frequency response of
the BMQCM to different dosage levels of APS
reductase. It is clear that the frequency change and
the rate of change increase with larger dosage rates,
as expected. The relative responses agreed

19

with those determined by the spectroscopically
measured optical density of the blue BCIP indigo
dimer deposited on the surface 18 of the quartz
crystal microbalance.

Table 2

APS reductase concentration (ng/mL)	Δ frequency/sec (Hz/sec)	Δ frequency in 30 min.
200	0.22	278
75	0.09	80
25	0.05	69
7.5	0.025	22

Example 3

Assay of human chorionic gonadotropin (hCG) using
horseradish peroxidase/hydrogen peroxide-iodide on
nylon membranes situated opposite to a quartz crystal
microbalance modified with polyvinylferrocene
modifying layers.

The procedure was performed according to
the mode illustrated in Figures 4 and 6, with a
polyvinylferrocene (PV-Fc) layer 28 on the quartz
crystal situated opposite to a nylon membrane 36
whose surface 30 was modified with capture reagent
The spacing of the compartment was approximately 1
mm. In this example, anti-hCG antibody was the

20

capture reagent 20, hCG was the analyte 22, anti-hCG
antibody with horseradish peroxidase (HRP) enzyme was
the conjugate 24, and a hydrogen peroxide-iodide
mixture was the substrate, S. The nylon membrane
served as a hydrophobic layer that adsorbed the
anti-hCG antibody, and presented the enzymatic
reaction product to the PV-Fc film. The
enzymatically catalyzed formation of iodine/triiodide
that occured when the conjugate was present resulted
in oxidation of the PV-Fc film, followed by
incorporation of triiodide into the film to maintain
electroneutrality. This led to an increase in mass
of the PV-Fc film and accordingly a large change in
the resonant frequency of the BMQCM device.

The first step was adsorption of the
anti-hCG antibody to the nylon surface 30 of membrane
36. This was performed by equilibration of the nylon
membrane 30 with 2 mL of 100 ug/mL anti-hCG antibody
in PBS buffer solution for 2 hours. The membrane
was then washed once with PBS buffer containing 0.1%
BSA and then 3 times with PBS buffer to remove excess
anti-hCG antibody and block any nonspecific binding
sites. The membrane was then exposed for thirty
minutes to different concentrations of hCG in PBS
buffer containing 0.1% BSA buffer solution. After
being washed with PBS buffer to remove
nonspecifically adsorbed hCG antigen, the membrane
was exposed for twenty minutes to 2 mL buffer
solution containing 20 mL of stock conjugate
comprised of anti-hCG antibody and HRP enzyme.
The membrane was then washed again with PBS/0.1% BSA
buffer solution.

21

The detection step was performed by placing
the membrane opposite to the quartz crystal modified
with the PV-Fc film, with a 1 mm diameter separator
between the two surfaces to form a reaction
compartment. The compartment was filled with a
pH=5.0 citrate/phosphate/iodide detection buffer.
After the frequency stabilized, 10 mL of a standard
solution of 0.01% hydrogen peroxide was added. The
enzymatically catalyzed reaction product
iodine/triiodide, P, diffuses across the compartment
to the PV-Fc film, resulting in oxidation of the
PV-Fc film by one-half of an equivalent of P. This
is subsequently followed by incorporation of an
equivalent of triiodide into the oxidized PV-Fc film,
resulting in an increase in mass of the film and a
corresponding decrease in resonant frequency of the
quartz crystal microbalance. Triiodide incorporation
results from the enzymatically catalyzed conversion
of iodide to iodine/triiodide, which, in turn, only
occurs if the HRP conjugate is present, which, in
turn, is possible only when the hCG antigen is
present.

Table 3

hCG concentration (ng/ml)	Δ frequency/sec (Hz/sec)	Δ frequency in 10 min.
0	< 0.005	< 3
600	0.01	48

22

The invention is defined by the following claims, although it will be appreciated by those skilled in the art that various modifications can be made without departing from the spirit thereof.

WHAT IS CLAIMED IS:

1.   In a process for detecting an analyte
suspected of being present in a liquid sample,. which
process comprises reacting the sample with a quartz
crystal microbalance (QCM) having an analyte capture
reagent bound to a surface thereof thereby binding
the analyte to the QCM by the analyte capture
reagent, the improvement comprising reacting the
bound analyte with (1) a conjugate comprising an
enzyme and either an anti-analyte reagent or the
analyte, and (2) a substrate which is capable of
being catalyzed by the enzyme to form a product which
is capable of accumulating on or reacting with the
QCM surface to induce a mass change, thereby leading
to a resonant frequency change of the QCM.

2.   The method of Claim 1 wherein the
conjugate comprises an enzyme and an anti-analyte
reagent.

3.   The method of Claim 1 wherein the
analyte capture reagent is an antibody, lectin,
chelating agent, binding protein, polynucleic acid
probe, or cell receptor.

4.   The method of Claim 3 wherein the
analyte capture reagent is an antibody or polynucleic
acid probe.

5.   The method of Claim 4 wherein the
analyte capture reagent is an antibody.

6.   The method of Claim 1 wherein the
anti-analyte reagent is an antibody, lectin,
chelating agent, binding protein, polynucleic acid
probe, or cell receptor.

7.   The method of Claim 3 wherein the
analyte capture reagent is an antibody or polynucleic
acid probe.

24

8. The method of Claim 4 wherein the analyte capture reagent is an antibody.

9. The method of Claim 1 wherein the enzyme is alkaline phosphatase and the substrate is 5-bromo-4-chloro-3-indolylphosphate.

10. The method of Claim 1 wherein the enzyme is horseradish peroxidase.

11. The method of Claim 1 wherein the surface is coated with a silane, polymer or organic thin film.

12. The method of Claim 11 wherein the polymer is polyvinylferrocene, polypyrrole, polythiophene, polyacetylene or phthalocyanine.

13. The method of Claim 12 wherein the enzyme is horseradish peroxidase and the substrate is hydrogen peroxide/iodide, urea peroxide/iodide or ferrocyanide.

14. The method of Claim 13 wherein the substrate is hydrogen peroxide/iodide.

15. A method for detecting an analyte suspected of being present in a liquid sample, comprising reacting the sample with a support surface having an analyte capture reagent bound thereon, said support surface being in close proximity to the surface of a quartz crystal microbalance, and reacting the support surface with a (1) conjugate comprising an enzyme and either an anti-analyte reagent or the analyte and (2) a substrate which is capable of being catalyzed by the enzyme to form a product which is capable of accummulating on or reacting with the QCM surface to induce a mass change, thereby leading to a resonant frequency change of the QCM.

16.   The method of Claim 15 wherein the
conjugate comprises an enzyme and an anti-analyte
reagent.

17.   The method of Claim 15 wherein the
analyte capture reagent is an antibody, lectin,
chelating agent, binding protein, polynucleic acid
probe, or cell receptor.

18.   The method of Claim 16 wherein the
analyte capture reagent is an antibody or polynucleic
acid probe.

19.   The method of Claim 17 wherein the
analyte capture reagent is an antibody.

20.   The method of Claim 15 wherein the
anti-analyte reagent is an antibody, lectin,
chelating agent, binding protein, polynucleic acid
probe, or cell receptor.

21.   The method of Claim 17 wherein the
analyte capture reagent is an antibody or polynucleic
acid probe.

22.   The method of Claim 18 wherein the
analyte capture reagent is an antibody.

23.   The method of Claim 15 wherein the
enzyme is horseradish peroxidase.

24.   The method of Claim 15 wherein the
quartz crystal microbalance surface is coated with a
silane, polymer or organic thin film.

25.   The method of Claim 24 wherein the
polymer is polyvinylferrocene, polypyrrole,
polythiophene, polyacetylene or phthalocyanine.

26.   The method of Claim 25 wherein the
enzyme is horseradish peroxidase and the substrate is
hydrogen peroxide/iodide, urea peroxide/iodide or
ferrocyanide.

27. The method of Claim 26 wherein the substrate is hydrogen peroxide/iodide.

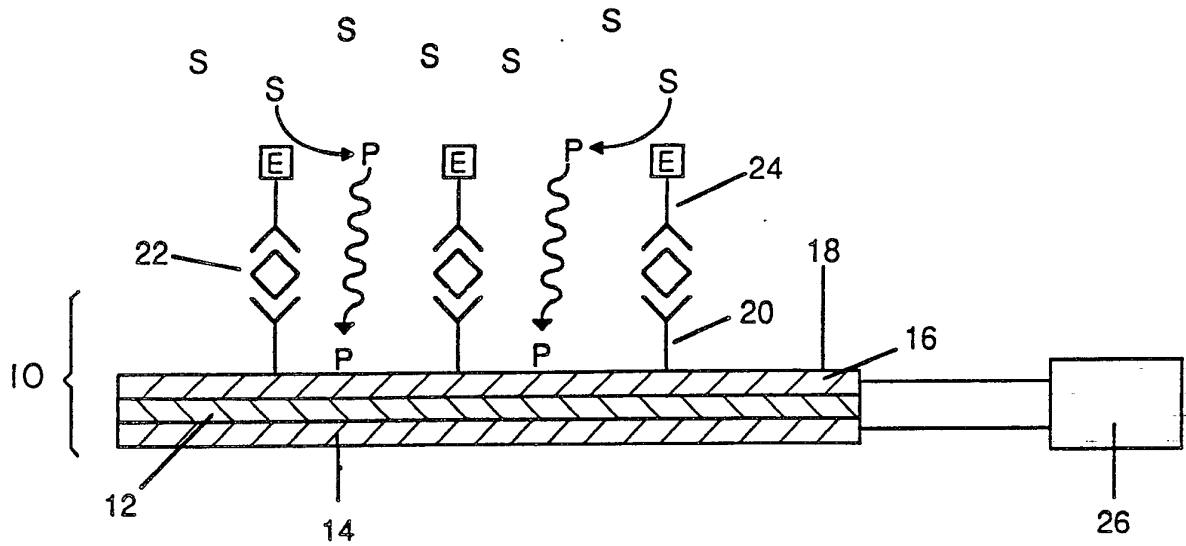28. The method of Claim 15 wherein the support surface is nylon or nitrocellulose.
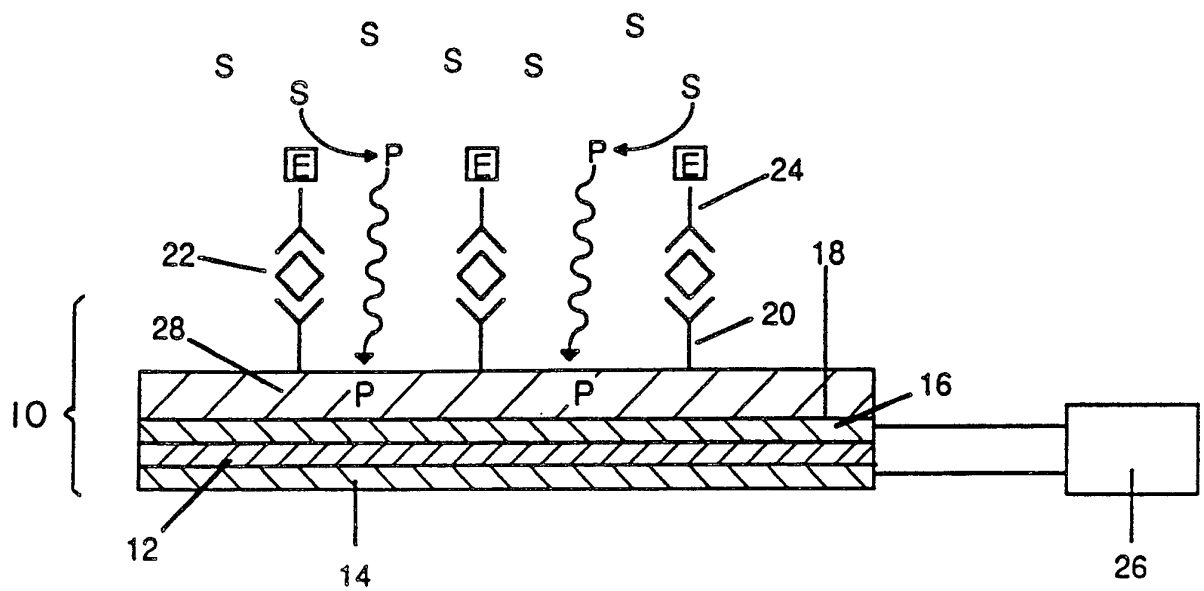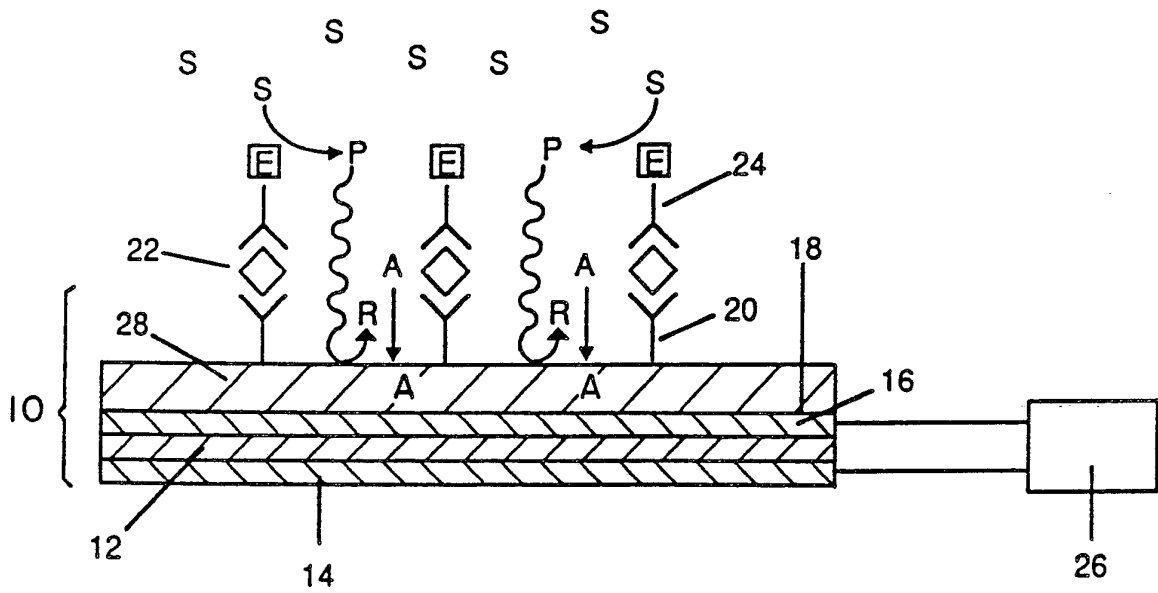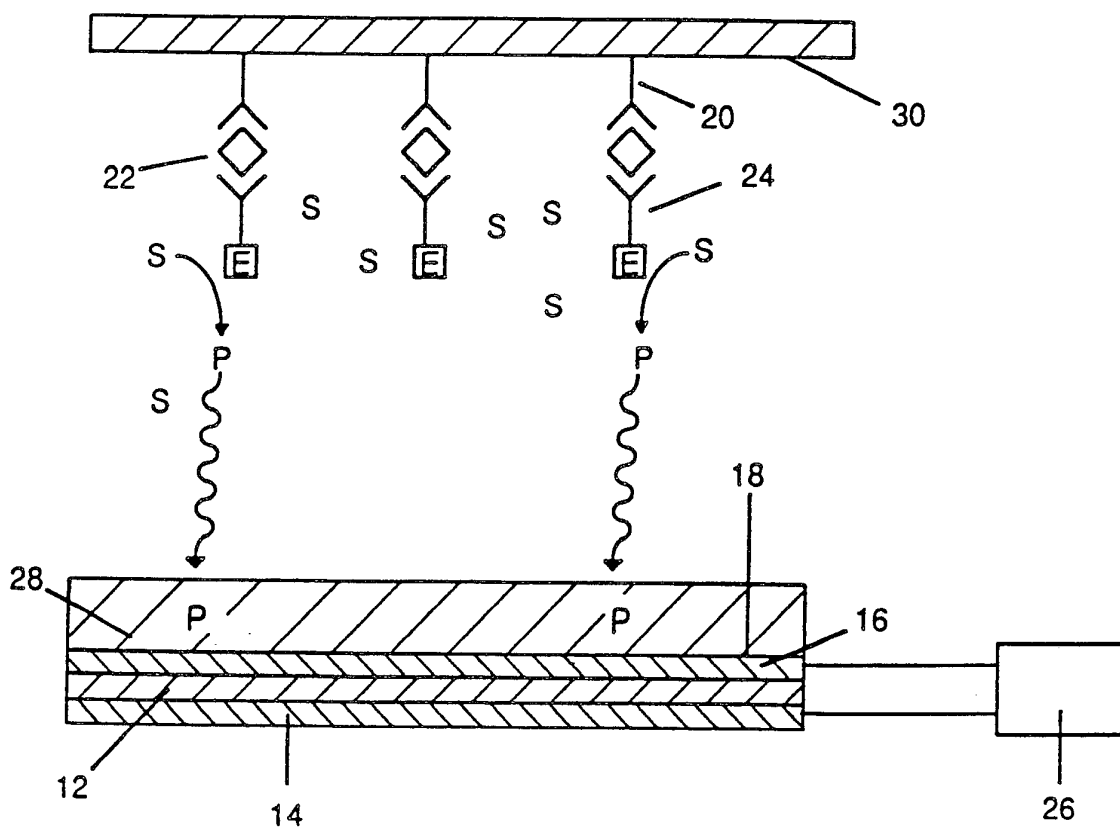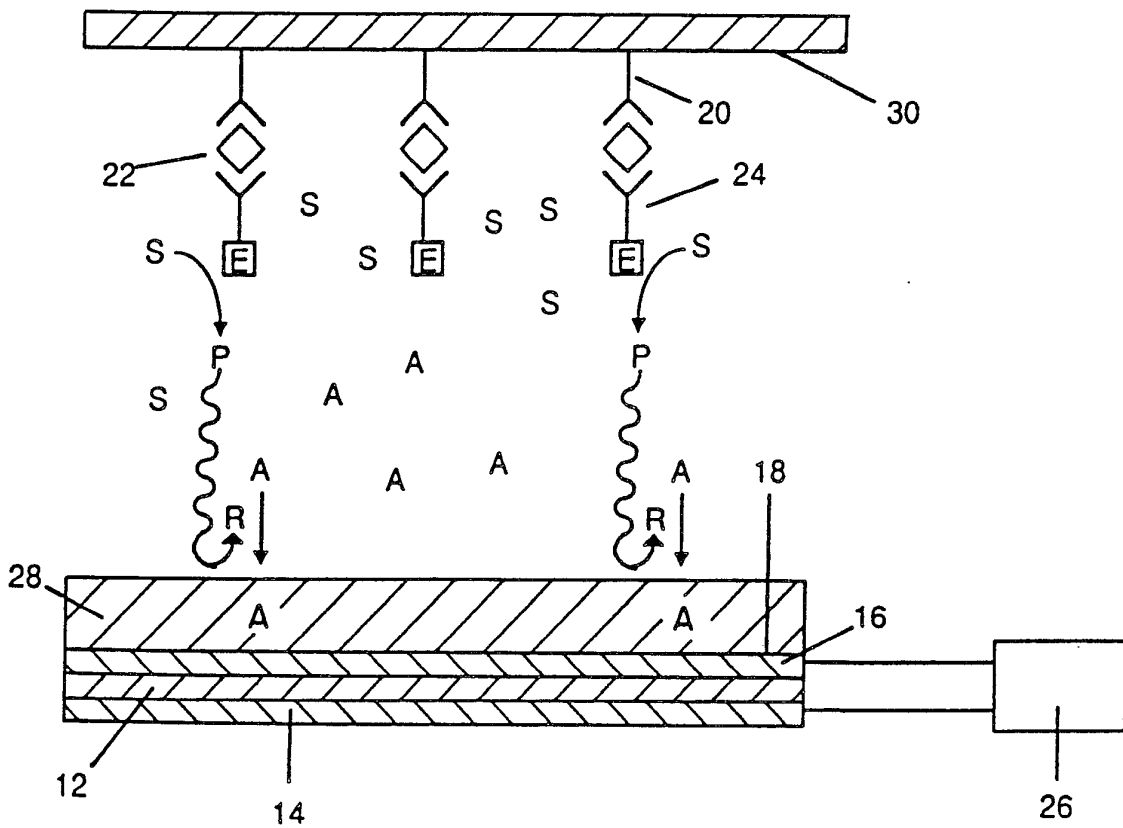
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F I G. 3

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F I G. 4

F I G. 5

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F I G. 6

F I G. 7

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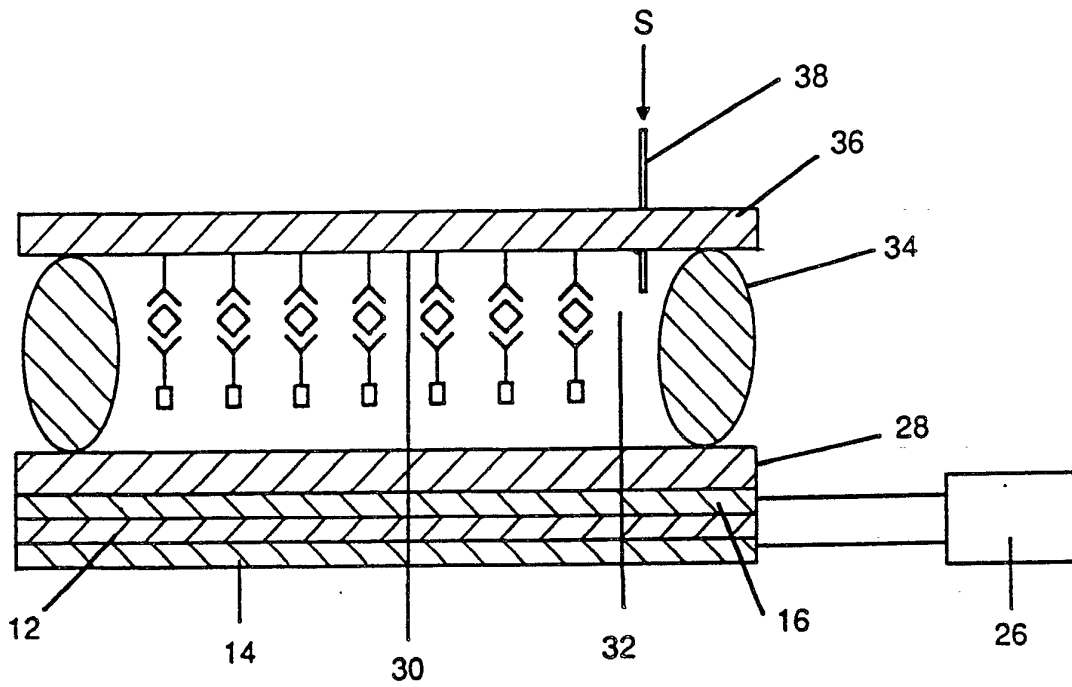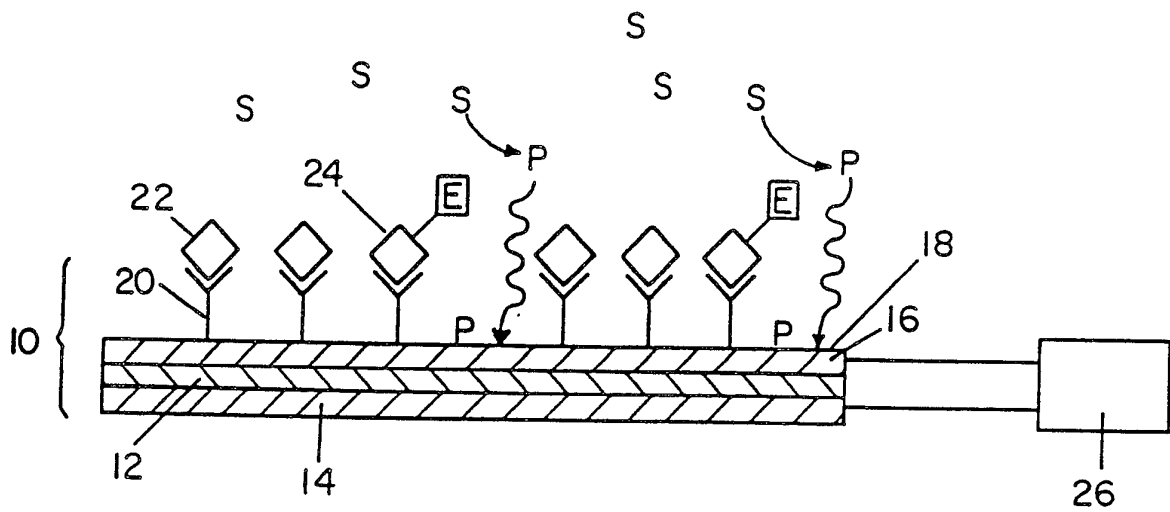
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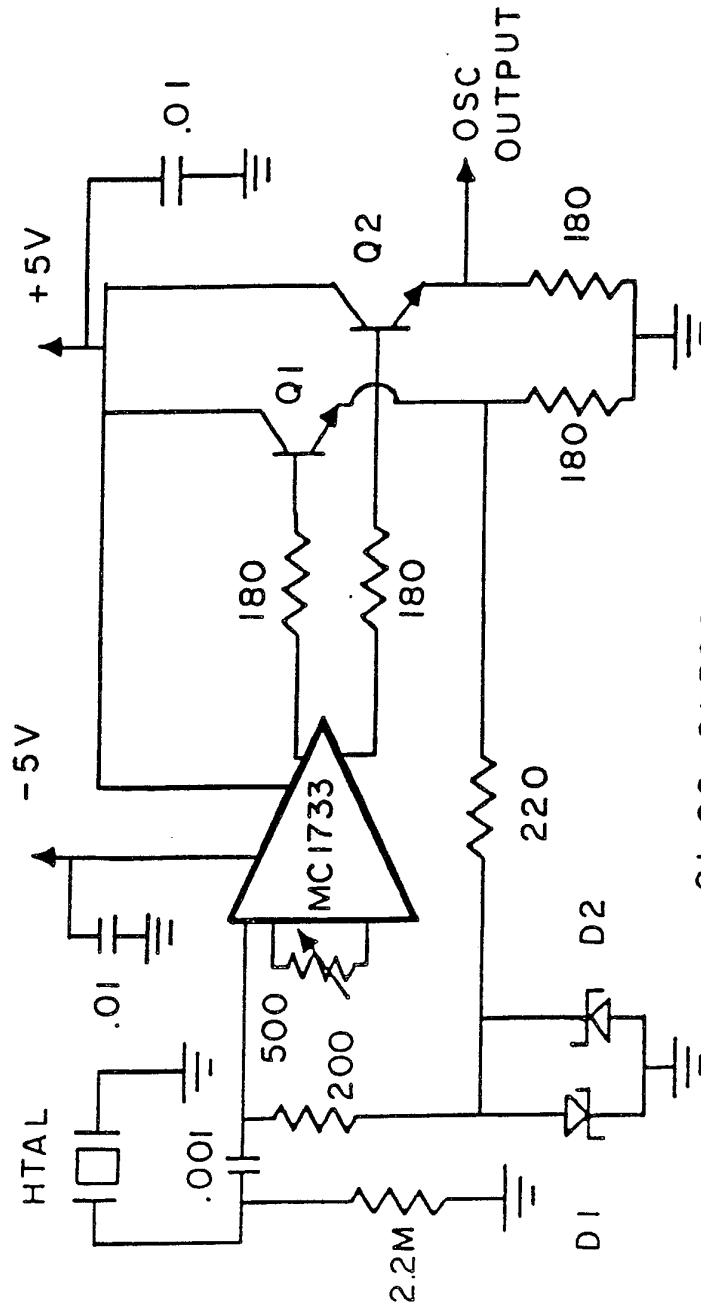
Q1, Q2 = 2N3904
D1, D2 = HP 5082-2811
SCHOTTKY DIODES

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC^4: G 01 N 33/542; 27/00; 33/58; 33/543

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
IPC^4	G 01 N; C 12 M

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched 8

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
X, P	Journal of the American Chemical Society, vol. 110, no. 26, 21 December 1988 The American Chemical Society (US) R.C. Ebersole et al.: "Amplified mass immunosorbent assay with a quartz crystal microbalance", pages 8623-8628 see the whole document	1-28
A	EP, A, 0215669 (SEIKO INSTRUMENTS & ELECTRONICS LTD) 25 March 1987, see abstract; page 2 (cited in the application)	1,3,6
A	WO, A, 87/02066 (THE UNIVERSITY OF SOUTHERN MISSISSIPPI) 9 April 1987, see the abstract	1,3,6

* Special categories of cited documents: 10

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
16th May 1989	1 2. U6. 89

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	P.C.G. VAN DER PUTTEN

Form PCT/ISA/210 (second sheet) (January 1985)

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 07/06/89
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		JP-A-	62064934	24-03-87
		JP-A-	63011835	19-01-88
		US-A-	4789804	06-12-88
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