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Practical Colorimeter for Direct Measurement of Microplates in Enzyme Immunoassay Systems

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A colorimeter capable of measuring results of enzyme-linked immunosorbent assay (ELISA) reactions directly in the wells of a microtiter plate is described. This colorimeter proved to be as accurate as a conventional spectrophotometer in assessing ELISA reactions, but had the advantage of not requiring transfer of the specimen to a separate chamber. With this colorimeter, 96 specimens can be read in approximately 5 min. A practical colorimeter such as this can make the use of ELISA tests more feasible for many laboratories.

The technique of enzyme-linked immunosorbent assay (ELISA) has proven to be an effective one for assessing serological response to a wide variety of infectious agents (3, 4, 8). In addition, ELISA has been used to detect the presence of antigen in serum and in stool specimens (9, 11, 12). ELISA is similar in design to radioimmunoassay, but uses an enzyme such as alkaline phosphatase or horseradish peroxidase in place of radioactive isotope as the immunoglobulin marker. Enzyme activity—and hence marker concentration—is assessed by measuring the change in optical properties of an appropriate substrate (8, 10). The advantage of this assay system is that it has sensitivity comparable with that of radioimmunoassay but without the problems of unstable reagents or radiation hazards (1, 6).

One problem inhibiting the widespread use of ELISA systems is the necessity of making objective readings of the amount of color of the substrate solution. Although readings can be done in a conventional spectrophotometer, the need to transfer the solution to the cuvette of the spectrophotometer increases the amount of time required for the test and the chance for error (2, 10). An additional problem is that ELISA assays are often carried out in microtiter systems with small volumes of substrate, thus making transfer of the solution to a spectrophotometer more difficult. Though color determinations can be made with the naked eye (11, 12), there is often a need for quantitative evaluation of the color density. This is especially true in ELISA systems that attempt to measure antibody amount on single dilutions of serum (7, 10).

While an automated system capable of making color determinations in a microtiter plate has been described (5), it is expensive and impractical for many diagnostic and clinical laboratories. This report describes a simple and relatively inexpensive instrument for measuring optical density (OD) in microtiter plates.

MATERIALS AND METHODS

Description of system. The system consists of three separate packages: (i) optical and mechanical unit; (ii) lamp power supply; and (iii) readout electronics. Figure 1 shows these three units as they are normally set up in operation.

Optical and mechanical unit. The optical system (Fig. 2) consists of a light source, condensing lens, infrared filter, light pipe, ultraviolet filter, and photodetector. The light source is a tungsten filament lamp, a no. 1634 microscope illuminator, with a nominal operating source of 20 V at 1 A. The condensing element is a 44-mm-diameter, 39-mm-focal length aspheric condensing lens. The light through the condensing lens is focused and contained by near-total internal reflection in a tapered-plexiglass light pipe with a diffusing exit port just above the microtiter tray. Below the tray the transmitted light is collected by a small-diameter aluminum light pipe, passes through an interference filter, and is detected by a silicon photodetector. The interference filter at either 400 or 488 nm is selected, depending on the enzyme involved. A 10-nm bandwidth appears to be near optimum.

One problem in designing a colorimeter capable of making OD determinations in a microtiter plate is that each well must be identically aligned with the optical system. Otherwise there will be variation in the readings due to refractive differences. In this system proper alignment is accomplished by means of a manual indexing system that accurately locates each of the 96 wells of a microtiter tray in the optical path. A grid (8 by 12 inches [ca. 20.3 by 30.48 cm]) of slots is milled into the table top. The microtiter tray fits into a carrier that has two rods affixed to the bottom orthogonal to each other. The milled slots and the rods are positioned so that the carrier can be located

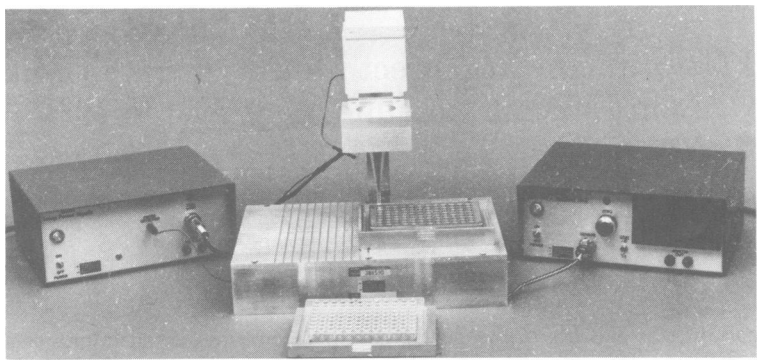

FIG. 1. *Photograph of ELISA colorimeter. Proper alignment with the optical system is obtained by advancing the metal microtiter tray holder along the grid matrix as shown.*

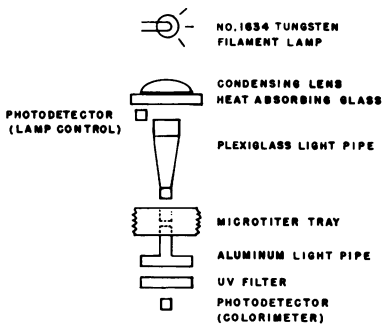

FIGURE 2. SIMPLIFIED DIAGRAM OF OPTICS SYSTEM

FIG. 2. *Schematic diagram of optics system.*

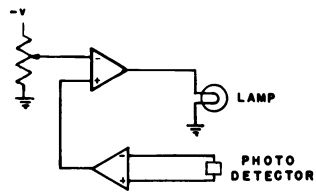

FIG. 3. *Block diagram of lamp power supply.*

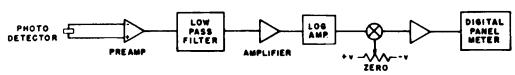

FIG. 4. *Block diagram of readout electronics.*

at any one of the 96 positions, each position placing one well in the optical system (Fig. 1).

Lamp power supply. The light source lamp is powered by a direct current (DC) power supply controlled by a feedback control signal proportional to the lamp-light level. A photodetector is placed just beneath the infrared filter that controls the lamp voltage around a preset level (Fig. 3). If the light output from the lamp varies for some reason (such as aging of the bulb), the voltage applied to the filament is adjusted to automatically compensate and hold the level of the light output relatively constant.

Readout electronics. The light transmitted through the sample and ultraviolet filter is detected by a UV-100B (EG&G, Inc.) silicon photodetector whose output current is directly proportional to the light level. A proportional voltage is developed by a preamplifier circuit in the base of the table. The signal is filtered to remove noise components greater than 2 Hz and log converted to provide an output voltage proportional to the OD of the sample (Fig. 4). The log convertor is operational over 4 decades of input voltage, which is the equivalent of 4 OD units. The final amplifier is a gain of ×1 (for a log convertor with a conversion factor of 1 V/decade) or ×16.66 (for a log convertor with a conversion factor of 60 mV/decade) and with an adjustable offset voltage input to produce a 0.0 OD reading when the reference sample is read.

The materials required to fabricate one of these units cost approximately $800.00. The fabrication time needed to machine and assemble the mechanical, optical, and electronics systems was approximately 100 h.

Operation. After zeroing the system on a blank well containing substrate, the carrier is moved to align the first test well with the optical system. The OD result of that well is displayed visually on a 3½ digit panel meter. The results can be recorded either manually or fed into an external data collection device such as a printer or a computer. The carrier is then moved to the next test well, and the process is repeated for the remainder of the test wells.

Methods of evaluation. The ELISA reader was evaluated by reading specimens in plates pretreated with guinea pig anti-rotavirus serum as is done in the ELISA assay for rotavirus antigen (12). Four types of plates were used: round- and flat-bottomed polyvinyl plates (Cooke microtiter plates) and round- and flat-bottomed hard plastic "substrate" plates (Cooke micro ELISA plates). For dilution experiments a solution of *p*-nitrophenylphosphate (Sigma 104 substrate) diluted in 10% diethanolamine (pH 9.8) was reacted with alkaline phosphatase (Sigma type VII), and the reaction was stopped with 3 M sodium hydroxide. The resulting solution, which had a strong yellow color, was diluted in 10% diethanolamine buffer for the de-

terminations. For variability experiments, 100-μl portions were put in 20 wells of each type of plate, using a micropipette (Eppendorf). The well-to-well variation was then measured. Comparison studies were performed on the same volume by a Beckman DB spectrophotometer, using a 1-ml microcuvette with a 10-mm pathlength. The cuvette was emptied by aspiration and washed once with saline solution between each reading. These studies were done at a wavelength of 400 nm in both the ELISA colorimeter and the spectrophotometer.

RESULTS

Comparison with spectrophotometer. Comparison experiments revealed the OD values determined by the ELISA reader, using polyvinyl round-bottomed plates, to be proportional to those obtained on the conventional spectrophotometer (Fig. 5). Similar results were obtained with the other types of plates. The ratio of the readings for round-bottomed wells on the 100-μl samples in the ELISA reader and the 10-mm-pathlength curve of the conventional spectrophotometer was approximately 0.7, indicating that the effective pathlength of 100-μl samples in the round-bottomed plates was approximately 7 mm.

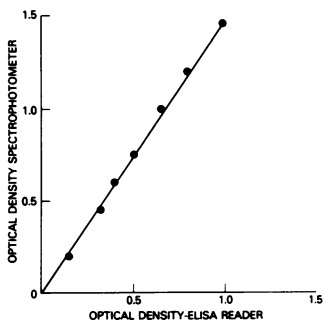

FIG. 5. *Comparison of OD readings performed in the ELISA colorimeter and a conventional spectrophotometer at a wavelength of 400 nm (see text).*

Variation experiments. The ELISA colorimeter proved to give consistent readings with all four types of microtiter plates for a range of ODs from 0.1 to 1.5 (Table 1). The standard deviation of 20 measurements was between 0.01 and 0.02 for the round-bottomed plates and between 0.01 and 0.03 for flat-bottomed plates. On the other hand, standard deviations of between 0.03 and 0.06 were noted in repeated measurements with the spectrophotometer.

Practical use. By means of the ELISA reader a plate of 96 specimens could be read and the results could be manually recorded in approximately 5 min. The same process took approximately 65 min when using a standard spectrophotometer. The ELISA reader proved to be of sufficient accuracy to diagnose the presence of human reovirus-like agents in human stools with the same sensitivity as electron microscopy and radioimmunoassay (12).

DISCUSSION

The above experiments indicate that the ELISA colorimeter is capable of measuring OD accurately on specimens in the wells of microtiter plates. The results obtained with the colorimeter were proportional to results obtained by a conventional spectrophotometer, with the difference in the actual OD values being due to differences in the optical pathlengths. Since most ELISA determinations are done by comparing a positive to a negative serum or stool specimen (8), this difference in pathlength would not be important. However, if direct comparisons with measurements made in the 10-mm-pathlength spectrophotometer cell are desired, the pathlength of the microtiter wells can be calculated by comparing the slope of dilution lines, such as those shown in Fig. 5, and the conversion can be made. The actual pathlength measured will be determined by the type of microtiter plate used and the volume of substrate used in the assay. Because the pathlength

TABLE 1. *Variability of OD readings of ELISA colorimeter and standard spectrophotometer*[a]

Dilution of substrate (solution)	ELISA colorimeter				Spectrophotometer
	Type of plate				
	Soft round	Soft flat	Hard round	Hard flat	
2^{-4}	1.39 ± 0.01	1.49 ± 0.01	1.34 ± 0.01	1.24 ± 0.02	ND
2^{-5}	0.72 ± 0.02	0.76 ± 0.03	0.68 ± 0.02	0.62 ± 0.01	1.1 ± 0.06
2^{-6}	0.36 ± 0.01	0.39 ± 0.03	0.31 ± 0.01	0.29 ± 0.02	0.46 ± 0.05
2^{-7}	0.17 ± 0.01	0.20 ± 0.01	0.14 ± 0.01	0.11 ± 0.01	0.28 ± 0.03

[a] Colorimeter readings represent the mean and standard deviation of 20 readings of 100 μl of the same solution in different wells of the microtiter plate. Spectrophotometer readings represent the mean and standard deviation of 20 readings of 100 μl of the same stock solution performed in a 10-mm-pathlength quartz cuvette. All readings were done at a wavelength of 400 nm.

[b] ND, Not determined due to reading being off the scale of the machine.

is dependent on the volume of liquid in the well, it is important that the amount of fluid added to each well be accurately measured. We found that this can be accomplished either by a standard micropipette (Eppendorf) or manifold (Cooke Microdoser II) system.

The well-to-well variation noted with the ELISA reader was less than the variation noted with repeated spectrophotometer readings. With the use of larger sample volumes, the spectrophotometer is capable of considerably more reproducible readings; therefore, most of the error noted was due not to the optics of the machine, but to the difficulties of preventing the introduction of air bubbles and obtaining complete removal of the previous specimens. These problems do not occur with a machine such as the ELISA colorimeter.

The ELISA colorimeter should be a useful adjunct to ELISA systems. It is hoped that with the use of machines such as this the advantages of ELISA systems can be brought to more laboratories and to more biological systems.

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