JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1985, p. 92–96
0095-1137/85/010092-05$02.00/0

Vol. 21, No. 1

Comparison of Enzyme-Linked Immunosorbent Assay with Enzyme-Linked Fluorescence Assay with Automated Readers for Detection of Rubella Virus Antibody and Herpes Simplex Virus

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Received 25 June 1984/Accepted 14 September 1984

The enzyme-linked immunosorbent assay (ELISA) was compared with the enzyme-linked fluorescence assay (ELFA) for the detection of rubella antibody and herpes simplex virus antigen. Test parameters, specimens, antigen or antibody, and conjugates for the two types of assays were identical except that *p*-nitrophenyl phosphate was used as the substrate for the ELISA and 4-methylumbelliferyl phosphate was used as the substrate for ELFA. Automated readers were used for both assays. Antibody titers and sensitivity of antigen detection were quite similar for ELISA and ELFA. ELFA for rubella antibody, however, could be conducted with less antigen or shorter substrate incubation time (5 min for ELFA versus 30 min for ELISA). For herpes simplex virus antigen detection, ELFA could also be read after a shorter substrate incubation time (15 min for ELFA versus 30 min for ELISA). Clear polystyrene microtiter plates routinely used for ELISA could be used for ELFA, but clear polyvinyl chloride plates had high background fluorescence. Black polystyrene and polyvinyl chloride plates gave lower background fluorescence than did clear plates. ELFA is of particular value as a substitute for ELISAs in which long substrate incubations are required or antigens of only low titer are available.

The enzyme-linked immunosorbent assay (ELISA) can be quantitated by using the enzyme alkaline phosphatase and a colorigenic substrate, *p*-nitrophenyl phosphate (PNP). A fluorogenic substrate, 4-methylumbelliferyl phosphate (4MUP), can be used with the same enzyme, and the assay is then called the enzyme-linked fluorescence assay (ELFA). The alkaline phosphatase label is detected at a concentration of 10^{-5} M by PNP, which it converts to the yellow *p*-nitrophenol, and at a concentration of 10^{-9} M by 4MUP, which it converts to the fluorescent 4-methylumbelliferone (5). The greater activity of the fluorescence substrate suggested that its use might substantially increase the sensitivity of the enzyme assay. Reports have appeared indicating that the fluorescence substrates offer greater sensitivity (1–4, 8, 11) or at least faster tests (10). Use of ELFA, however, has been limited by the lack of convenient, rapid methods for evaluating the tests. Recently, a new instrument, the MicroFLUOR reader (Dynatech Laboratories, Inc., Alexandria, Va.), which automatically measures fluorescence in a 96-well microtiter, has become available. We have used this instrument and a Dynatech MicroELISA reader (model MR580) to compare the ELISA and the ELFA.

Flat-bottomed clear polystyrene (PS) microtiter plates were used in the ELISAs. For the ELFAs, clear PS and polyvinyl chloride (PVC) microtiter plates were compared with black PS and PVC plates specially produced for use with the MicroFLUOR reader.

MATERIALS AND METHODS

MicroELISA autoreader. The MR580 MicroELISA reader (Dynatech Laboratories) is a dual wavelength, through-the-plate reader. The vertical beam from a halogen cycle lamp is projected through the sample and two filters to a silicon photovoltaic cell. The instrument is equipped with four narrow-band pass filters, 410, 450, 490, and 570 nm. Artifacts in the plastic, fingerprints, etc., can be compensated for by using two different filters, one giving the wavelength optimal for reading the substrate color and one reference filter with a different wavelength. Blanking is done automatically on well A1 or manually on any other well selected by the user. This spectrophotometer automatically reads a 96-well plate in ca. 1 min and prints the results in optical density (OD) units with a built-in alphanumeric printer.

MicroFLUOR reader. The MicroFLUOR reader is designed to measure relative fluorescence emitted by samples in a microtiter plate well and is equipped with a light source and filters to optimize readings of 4-methylumbelliferon. A schematic diagram of the instrument is presented in Fig. 1. A low-pressure mercury vapor lamp projects a beam of light through a lens to a 365-nm broad-band excitation filter. The excited beam is directed into the sample well by means of a mirror causing the substrate-enzyme complex to fluoresce. The emitted light passes upward and is reflected by a second mirror through two emission filters, one 450-nm narrow-band interference filter and one 400-nm UV blocking, non-fluorescent filter, and through another lens into the photomultiplier tube and the amplifier. The data, in relative fluorescence units (FU), are recorded on an external thermal printer. A 96-well microtiter plate can be read in 1 min.

Solid-phase carriers. Dynatech PS Immulon I (lot 112079) microtiter plates are used routinely in our laboratory for rubella antibody tests by ELISA. Costar PS EIA plates (lot 777) are used for the biotin-streptavidin ELISA for herpes simplex virus type 1 (HSV-1) and HSV-2 antigen. To determine whether these plates could be used with the MicroFLUOR reader for ELFAs, they were compared with clear PVC plates and with black PS and PVC plates (Mi-

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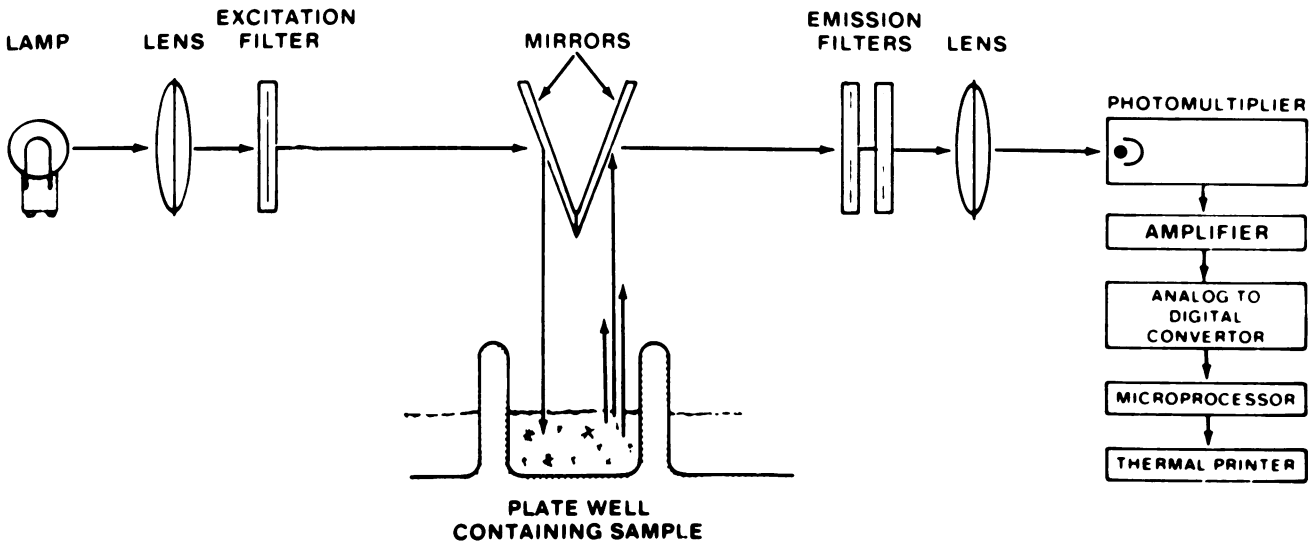

FIG. 1. Diagram of MicroFLUOR reader operation.

croFLUOR B plates; Dynatech Laboratories) in an ELFA for human gamma globulin. Pentex human gamma globulin (100 ng/ml) was adsorbed (200 μl per well) to the wells of the plate at 4°C overnight. Plates were washed three times with phosphate-buffered saline containing 0.05% Tween 20 and tapped dry. Conjugate, alkaline phosphatase-labeled anti-human immunoglobulin G (no. 5615; Microbiological Associates), was added. After 1 h at 37°C, the plates were again washed and 100 μl of 4MUP (25 μg/ml; Sigma Chemical Co., St. Louis, Mo.) in diethanolamine buffer (pH 9.8) was added to each well. Plates were read on the MicroFLUOR reader at 5-min intervals for 1 h. From these comparisons it was determined that the clear PS plates could be used for both ELISA and ELFA. Adjustment for background fluorescence inherent to the plates was made by blanking the reader on a well with only antigen and substrate added. This reading was automatically subtracted from all subsequent readings on that plate.

Rubella antibody assay. (i) ELISA. An ELISA for rubella antibody previously described (9) was used to compare the ELISA and ELFA systems. Briefly, sucrose-gradient-purified rubella virus (HPV77) in carbonate buffer (pH 9.6) was adsorbed to Dynatech Immulon plates at 4°C overnight. Positive and negative sera in twofold dilutions from 1:8 to 1:8,192 in phosphate-buffered saline with 1% bovine serum albumin and 0.05% EDTA were added to the sensitized wells. After 90 min of incubation at 37°C, the plates were washed and conjugate was added. After 1 h at 37°C, unbound conjugate was washed away and 100 μl of PNP (1 mg/ml of substrate 104-105; Sigma) in diethanolamine buffer was added. The plates were incubated at 37°C for 30 min, and the color change was measured with an MR580 Micro-ELISA reader.

(ii) ELFA. The same procedure was used for ELFA, except that 4MUP (25 μg/ml) was used (100 μl per well) as substrate. Readings were made every 5 min for 1 h on a MicroFLUOR reader. A block of 100 samples, previously tested by hemagglutination inhibition (HI) and selected for evaluating rubella antibody assay methods, were tested by the ELISA and the ELFA (9). The test panel, consisting of sera from laboratory personnel and patients requiring rubella serology, was heavily weighted with negative and low-positive samples as indicated by HI titers.

A similar assay with rubella antigen dilutions from 1:1,500 to 1:8,000 served to determine the optimum antigen concentration relative to the substrate incubation time for the ELFA.

HSV-1 and HSV-2 antigen detection. (i) ELISA. A "capture" ELISA for HSV-1 and HSV-2 antigens, which involved the use of the biotin-streptavidin technique previously described (7), was also assessed by the ELFA. For the ELISA, rabbit anti-HSV-1 and anti-HSV-2 globulins were bound to microtiter plate wells by incubation overnight at 4°C. The plates were washed three times and tapped dry. Stock virus or clinical specimen was added, and the plates were incubated at 37°C in 5% CO_2 for 2 h. The plates were washed three times, and biotin-linked antibody was added. After incubation for 1 h at 37°C, the plates were again washed. Streptavidin linked to alkaline phosphatase was added, and the plates were incubated at 37°C for 30 min. Unbound conjugate was washed away, PNP was added, and a color change was recorded after 30 min of incubation at 37°C.

(ii) ELFA. The same procedure as for the ELISA was followed, except that the substrate 4MUP (25 μg/ml) was used and fluorescence was measured every 5 min for 1 h.

Twenty samples collected on vaginal swabs and found to be positive by tissue culture were screened for antigen in duplicate or triplicate by ELISA and by ELFA. Replicate readings of viral collection medium and buffer were used as negative controls. A known dilution of stock virus was the positive control. Eight replicates of viral control medium were averaged, and the standard deviation for the test on that day was determined. Samples were considered positive if their average absorbance value was equal to or above the predicted limit of absorbance as determined by the 95% prediction-bound formula (7).

RESULTS

The comparison of five plates in an ELFA for detection of human gamma globulin is presented in Fig. 2. Background fluorescence was lower, between 600 and 800 FU, for the two MicroFLUOR B plates. For the two clear PS plates the background was somewhat higher, between 1,000 and 1,300 FU, but for the clear PVC plates it was very high, 1,800 to

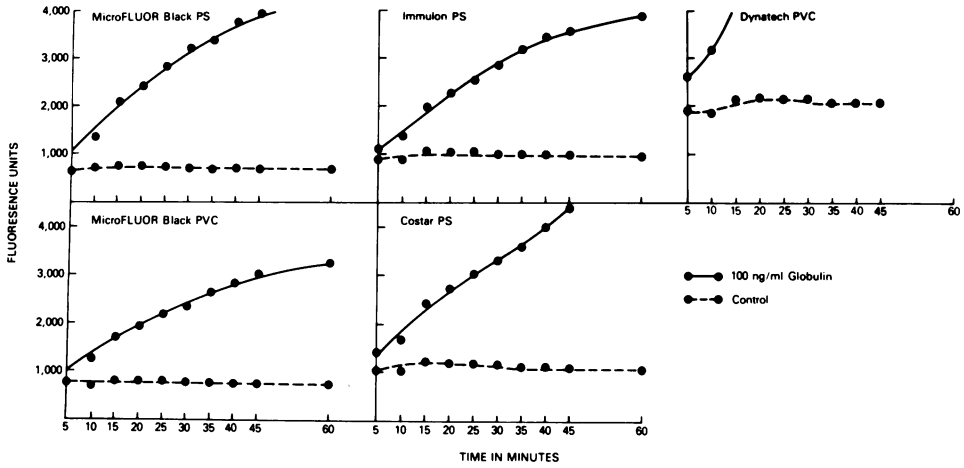

FIG. 2. Comparison of five microtiter plates in ELFA for detection of human gamma globulin.

2,100 FU. Since the background fluorescence for the two clear PS plates could be removed by the blanking mechanism of the MicroFLUOR reader, these plates were selected for use in comparisons of the ELISA and ELFA.

A summary of the results of a comparison of ELISA and ELFA with HI assays for the measurement of rubella antibody is presented in Table 1. Samples with HI titers of >8 were all found to be positive (>128) by both ELISA and ELFA. Among the samples with HI titers of <8, ca. 40% had titers of >8 by ELISA and ELFA. Three samples with titers of <8 by ELISA had titers of >8 by ELFA.

A comparison of the titers of the 100 sera tested for rubella antibody by ELISA and ELFA is shown in Fig. 3. When two standard deviations above the mean FU or OD for the negative control sera is used as endpoint, titers of these sera obtained by ELISA and ELFA are not appreciably different.

A comparison of the ELISA and ELFA for detecting HSV-1 and HSV-2 antigens by using a biotin-streptavidin system was conducted. Results of this test are presented in Table 2. Of the 20 samples positive by tissue culture, 19 were positive for at least one of the HSV antigens by both ELISA and ELFA. Sample 18 was negative by both assays. With the fluorescence substrate, readings could be made after a 15-min incubation easily, whereas at least 30 min were required for the colorigenic substrate. The sensitivity was not increased by the fluorescence substrate, even with prolonged incubation, although the actual numbers were larger.

The rubella antibody ELFA read at 5-min intervals after substrate addition indicated that the ELISA parameters for rubella antibody should be adjusted if the fluoresence substrate was to be used, since after 10 min of incubation the signal was beyond the capacity of the instrument to measure directly. A comparison of ELISA and ELFA readings made in tests with increasing antigen dilution is presented in Table 3. The optimal antigen dilution for ELISA was 1/1,500 for 30 min of substrate incubation, 1/2,000 for 45 min, and 1/3,000 for 60 min. With this antigen, a dilution of 1/8,000 would be sufficient for a 30-min incubation with 4MUP, and a dilution of 1/6,000, or even 1/8,000, would be adequate for a 10-min substrate exposure. The 5-min incubation tended to show more variation, probably because of the great speed of the reaction at this point.

The capacity of ELFA to function with much less antigen was shown in a comparison of ELFA and ELISA results by using a rubella antigen rejected for laboratory use because the slope of the ELISA curve for the positive control was too shallow (Fig. 4). When the test was repeated with ELFA, the signal was substantially increased. The endpoint remained the same, but there was a 10-fold difference in the OD and FU readings of dilutions with antibody.

A comparison of ELFA readings for blank wells (rubella antigen and substrate only), negative serum, and positive

TABLE 1. Comparison of ELISA and ELFA with HI assays for the measurement of rubella antibody in 100 serum samples

HI titer (no. of samples)	ELISA[a]						ELFA[b]					
	No. of samples with titers of:						No. of samples with titers of:					
	<8	8	16	32	64	≥128	<8	8	16	32	64	≥128
<8 (50)	31	8	2	1	2	6	29	8	5	1	1	6
8 (3)						3						3
≥16 (47)						47						47

[a] ELISA readings made after 30 min.
[b] ELFA readings made after 10 min.

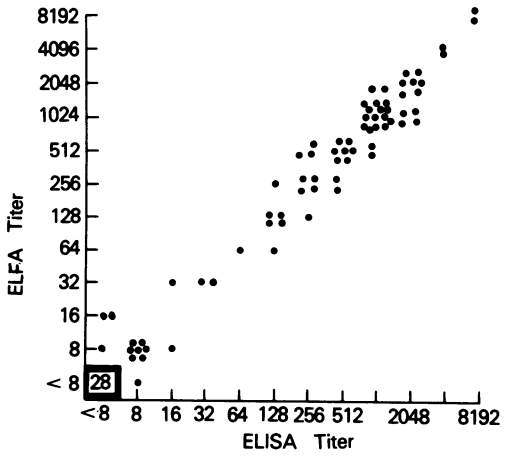

FIG. 3. Correlation between rubella antibody titers from ELISA and ELFA.

TABLE 2. Comparison of ELISA and ELFA results in antigen capture assay for HSV

Sample no.	ELISA results (OD × 10^3)[a] for:		ELFA results (FU)[b] for:	
	HSV-1	HSV-2	HSV-1	HSV-2
1	105	426	1,350	2,253
2	068	244	1,257	1,817
3	198	1,375	1,773	—
4	—[c]	—	—	—
5	750	—	3,346	—
6	061	294	1,223	1,957
7	—	—	—	—
8	172	581	1,683	3,070
9	—	1,261	—	—
10	—	708	—	3,071
11	—	—	—	—
12	—	—	—	—
13	68	402	1,189	2,223
14	17	173	1,104	1,587
15	—	—	—	—
16	19	126	1,189	1,529
17	—	—	—	—
18	0	15	1,046	1,097
19	666	313	3,220	1,918
20	007	87	1,153	1,431
Blank	7 ± 2	15 ± 2	1,059 ± 50	1,237 ± 100

[a] Read after 30 min of substrate incubation.
[b] Read after 15 min of substrate incubation.
[c] —, Greater than OD 1,500 or FU 4,094. Samples with an OD or FU greater than the average of the control + 2 SDs were considered positive.

serum with time is presented in Table 4. Over a period of 60 min, readings of the blank wells remained essentially unchanged. By use of the same plate, sera negative for rubella antibody diluted 1:8 showed slowly increasing FU readings with time. Positive sera gave strongly positive readings as early as 5 min after substrate addition. From these observations, we concluded that positive samples should be evaluated relative to a negative serum rather than to a blank well. By using a negative serum blank, we achieved a very close correlation between the ELISA and ELFA endpoints (Fig. 3).

We also found that an assay could be evaluated first by a colorigenic substrate (PNP) and, if the reaction was not stopped with NaOH, substrate could be removed by washing and a reading could be made with a fluorogenic substrate, 4MUP, without appreciable loss in titer. Readings with the two substrates could be made in either order. The same relative differences between substrates were evident whether they were tested on separate plates or on the same plate.

DISCUSSION

The availability of ELISAs (colorigenic) and ELFAs (fluorescent) read on automated readers has made it practical to compare the two assays for sensitivity and speed. We evaluated the two methods for both antibody and antigen detection. Repeated readings with time in duplicate assays differing only in the substrate used showed that the ELFA was much more rapid than the ELISA.

Similar endpoint titers were obtained with ELISA and ELFA if each substrate was incubated for an appropriate time, indicating, as stated previously (5, 6, 10), that sensi-

TABLE 3. Comparison of ELISA and ELFA for rubella antibody: determination of optimum concentration of rubella antigen

Time (min) of substrate incubation for:	Titer[a] at rubella antigen dilutions of:											
	1/1,500		1/2,000		1/3,000		1/4,000		1/6,000		1/8,000	
	+[b]	−	+	−	+	−	+	−	+	−	+	−
ELISA												
30	726	15	428	14	399	14	252	12	153	9	142	12
45	1,234	25	716	26	666	30	445	27	249	19	232	26
60	—[c]	38	961	39	893	44	602	37	324	26	312	30
ELFA												
5	1,464	96	966	38	849	88	587	48	364	27	280	1
10	2,490	147	1,811	78	1,599	151	1,146	114	708	104	501	3
15	—	166	2,693	93	2,593	222	1,840	160	1,189	278	843	129
20	—	221	—	158	—	246	2,365	385	1,438	283	1,064	166
25	—		—		—	269	>2,458	212	1,763	314	1,323	345
30	—	289	—	218	—	335	>3,400	262	2,051	472	1,749	345
45	—		—		—		—		>2,535		2,514	459

[a] Average of eight readings were taken for each sample. ELISA titers were measured in OD units × 10^3; ELFA titers were measured in FU.
[b] 1:128 Dilution of either positive (+) or negative (−) serum was used.
[c] —, Greater than OD 1,500 for ELISA; greater than FU 4,094 for ELFA.

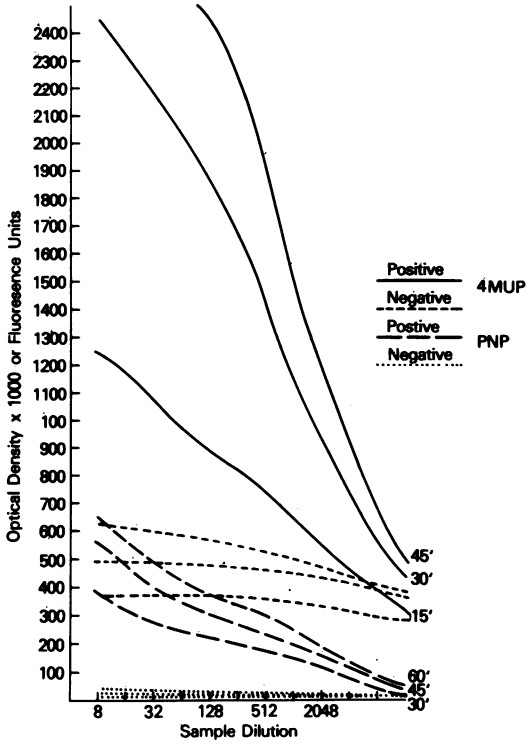

FIG. 4. Comparison of PNP and 4MUP in rubella antibody titration.

tivity of the ELISA rests not with the detection of the label but rather with the primary antigen-antibody reaction. Future use of highly avid monoclonal antibodies may overcome this problem and allow the development of more sensitive assays.

The increased reactivity of the ELFA substrate facilitates the evaluation of assays in which very little antigen (such as immunoglobulin E, HSV, etc.) is available, since substrate incubation of 1 h or less instead of overnight is possible. On the basis of this finding, miniaturization of ELFA has been reported by Labrousse et al. (6). Reactions barely detectable with a spectrophotometer are clearly detectable with the fluorometer. Thus, within a fixed time frame, ELFA is more sensitive than ELISA.

The microFLUOR reader provides convenient and rapid (96 well per min) evaluation of fluorescence assays in the microtiter plate format. The fluorescence substrate is inexpensive, safe, and stable, has a long shelf life, and can be used as an alternative to PNP, giving comparable sensitivity with a shorter substrate incubation time.

The MicroFLUOR B plates designed to reduce background and enhance fluorescence were shown to have lower background fluorescence readings than the clear plates routinely used for ELISA. Most plastics and body fluids have a certain amount of inherent fluorescence, and this must be accounted for with controls in ELFA.

TABLE 4. ELFA for the measurement of rubella antibody: comparison of fluorescence readings for blanks, negative sera, and positive sera with time[a]

Time (min)	Fluorescence reading (FU) for:								
	Blanks			Negative sera			Positive standards		
	1	2	3	1	2	3	1	2	3
5	386	375	372	70	109	82	421	415	440
10	371	370	355	205	260	198	966	960	970
15	383	380	358	375	428	302	1,489	1,510	1,514
20	376	367	354	502	556	475	2,025	2,029	2,051
30	360	359	350	731	801	729	2,780	2,800	2,832
60	359	360	333	1,364	1,413	1,321	—[b]	—	—

[a] Blank wells were incubated with antigen and substrate only. Negative serum was tested at a 1:8 dilution. Positive serum was tested at a 1:4,096 dilution because after 10 min, lower dilutions gave FU readings of >4,094, which is the upper limit of the instrument. Readings were made on three plates for each group.

[b] —, Greater than FU 4,094.

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