Rapid and Inexpensive Microcapillary Immunodiffusion Assay Technique

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A simple, rapid, and inexpensive single-gel immunodiffusion technique requiring but 1 μ l of sample for analysis and an incubation period of only 2 h is described.

Oudin (4) developed a single immunodiffusion technique for antigen analysis in which melted agar was mixed with antibody in a tube, allowed to gel, and overlaid with antigen. The precipitation band that formed migrated through the agar at a rate directly proportional to the antigen concentration. Recently, Weirether et al. (5) determined the influence of various factors on the tube assay technique. A newer modification of this technique has been the one-dimensional single electroimmunodiffusion method described by Gasper et al (3).

In all published reports to date, a minimum antigen volume of approximately 10 μ l and a minimum incubation period of 3 to 4 h have been required for a readable and reproducible test. The simple, reproducible, and inexpensive assay method described below requires but 1 μ l of antigen for analysis and an incubation period of only 2 h.

Solutions of bovine serum albumin (BSA), powder fraction V (lot 40901; General Biochemicals, Chagrin Falls, Ohio), were prepared in phosphate-buffered saline (0.02 M, pH 7.4) (5) in concentrations ranging from 25 to 400 μ g/ml. Canine serum albumin, fraction V (lot 82151; Pentex, Inc., Kankakee, Ill.), was dissolved in a separate portion of phosphate-buffered saline in a concentration of 400 μ g/ml.

Antiserum was prepared by injecting rabbits with BSA twice a week for 3 weeks by subcutaneous and intramuscular routes. Each injection consisted of 0.39 ml of an 0.85% saline solution of BSA (18 mg/ml) emulsified in an equal volume of Freund complete adjuvant. Animals were bled 10 days after the last injection. Partial purification of antibody globulin was accomplished by the ammonium sulfate precipitation method of Coons (1).

Solutions of washed Noble agar (0.3 and 0.6%; Difco, Detroit, Mich.) were prepared in phosphate-buffered saline by heating on a steam table. Solutions were filtered through no. 1

Whatman filter paper and cooled to 46°C in a water bath. Antibody globulin solution was diluted 1:15 (vol/vol) in phosphate-buffered saline. The diluted globulin was heated to 46°C and mixed with an equal volume of 0.6% agar solution. This final dilution of globulin (1:30) is the highest that gave a dense, sharp band with antigen.

A disposable micropipette, 1-mm ID, 1.27-mm OD, and 35 mm in length (Bolab, Inc., Reading, Pa.), served as the gel diffusion tube. Tubes were first coated with 0.3% agar to prevent antigen from penetrating between the antibody agar and the wall of the tube. Melted agar was drawn into the tube and expelled using the rubber bulb provided. The agar coating was dried by placing the tube in a vertical position in a desiccator jar over anhydrous calcium sulfate for at least 24 h.

Antibody agar was added to coated tubes by capillary attraction. The meniscus of a 7- to 9-mm melted agar plug was rapidly positioned about 1 mm from one end of the tube by small quick jerks of the wrist. Tubes were incubated for a few minutes unsealed, horizontally, until gelling was complete.

Test antigen was added to duplicate gel diffusion tubes with the aid of disposable micropipettes of 3- μ l capacity, 55 mm in length (Bolab, Inc., Reading, Pa.). Approximately 1 μ l of antigen solution was added to the micropipette by capillary attraction. The gel diffusion tube was held at an angle approximately 30° to the horizontal. The tip of the micropipette was placed just inside the lip of the tube, and a small amount of antigen was expressed to wet the walls of the tube above the agar meniscus. The micropipette was then withdrawn, and its end was placed against the end of the tube. The antigen well was then quickly filled by expressing the remaining antigen down the inner wall of the tube. Residual antigen on the outer lip of the gel diffusion tube was blotted away. Evaporation of antigen was prevented by layering a

small amount (0.2 to 0.5 μ l) of light mineral oil over the surface of the antigen. The gel diffusion tube was returned to the stoppered test tube and incubated at room temperature for 2 to 48 h in a horizontal position.

Precipitin bands were measured under 50 $\times$ magnification with a standard binocular microscope equipped with a mechanical stage and a vernier scale reading to 0.1 mm. One ocular contained a cross-hair reticle. A convenient and inexpensive device for supporting the gel diffusion tube on the microscope stage was made by constructing a trough from 2.5-by-7.5-cm microscope slides. Sides of the trough were made by placing two slides face down with long edges parallel and separated by a distance slightly greater than the outside diameter of the tube. A third slide placed over the gap between the two formed the bottom of the trough. The slides were fastened together with transparent adhesive tape. A strong shielded light source was positioned obliquely to the plane of the stage. The distance of precipitin band migration was measured to the nearest 0.1 mm from the antibody agar meniscus to the leading edge of the band. Average values for duplicate tubes were plotted against antigen concentration on semilog paper.

Figure 1 illustrates a representative series of precipitin bands formed by BSA solutions ranging from 25 μ g/ml (tube F) to 400 μ g/ml (tube B) against specific BSA antiserum (1:30) after 2 h of incubation at room temperature. Canine serum albumin served as specificity control (tube A). Antigen solutions and mineral oil overlays occupy portions of the tubes in approximately the upper half of the figure. Precipitin bands appear uniformly dense and sharp. No cross-reacting bands appear in the tube containing canine serum albumin.

A straight-line relationship between distance of band migration and antigen concentration existed when values were plotted on semilog paper (Fig. 2). Extending the incubation time to 21 and 48 h produced two straight-line slopes for each of these two incubation periods. Antigen concentrations greater than 50 μ g/ml produced one slope, and concentrations less than 50 μ g/ml produced the other. A similar situation was reported by Fung and Wagner (2) for the assay of staphylococcal enterotoxins by the capillary tube single-gel diffusion technique. Capillary action may account for this phenomenon. The leading edge of the precipitin band formed at 48 h by antigen at 400 μ g/ml was too diffuse to be measured accurately, and therefore was not plotted.

The results of six assays run on successive

FIG. 1. Precipitin bands formed in capillary tubes containing agar overlaid with: (A) 400 μ g of canine serum albumin per ml; (B) to (F) 400, 200, 100, 50, and 25 μ g of BSA per ml, respectively.

FIG. 2. Titration curves produced by incubation times of 2 h (●—●), 21 h (□—□), and 48 h (▲—▲). Results are averages of duplicate tubes.

days are shown in Table 1. The relatively small standard deviation in distance of band migration (0.04 to 0.09 mm) supports the reproducibility of this method.

Once such a reference curve has been plotted, the concentration of antigen in an unknown sample can then be determined simply by taking the value from the reference curve and correcting for dilution. The assay requires, however, that a monospecific antiserum be used to avoid multiple antigen-antibody systems, which would appear as distinct precipitin bands that may be misinterpreted.

A comparative study was done to determine

TABLE 1. *Reproducibility of band migration with the microcapillary immunodiffusion assay technique after 2 h of incubation*

BSA concn. (μg/ml)	Band migration (mm) in assay no.						$\bar{X}$	SD ^a
	1	2	3	4	5	6		
25	0.15	0.05	0.05	0.10	0.15	0.10	0.10	0.04
50	0.45	0.40	0.40	0.45	0.35	0.35	0.40	0.04
100	0.65	0.75	0.70	0.80	0.75	0.65	0.72	0.06
200	0.95	1.15	1.10	1.15	1.00	1.05	1.07	0.08
400	1.20	1.45	1.35	1.40	1.40	1.35	1.36	0.09

$$^a \text{SD, Standard deviation} = \sqrt{\frac{\sum(X - \bar{X})^2}{N}}$$

the relative sensitivities of the single-gel diffusion assay described here and one performed in large test tubes (4.5-mm ID, 6-mm OD, and 50 mm in length). Except for the larger volumes of antibody agar, antigen, and mineral oil overlay required for the test tube method, the experimental conditions and method for measurement of band migration used were the same as those described above. Titration curves produced at 2, 21, and 48 h of incubation were almost identical

in both assays. The sensitivity in both cases appears, therefore, to be the same.

This technique should provide a simple, rapid, and inexpensive method for quantitating precipitable antigens when the volume of biological sample containing the antigen is critically small.

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