

Evaluation of Performance Parameters of a Membrane-Based Dot Immunoassay for Meningococcal Polysaccharide

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Increasingly, membrane-based enzyme immunoassays are being developed as the preferred solid-phase enzyme immunoassay format. We describe the rate kinetics of a polyvinylidene difluoride membrane-based dot immunoassay for meningococcal group A polysaccharide. Antigen detection sensitivity decreased logarithmically with linear decreases in incubation time. The sensitivity of a 30-min assay (5-min incubation steps) was increased to nearly the level of the standard assay (1-h incubation steps) by increasing the concentration of assay reagents fourfold. These results support the idea that existing microtiter plate assays can be transferred to rapid dot immunoassay formats with little or no loss of sensitivity.

Enzyme immunoassays (EIAs) are commonly used for rapid diagnosis of infectious disease by both antigen and antibody detection. Solid-phase formats include microtiter plate and membrane-based assays (16). Successful tests have been developed for viruses (4, 8, 13) as well as bacteria and other agents (1, 9, 10, 15). The sensitivity and specificity of EIAs have been shown to be comparable to those of fluorescent-antibody (4), radioimmunoassay (12), and hemagglutination inhibition (2) tests.

Optimizing assays for rapid diagnostic performance is largely done on a trial-and-error basis with few general principles. Few existing microtiter plate EIAs have been transferred to membrane-based formats. In this report, we describe the rate kinetics of a membrane-based dot immunoassay (dot IA), using a novel membrane, polyvinylidene difluoride (PVDF; Immobilon Transfer; Millipore Corp., Bedford, Mass.), on which a monoclonal antibody was immobilized for antigen capture. The model used was an assay for *Neisseria meningitidis* group A polysaccharide, a system based on a previously reported microtiter plate EIA (9). The sensitivity of the dot IA was compared with that of the analogous microtiter plate EIA.

The feasibility of detecting meningococcal polysaccharides in cerebrospinal fluid and the utility of such EIAs for diagnosis have been demonstrated previously (1, 9). The test described here is an antigen capture assay for the detection of *N. meningitidis* group A polysaccharide. A monoclonal antibody was immobilized on a PVDF membrane, which is hydrophobic and inert. Meningococcal polysaccharide group A was captured and subsequently detected with a second polyclonal antibody. *N. meningitidis* polysaccharides and antibodies are well characterized and available commercially in purified form. This ensured a well-defined system in which to evaluate dot IA performance.

MATERIALS AND METHODS

Reagents. *N. meningitidis* polysaccharide groups A and C were obtained from the American Type Culture Collection, Rockville, Md.

An immunoglobulin M mouse monoclonal antibody (no. 1622) to meningococcal group A polysaccharide was generously provided by H. Feindt, Becton Dickinson Advanced Diagnostics, Baltimore, Md. Hyperimmune rabbit sera to *N.*

meningitidis polysaccharide group A was purchased from Difco Laboratories, Detroit, Mich.

EIA. Optimal antibody and conjugate dilutions were first determined by block titrations. Assay negative controls were normal rabbit sera and *N. meningitidis* group C polysaccharide at concentrations equivalent to those of the material tested.

An antigen capture microtiter plate-based EIA was prepared essentially as described elsewhere (11). Briefly, Immunolon I microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.) were coated with purified anti-*N. meningitidis* group A mouse monoclonal antibody 1622. Plates were then incubated with successive 10-fold concentrations, from 10 pg/ml to 100 ng/ml, of *N. meningitidis* polysaccharide antigen for 1 h at 37°C. After plates were washed with phosphate-buffered saline (PBS)-Tween 20, anti-*N. meningitidis* group A rabbit serum was added and incubation was continued at 37°C for 1 h. Plates were washed as before, and pretitrated horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) was added; incubation was continued at 37°C for 1 h. 2,2'-Azino-di(3-ethylbenzthiazoline sulfonate) was used as substrate, after a final wash.

Plates were optically scanned with a Microelisa Auto Reader. Controls included sera from nonimmunized rabbits and meningococcal group C polysaccharide as negative antigen. Positivity was defined as color development equal to or greater than the mean plus 2 standard deviations of the negative antigen control.

Dot IA. Assays were performed on PVDF membranes. A 1- μ g portion of anti-meningococcal group A monoclonal antibody 1622 in 100 μ l of sterile distilled water was spot filtered through the membrane. Treated membranes were then allowed to dry at room temperature for at least 1 h. These membranes were wetted in a solution of PBS with 0.1% Tween 20, and then nonspecific binding sites were blocked by incubating the membrane in a solution of 5% nonfat dry milk in PBS-0.01% Tween 20 for 1 h at room temperature. Prepared membranes could be dried and stored at 4°C, at this point, for later use. Membranes must be rewetted before use by immersing them in a solution of PBS-0.1% Tween 20 prior to initiating the assay.

Assays were performed by dipping PVDF strips, spotted with monoclonal antibody, into Eppendorf centrifuge tubes containing 100 μ l of meningococcal polysaccharide in a

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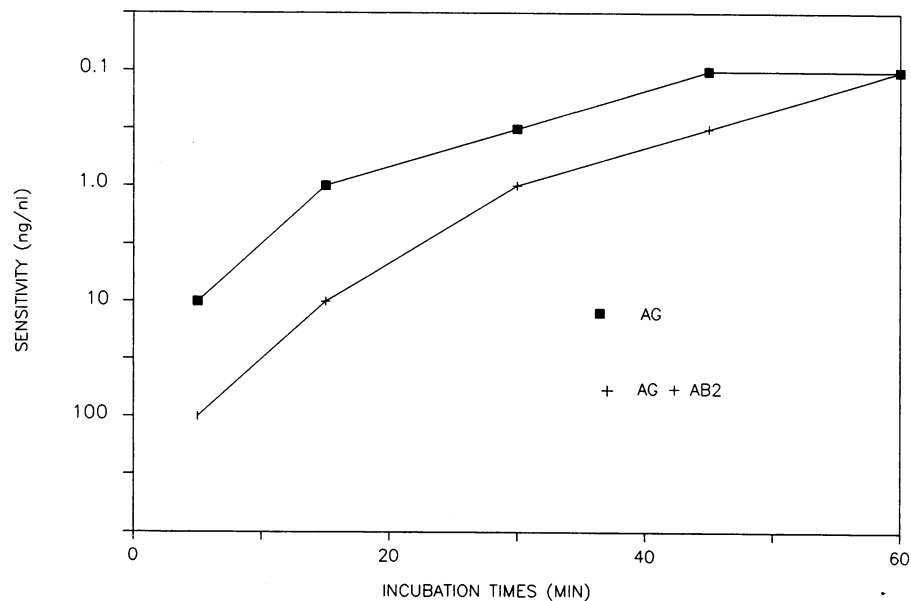


FIG. 1. Relationship of dot IA sensitivity to incubation time. Values are for various incubation times for the antigen (AG) step and for both the antigen and second-antibody (AG + AB2) steps. All assays were incubated at 37°C. The final enzyme-conjugated antibody step was a constant 60 min for all assays. Each point represents a concordant value for the assay done in triplicate.

diluent of PBS–0.01% Tween 20–1% nonfat dry milk. Concentrations of antigen ranged from 10 pg/ml to 100 ng/ml in 10-fold increments. After this first incubation, membrane strips were washed in PBS–0.01% Tween 20 for 1 min. The second incubation was with anti-meningococcal group A rabbit serum, and strips were washed again. PVDF strips were incubated with peroxidase-conjugated anti-rabbit immunoglobulin (Kirkegaard and Perry Laboratories) for 1 h at 37°C. PVDF strips were then washed as before and placed in a final wash of PBS for 1 min. After this, assay strips were placed in a substrate solution of 4-chloro-1-naphthol and color development was allowed to occur for approximately 30 min at 37°C. All assays were repeated at least three times. A negative sample yielded essentially no color on the membrane; a sample yielding a detectable (by eye) color was considered positive.

Length of incubation, at a constant temperature of 37°C, was varied for antigen in one group of assays and for both antigen and second antibody (rabbit) in another set of assays. Incubation temperature, for 1-h incubation periods, was varied for both antigen and second antibody in another experiment. Incubation with conjugate was constant for 1 h at 37°C for both of the above experiments. In a final experiment, incubation times for all steps were reduced to 5 min (incubation in substrate was for 15 min) and performed at room temperature. To increase sensitivity in this assay, second antibody (rabbit serum) and enzyme conjugate concentrations were increased two- and fourfold, respectively. Antigen concentrations were as described above.

RESULTS

The dot IA protocol was shown to detect antigen with a sensitivity of 100 pg/ml when incubation periods were 1 h at 37°C (standard assay). The sensitivity of a similar microtiter plate EIA protocol was equivalent. Dot IA sensitivity was increased to 10 pg/ml when all incubations were performed at 42°C for 1 h. Optimal capture antibody (no. 1622) concentration, on the membrane, was found to be 15 µg/cm². Dot

IA results were highly consistent through all test replicates. Maximal color saturation was reached at an antigen concentration of 100 ng/ml. Background in the standard dot IA format was very low; concentrations of 10 to 100 ng of negative control antigen per ml produced no color development on the membrane.

The correlation between sensitivity and time of incubation for antigen alone and antigen as well as second antibody is illustrated in Fig. 1. Sensitivity decreased fivefold with a decrease in antigen incubation time to 30 min. From there, the drop in sensitivity with time was greater. When incubation with antigen was reduced to 5 min at 37°C, the sensitivity of the assay was only 10 ng/ml (Table 1). The sensitivity decreased at a logarithmic rate for a decrease in both antigen and second-antibody incubation times. Antigen detection dropped to 100 ng/ml for an assay with 5-min incubation times for both antigen and second antibody. A rapid assay (5-min incubation times) was therefore 1,000 times less sensitive than the standard assay.

TABLE 1. Dot IA sensitivity for antigen detection when time and temperature were varied

Incubation time and temp	Sensitivity (ng/ml)	
	Ag ^a	Ag + Ab2 ^b
Time (min)		
60	0.1	0.1
45	0.1	0.5
30	0.5	1.0
15	1.0	10.0
5	10.0	100.0
Temp (°C)		
42		0.01
37		0.1
25		0.1
18		0.1

^a Antigen (Ag) incubation step only.

^b Antigen and second-antibody (Ab2) (rabbit) steps.

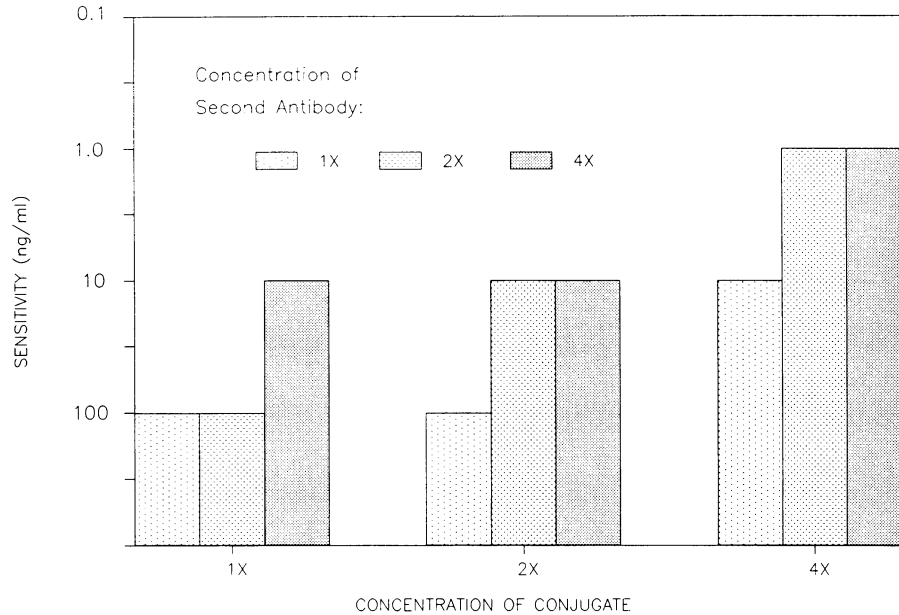


FIG. 2. Effect of concentration on sensitivity. Incubations in this assay were carried out for 5 min at room temperature for all steps (total assay time, including color development in substrate, was 30 min). A 1× value is for a concentration of reagent equal to that used in 60-min-incubation step assays. Second-antibody and enzyme-conjugated antibody concentrations were increased twofold (2×) and fourfold (4×). Each bar represents a concordant value for the assay done in triplicate.

Assays could be performed at a wide range of temperatures down to room temperature with no decrement in sensitivity (Table 1). An improvement in antigen detection to a sensitivity of 10 pg/ml was obtained, however, by increasing incubation temperatures to 42°C.

Dot IA performance was evaluated for a rapid format with incubation times of 5 min at room temperature for all reagents (Fig. 2). Total assay time for this test was 30 min, including color development in substrate (15 min). Antigen detection sensitivity was 100 ng/ml in this assay, using reagent concentrations equivalent to those of the standard assay. Logarithmic increases in sensitivity were achieved by increasing the concentrations of both second antibody and enzyme conjugate two- and fourfold, respectively (Table 2). A fourfold increase in reagent concentration yielded an antigen detection level of 1 ng/ml, which was a 100-fold increase. An increase in reagent concentration beyond a fourfold limit brought a concomitant increase in background.

DISCUSSION

The influence of time or temperature or both on assay sensitivity has been described for a variety of microtiter plate and nitrocellulose membrane EIA formats (17). Similar

TABLE 2. Effect of increasing concentration of assay reagents on sensitivity of antigen detection for 5-min incubations at room temperature

Concn of second antibody ^a	Sensitivity (ng/ml) at given concn of enzyme-conjugated antibody ^a		
	1×	2×	4×
1×	100	100	10
2×	100	10	1
4×	10	10	1

^a From standard assay.

results relating to the Law of Mass Action have been reported, with sensitivities in the nanogram- to picogram-milliliter range (1, 14). Our data confirm these observations for a membrane-based EIA, using a hydrophobic inert substrate onto which a monoclonal capture antibody is immobilized. Use of PVDF allowed the binding of a high concentration of specific protein. This is due to a strong ionic interaction between proteins and the hydrophobic fibers of which the membrane is made (3, 6, 7). This results in higher signal/noise ratios and thus the ability to better discriminate between positive and negative samples. Binding a high concentration of capture antibody to the membrane eliminated this component as a rate limiter for antigen detection.

It was found that sensitivity decreased logarithmically with a decrease in incubation time for both antigen and second antibody. Assay performance was not as affected by changes in incubation temperature. An assay with 30-min incubation times (total assay time, 2.5 h) could detect 1 ng of antigen per ml. While this level was 10-fold lower than the standard assay, it would still be acceptable for clinical diagnosis (9, 11). From 30 min to 5 min, the sensitivity of the dot IA decreased 100-fold to 100 ng/ml. This is contrary to previously published reports for microtiter plate-based EIAs for other meningococcal polysaccharides, in which sensitivity did not decrease as greatly (17). The discrepancy in these data may be due to the increased surface area of the membrane, intensifying the effect of a reduction in incubation time.

Assay sensitivity was restored by increasing the concentration of reagents. To develop a feasible rapid assay, second antibody and enzyme conjugate were both increased fourfold over standard assay concentrations. Antigen detection was increased to 1 ng/ml for a rapid assay format with incubations at room temperature for 5 min. Therefore, a linear increase in reagent concentration increased assay sensitivity exponentially. Further incremental increases in reagent concentration and incubation time resulted in anti-

gen detection limits of 100 pg/ml. Total assay times were still under 1 h.

Our data indicate that dot IA sensitivity is equivalent to microtiter plate sensitivity. Standard dot IAs can be improved to be used in a rapid diagnostic format. Immunoassay rate kinetics are largely influenced by the diffusion rate of the assay reagents (14). An increase in reagent concentration would increase this rate. Dot IA performance, in a rapid format, was improved to nearly the sensitivity of the standard assay when second-antibody and conjugate concentrations were both increased. The large surface area of a membrane-based system may enhance the effect of incubation time or reagent concentration or both on test sensitivity.

The dot IA protocol, using PVDF membranes, has been found to work well as a general immunoassay method for other antigen-antibody systems (5). Existing microtiter EIAs may be transferred to a dot IA format, and, with a fourfold or greater increase in reagent concentration, more rapid and logistically simpler tests may be developed.

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