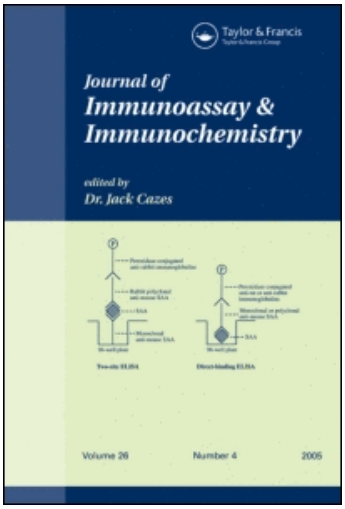

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A Computerised ELISA System for the Determination of Total and Antigen-Specific Immunoglobulins in Serum and Secretions

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A COMPUTERISED ELISA SYSTEM FOR THE DETERMINATION OF TOTAL AND ANTIGEN-SPECIFIC IMMUNOGLOBULINS IN SERUM AND SECRETIONS

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ABSTRACT

A computerised micro-ELISA system is described which features rapid and simple procedures for sample storage and transfer, plate washing and calculation of results. Calculations are performed by a Commodore Pet desk computer interfaced with a Titertek Multiskan micro-ELISA plate reader. Up to 1200 analyses per day can be performed by one person. Its application to the measurement of total immunoglobulin isotype and class specific antibody to parainfluenza type 1 (Sendai virus) in mice is described.

KEY WORDS: ELISA, Computer, Parainfluenza, Sendai.

INTRODUCTION

The use of ELISA for the measurement of antibody in a variety of biological fluids is now well documented (1-6). One of the main advantages of the technique is that the equipment necessary to carry out basic assays can be both simple and cheap, with end points that may be read semiquantitatively by eye. In

order to increase the sensitivity and accuracy a spectrophotometer must be used to measure optical density (7). Attempts have been made to automate the complete assay system using one piece of equipment (8). There are three time-consuming steps in the procedure: i) sample addition and dilution, ii) plate washing, and iii) reading and calculation of results. We have investigated each of these and describe how to increase the number of analyses that can be carried out in a day, without loss of precision.

The system has been developed for the analysis of antigen specific immunoglobulins to a wide variety of viral, bacterial and pure protein antigens. These include antibodies to Streptococcus mutans and Escherichia coli, toxoids of Vibrio cholera, Corynebacterium diptheria and Clostridium tetani and pure protein antigens such as human serum albumin (H.S.A.).

The methods illustrated below refer to the detection of specific IgG class antibodies to Sendai virus and the measurement of total immunoglobulin A in mouse serum.

MATERIALS AND METHODS

Antigens

1) Concentrated parainfluenza type 1 Sendai (D/52 strain) antigen was prepared by ultracentrifugation of infected egg allantoic fluid (200ml) at 27,000g for 1 hour. The virus

concentrates were washed twice with saline (2 x 200ml), reconstituted in 5ml of phosphate buffered saline(P.B.S.) and then dispersed by ultrasonicating for 30 seconds. The preparation had a haemagglutination titer of 1/20,000 (per 0.025ml). Control antigen was prepared in the same way but using non-infected allantoic fluid.

2) Purified virus antigen was prepared according to the method of Ertl and co-workers (9).

Samples

Sera and secretions from Swiss white mice (Alderley Park strain) were stored in 'special' polystyrene tubes in a transfer tube strip holder (Flow Labs, Irvine, Scotland). Eighty-eight samples are stored on the equivalent of one microtitre plate in this way. Stock dilutions (if required) were made by transferring a small volume of sample into 250µl of diluent (PBS/0.05% Tween 20) in a microtitration plate. A 12 channel variable volume micropipette (5-50µl, Flow Labs) was used to transfer each row of samples. Stock dilutions and sample plates were sealed with adhesive tape or cap strips (Flow Labs) and stored at -20°C.

Standards

Sendai-virus specific immunoglobulins

Mouse sera having an antibody titre of greater than 1/50,000, previously determined by ELISA were pooled, dispensed in 0.5ml aliquots, lyophilised and stored at -20°C.

Total Immunoglobulins

Commercially prepared mouse immunoglobulin standard containing IgM, IgA and IgG at known concentrations was obtained from Meloy Labs (Springfield, Virginia, USA).

Antisera and Conjugates

Rabbit antiserum to mouse IgG and goat antiserum to mouse IgA were obtained commercially (Nordic Imm., Maidenhead, Berks.; Meloy Labs.). Rabbit antiserum to mouse IgA was a gift from Dr. H. Gregory.

Goat anti-rabbit (GAR) IgG peroxidase-labelled antiserum was obtained from Nordic Imm.

Substrate

O-phenylene diamine (OPD) (0.4mg/ml)/urea peroxide (0.23 mg/ml) was freshly prepared in PBS/Tween.

Enzyme-linked immunosorbent assay (ELISA)

Microelisa plates (Greiner M 129 B, Dynatech Labs Ltd., Billingshurst, Sussex) were coated with purified or crude virus antigen. For total immunoglobulin determination specific goat antiserum to IgA was coated onto the plate. Antigen (250µl) was diluted 1/2000 in coating buffer (0.05M carbonate - bicarbonate buffer pH 9.6) or goat antiserum to mouse IgA diluted 1/500, was placed into each well and left overnight at +4°C. Plates were washed by total immersion in a bath of tap water for 2 minutes (10). A continuous stream of fresh water was supplied to the bath and excess water was allowed to overflow to waste. The

plates were manually agitated for 5 seconds to remove all air bubbles. We have compared this method with standard washing procedures (5) and have found no evidence of non-specific adsorption. Washed plates may be stored at +4°C after through drying.

Transfer of diluted samples onto pre-coated plates was facilitated by the use of the 12-channel micro-pipette. All dilutions were made in PBS/Tween. Duplicate samples were analysed at a single dilution.

A standard curve was prepared by making serial dilutions of the appropriate standard and transferring 200µl volumes into the wells on row 12 of each plate. The plates were then left for two hours at room temperature in a humid box.

After another washing step, 200µl of the appropriate diluted rabbit antiserum was added and incubated for one hour at room temperature in a humidified chamber. After a further washing step GAR IgG enzyme conjugated antiserum (200µl at 1/2000 dilution) was added and again incubated for one hour at room temperature in a humidified chamber.

After a final washing step, 200µl of freshly prepared O-phenylene diamine substrate was added to each well. The resulting colour reaction was allowed to develop in the dark for 45 minutes. The enzymatic activity was then inhibited by the addition of 50µl of 25% sulphuric acid and the plates were agitated for 30 seconds on a microtitre shaker.

Optimum dilutions of all antigens and antisera used were determined using checkerboard type assays on positive and negative samples (5,6).

Reading and Calculations

Optical density determinations at 492nm and calculation of results were carried out automatically using a Titertek Multiskan reader (Flow Labs) interfaced with a Commodore Pet desk computer and printer (Cytek UK Ltd., Old Trafford, Manchester), using a Titertek Multiskan interface (Flow Labs) and an RS 232C serial interface type B (Small Systems Engineering Ltd.) (Figs. 1 and 2). The plate reader is first blanked using substrate/acid solution (250µl). Information concerning experimental details is typed into the computer in response to pre-programmed questions displayed on the visual display unit (VDU). The plate is then read automatically and optical densities and information are printed out.

The computer programme which was designed and written by the authors fits data from the standard curve to a second degree polynomial equation (11). The standard curve computer plot is displayed on the VDU and printed on the results sheet. Optical density versus concentration of standard, or reciprocal of dilution of standard is used in the equation:

$$y = a + b\ (\log x) + c\ (\log x)^2$$

where y = optical density and x = concentration of standard or reciprocal of dilution of standard.

Figure 1 Photograph showing computer, printer and Multiskan spectrophotometer together with interface.

Sample immunoglobulin concentrations are determined directly from the equation. Antibody titres are determined by comparing the optical density of the sample to the standard curve and extrapolating to predict an end point titre (that is the dilution of the sample giving an optical density of 0.1).

RESULTS

To assess the reproducibility and precision of the method different dilutions of the standard serum pool were analysed several times on one microtitration plate for Sendai virus IgG antibody (Table 1). One of these sets of analyses was taken as a standard (Column 6, table 1) and used to calculate the others. End point titres (Table 2) were predicted by the computer using this standard curve (Fig. 3). The results compare

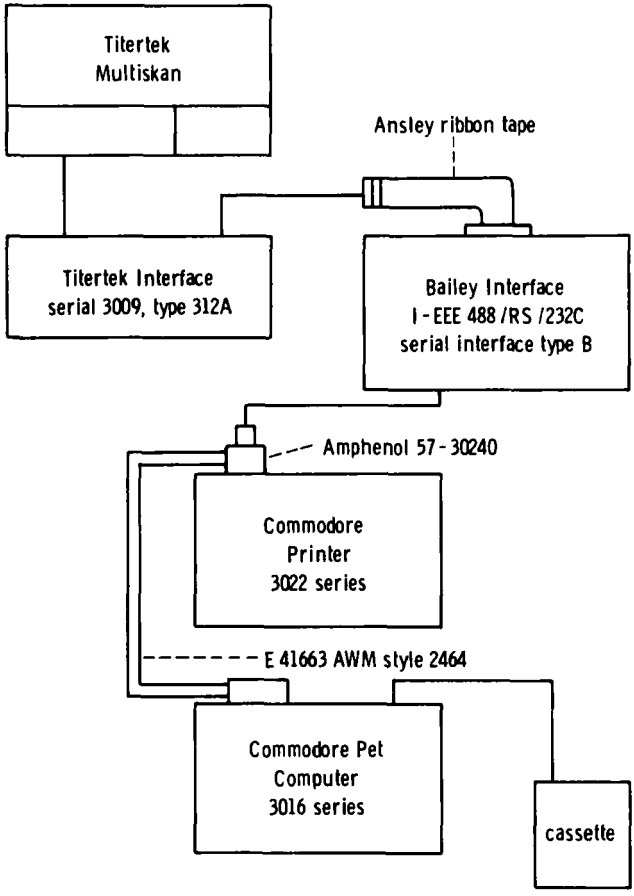

Figure 2 Interfacing of Titertek Multiskan with computer and printer.

favourably with the titre of 1/289,500 determined from the serial dilution of the standard. The coefficient of variation for the predicted titres was 3.7% over the range of optical densities from 0.078 to 1.84.

TABLE 1.

OPTICAL DENSITIES OBTAINED from ELISA ANALYSIS of REPLICATE SERUM STANDARDS for SENDAI VIRUS IgG ANTIBODY.

Serum Dilution	Optical Density				Standards
1/625	1.813	1.819	1.840	1.745	1.788
1/3,125	1.183	1.179	1.200	1.166	1.173
1/15,625	0.592	0.603	0.556	0.547	0.578
1/78,125	0.232	0.240	0.201	0.212	0.263
1/390,625	0.078	0.080	0.087	0.081	0.095
1/1,953,125	0.042	0.030	0.029	0.031	0.044
1/9,765,625	0.013	0.004	0.007	0.012	0.008

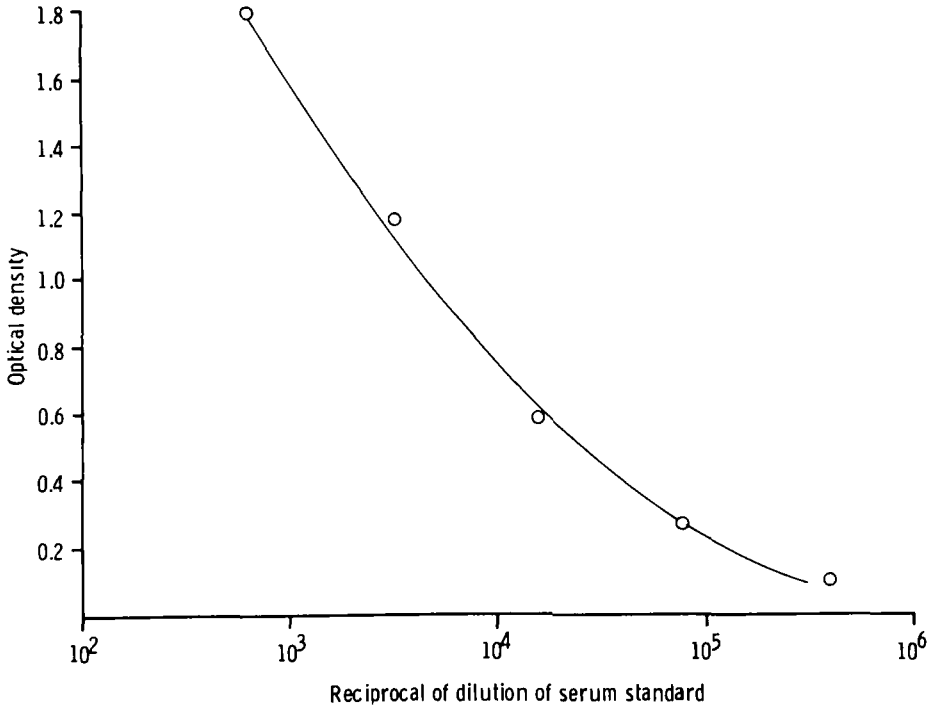

Figure 3 Computer plot of ELISA results for Sendai virus IgG antibodies in the standard serum pool.

TABLE 2.

COMPUTER PREDICTED END POINT TITRES for RESULTS SHOWN in TABLE 1.

Serum Dilution	Predicted End Point Titres			
1/625	295,937	299,750	313,375	255,750
1/3,125	334,687	331,250	350,000	320,000
1/15,625	262,500	275,000	228,198	220,380
1/78,125	234,450	242,265	187,560	203,190
1/390,625	273,525	273,525	312,600	273,525

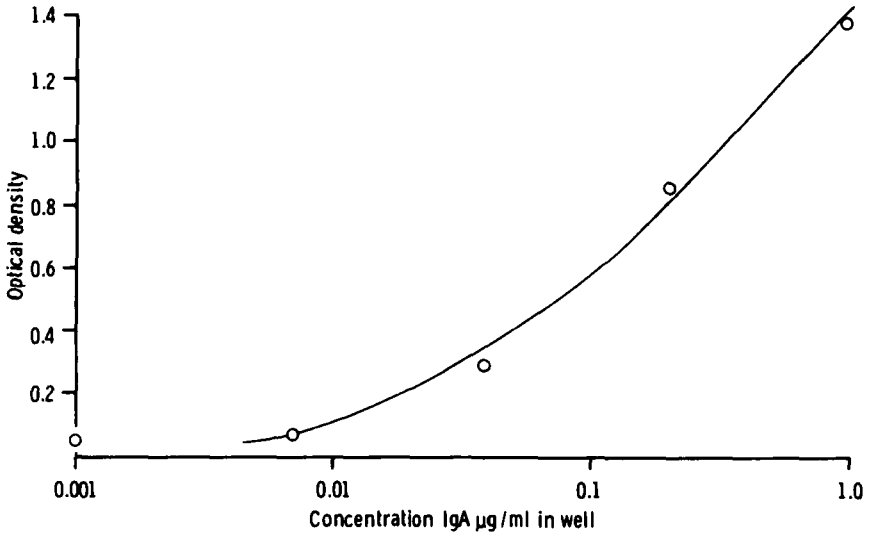

Figure 4 Computer plot of ELISA results for IgA standard curve.

Replicate dilutions of the IgA standard were made and analysed for IgA content in the same manner as described above. Known concentrations of the same standard were used to construct a standard curve (Fig. 4). The results are shown in Tables 3 and 4 for optical densities and calculated IgA concentrations respectively. The coefficient of variation for the calculated immunoglobulin concentrations was found to be 7.0% over the range of optical densities from 0.052 to 1.41.

DISCUSSION

Reading, calculation and tabulation of results from one plate takes less than two minutes to complete. Using a single dilution of sample stored and analysed as described it is

TABLE 3.

OPTICAL DENSITIES OBTAINED from ELISA ANALYSIS of REPLICATE SAMPLES of KNOWN IgA CONCENTRATIONS.

IgA Conc^{n} µg/ml	Optical Density				Standards
0.96	1.33	1.41	1.260	1.396	1.384
0.19	0.825	0.91	0.830	0.865	0.875
0.038	0.329	0.258	0.293	0.274	0.271
0.007	0.052	0.065	0.075	0.062	0.072
0.001	0.040	0.029	0.037	0.052	0.031
0.0002	0.000	0.000	0.000	0.000	0.000

TABLE 4.

COMPUTED IgA CONCENTRATIONS from RESULTS SHOWN in TABLE 3.

IgA Conc^{n} µg/ml	Calculated IgA Concentration (µg/ml)			
0.96	0.785	0.939	0.668	0.910
0.19	0.217	0.276	0.221	0.244
0.038	0.037	0.026	0.031	0.028
0.007	0.005	0.006	0.007	0.006
0.001	0.004	< 0.001	0.004	0.005

possible for one person to process 14 plates (1200 samples) in one day. The system was designed for use in a high throughput screen but greater precision would be obtained by using replicate points on the standard curve. It should be appreciated that by extrapolating from a pooled serum standard we are assuming that the curves are the same for all sera and secretions. This method does have the advantage that only one dilution of the sample is required yet results are expressed in a linear form covering a large range of values as opposed to optical density measurement alone (12).

Sensitivity of the assay for mouse IgA is of the order of 10ng/ml. The use of a second antibody which is enzyme conjugated (goat anti rabbit peroxidase) has given us greater freedom to determine different immunoglobulin types against any antigen without the need for time consuming conjugation of the specific antisera.

The reader-computer linkage can be used not only for a large variety of ELISA assays but is adaptable for other assays (minimal inhibitory concentrations (MIC), interferon).

The ELISA technique described is used routinely in our laboratory and represents a logical progression towards a new generation of computerised ELISA systems.

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