

Chapter 26

The Multiple Antigen Blot Assay: A Simple, Versatile and Multipurpose Immunoenzymatic Technique

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Summary

This technique is based on the sensitization of different antigens in a single nitrocellulose strip, which react when exposed to an immune serum and thereafter with the appropriate peroxidase conjugate and the corresponding substrate. Signals in those reactive spots are recorded as black squares in a negative photographic film, using a chemiluminiscent substrate or as blue spots when a precipitable colorimetric substrate is used. This technique allows the simultaneous demonstration of antigenicity of different antigens (peptides, recombinant molecules, and crude preparations), with a high sensitivity and specificity. Its major value is based on its versatility, since it is possible to rapidly evaluate and to compare various antigenic preparations and to use it for diagnosis of different infectious, allergic and autoimmune diseases, at a low cost.

Key words: Multiple, Blot, Antigen, MABA, Immunochromatographic, Enzyme-immunoassay, Immunodiagnosis, Luminescent, Colorimetric

1. Introduction

Three diagnostic techniques comply with the concept of simultaneous multidiagnosis: the antigen/antibody microarrays (1), the multiple laser detection beads (Multiplex®) (2), and the multiple antigen blot assay (MABA) (3). The first two have been used for the detection of antigens, antibodies, or nucleic acids, while the third one only for antibodies, so far.

The occurrence of simultaneous infections is not an infrequent finding worldwide; therefore, it would be ideal for the

health personnel to be aware about the patient's current or past history of contact with the most important pathogens, as well as his immune status, to have a more integral knowledge of each patient for a more rationale medical practice. With this purpose, MABA can be implemented and adapted for particular populations and institutions, such as blood banks, pediatric diseases, immunocompromised patients, vaccine evaluation, etc., being a major line of research of our laboratory. It is also a practical, rapid, reproducible, sensitive, and cheap technique for the identification and evaluation of antigens (antigenicity) and sera. This can be done with crude, chemically synthesized and recombinant antigens (4–8). Allergic conditions detecting specific IgE have been successfully evaluated with this technique (9). Another advantage is that a single MABA that allows the evaluation of at least 28 different sera against 28 different antigens is equivalent to eight conventional ELISA plates. The cost of evaluation of each antigen per patient is 0.078\$ by MABA vs. 0.114\$ by a conventional ELISA.

2. Materials

1. Carbonate-bicarbonate buffer, pH 9.6: 1.59 g sodium carbonate (Na_2CO_3) (0.015 M); 2.93 g sodium bicarbonate (NaHCO_3) (0.03 M). Adjust pH to 9.6 and with distilled water complete to a final volume of 1,000 mL. Store at 2–8°C.
2. Phosphate buffered saline (PBS)-0.05% Tween-20 (PBST), pH 7.5: Solution **A** (0.2 M NaH_2PO_4): 24.0 g NaH_2PO_4 (anhydrous) in 1,000 mL distilled water; solution **B** (0.2 M Na_2HPO_4): 5.68 g Na_2HPO_4 (anhydrous) in 200 mL distilled water; 13 mL solution **A** + 87 mL solution **B** + 8.76 g NaCl + up to 800 mL distilled water. Adjust pH to 7.5 with solutions **A** or **B**. Then, complete with distilled water up to 1,000 mL and add 0.5 mL of Tween-20. Once prepared, keep at 4°C.
3. Blocking solution (5% non-fat milk in PBST): 5 g nonfat milk in 100 mL PBST. Prepare freshly.
4. SuperSignal® (West Pico Chemiluminescent Substrate, Pierce, USA, Cat. # 34077). The two solutions should be mixed 1:1. Prepare freshly.
5. Acetate buffer 50 mM: Solution **A**: acetic acid 200 mM: 2 mL acetic acid + 172 mL distilled water; Solution **B**: 5.44 g sodium acetate in 200 mL distilled water. The buffer may be stored at 2–8°C for up to 1 month; 110 mL Solution **A** + 15 mL Solution **B** + distilled water up to a volume of 500 mL.

6. TMB Membrane Peroxidase Substrate (KPL; Gaithersburg MD, USA, Cat. # 50-77-03): This substrate contains 3,3', 5,5' - tetramethylbenzidine in acidic buffer. The concentration of hydrogen peroxide (H_2O_2) is 0.02%. It is a very sensitive chromogenic substrate for peroxidase detection. It develops a deep blue color at the reaction sites labeled with horseradish peroxidase-labeled conjugates. No reagent preparation is needed.
7. Sodium metaperiodate-SMP (Sigma Chemical Company, St. Louis, MO, USA, Cat. #S-1878) (1 mM) in acetate buffer: 4.28 mg SMP in 20 mL acetate buffer. Prepare freshly.
8. 50 mM sodium borohydride ($NaBH_4$) (Sigma Chemical Company, St. Louis, MO, USA, S-9125) in PBS: 0.189 g $NaBH_4$ in 100 mL PBS. Prepare freshly.
9. Anti-human IgG (Fc specific) peroxidase antibody produced in goat (Sigma A-0170). For continuous use, store at 2–8°C up to 1 month. For extended storage, the solution may be frozen in working aliquots at –20°C, since repeated freezing and thawing is not recommended. Dilute to 1:30,000 in blocking solution. Prepare freshly.
In some cases, the quality of the water is critical. In those situations it is necessary to use Milli Q water.

3. Methods

3.1. Luminescent MABA (3)

1. A rectangle of nitrocellulose (NC) sheet (BIORAD® 0.45 μm Cat. 162-0115) 7.5 × 10 cm is cut (**Fig. 1**) and labeled with an indicator line in one of the borders for reference, done with a fine ball point-water resistant pen (**Fig. 2**).
2. The paper is soaked in distilled water for 5 min (**Fig. 3**).
3. The wet paper is aligned in the upper part of Miniblotter® 28SL (Immunetics Inc, Cambridge, MA, USA), with the reference line parallel to channel 1 (**Fig. 4**). Then, a plastic cushion is placed onto the nitrocellulose sheet (**Fig. 5**).
4. The Miniblotter® is closed with the lower piece and tightened with the two provided screws (**Fig. 6**). Avoid membrane damage by excessive tightening. Remaining water in the parallel channels is removed by aspiration with an Eppendorf-type pipette (**Fig. 7**). Next steps should be carried out in less than half an hour, to prevent drying and possible cracking of the paper.



Fig. 1. Cut a rectangle of NC sheet 7.5×10 cm.



Fig. 2. Reference line in one of the borders.



Fig. 3. NC paper is soaked in distilled water for 5 min.

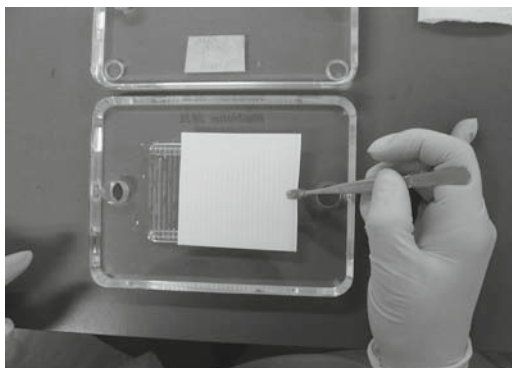


Fig. 4. NC is aligned in the upper part of Miniblotter® with the reference line parallel to channel 1.



Fig. 5. A plastic cushion is placed onto the NC.

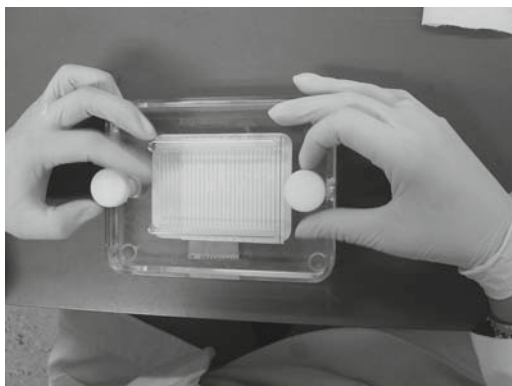


Fig. 6. The Miniblotter® is closed with the lower piece and tightened with the two provided screws.

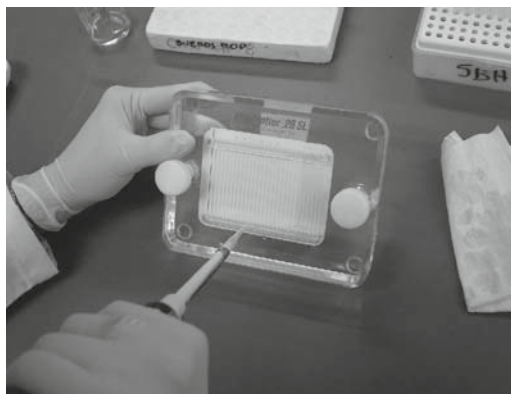


Fig. 7. Remaining water in the channels is removed by aspiration with an Eppendorf-type pipette.

5. The different antigen preparations (crude, synthetic peptides, and recombinant molecules) up to a maximum of 28 can be analyzed in each NC sheet, at a concentration between 10–50 $\mu\text{g}/\text{mL}$ in a carbonate-bicarbonate buffer, pH 9.6.
6. To sensitize the NC sheet, 60 mL of each antigen are introduced in each groove of the Miniblotter[®] (**Fig. 8**), avoiding the formation of bubbles, and incubated for 60 min on an orbital or horizontal shaker, at room temperature (RT) (**Fig. 9**).
7. The antigen solutions are removed by washing with 40 mL PBST, using a washing bottle (**Fig. 10**) or the manifold provided with the equipment (**Fig. 11**).
8. Then the Miniblotter[®] is opened and the NC sheet is washed three times with 10 mL PBST on a shaker for 10 min each.
9. Blocking is achieved by immersing the NC in 5% nonfat milk in PBST for 2 h on a shaker, at RT (**Fig. 12**).
10. Once blocked, the NC sheet could be processed immediately or stored wrapped in filter paper and inside a plastic bag at -20°C .
11. Numbered 2 mm wide strips are cut perpendicular to the channels and the reference line, with a scalpel (**Fig. 13**). Therefore, each strip would contain a row of square spots corresponding to a maximum of 28 different antigens. Shorter strips with less spot rows can be designed.
12. Strips are immersed individually in the troughs of an incubation tray, in serum diluted (1:100 or 1:200) in blocking solution (**Figs. 14–16**) and incubated for 90 min at RT on a shaker (**Fig. 17**).
13. Strips are washed 3–5 times depending on the background of each system, for 10 min each, with PBST (**Fig. 18**), and then incubated for 90 min with the corresponding

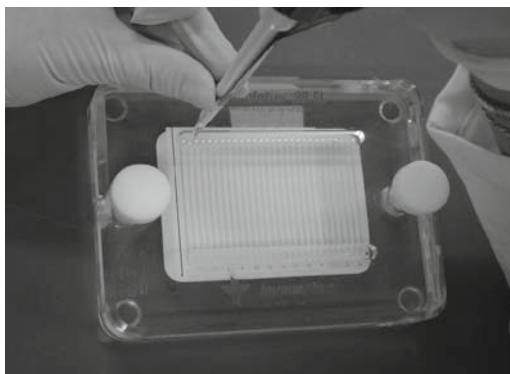


Fig. 8. Sixty microliter of each antigen are introduced in each groove.



Fig. 9. Incubation for 60 min on an orbital or horizontal shaker.

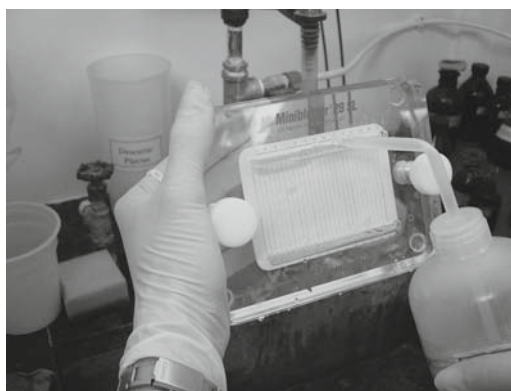


Fig. 10. Antigen excess is removed by washing with 40 mL PBST using a washing bottle.



Fig. 11. Antigen excess can also be removed by washing with 40 mL PBST using the manifold provided with the equipment.



Fig. 12. Blocking is achieved by immersing the NC sheet in 5% nonfat milk in PBST for 2 h on a shaker.

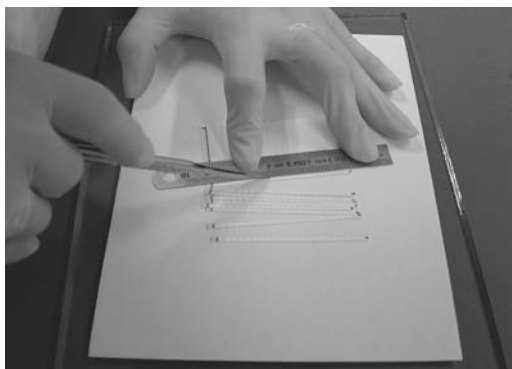


Fig. 13. Numbered 2 mm wide strips are cut perpendicular to the channels and the reference line, with a scalpel.



Fig. 14. Add 600 μ L of blocking solution.



Fig. 15. Three to six microliter of sera (dilution of 1:200 or 1:100, respectively) in blocking solution.

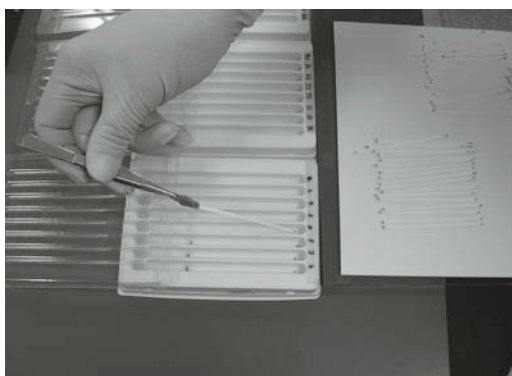


Fig. 16. Strips are immersed individually in the troughs of an incubation tray.



Fig. 17. Incubation for 90 min at RT on a shaker.

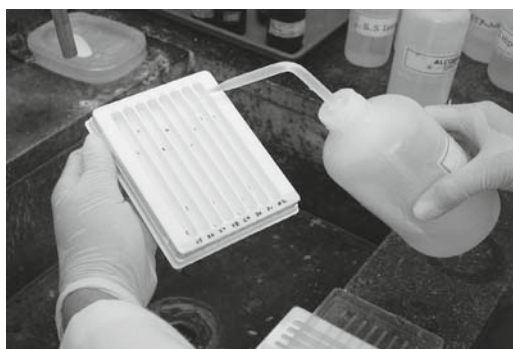


Fig. 18. Strips are washed 3–5 times for 10 min each, with PBST on a shaker.

anti-serum horseradish peroxidase labeled conjugate (**Fig. 19**), diluted (1:1,000–1:50,000) in blocking solution at RT, on a shaker.

14. Three washes of strips are carried out for 10 min each with PBST, as in **Fig. 18**.
15. All strips are put together in a single tray. In case of using a chemiluminescent substrate, strips are immersed in approximately 1 mL of SuperSignal® solution for at least 1 min (**Fig. 20**) and arranged in parallel onto a glass (**Fig. 21**) and covered with a plastic wrap (**Figs. 22 and 23**).
16. Finally, the strips are exposed to Hyperfilm® (Amersham, UK, Cat. # RPN3103K), under darkness and developed photographically (**Fig. 24**). Routinely, two different exposure times are carried out: 5 and 20 s, depending on the intensity of the signal for each condition. For each antigen and antibody systems, all steps must be standardized. If it is available a luminescent detection system (ChemiDoc™, BIO-RAD Laboratories,



Fig. 19. Incubation for 90 min with the corresponding anti-serum horseradish peroxidase-labeled conjugate.

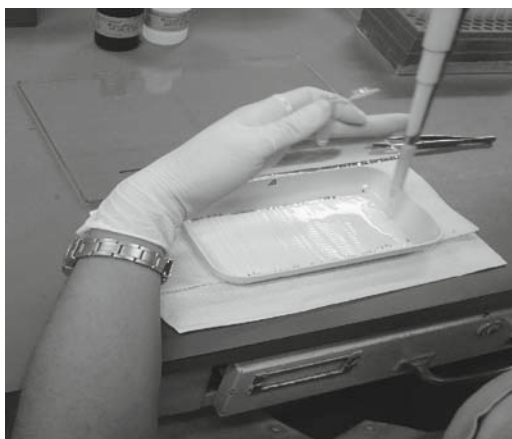


Fig. 20. Strips are immersed in approximately in 1 mL SuperSignal® for at least 1 min.

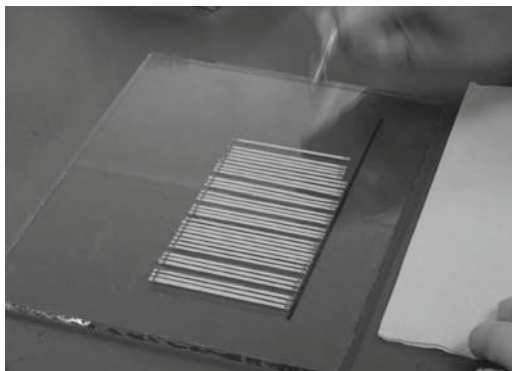


Fig. 21. NC strips are arranged in parallel onto a glass.



Fig. 22. Strips are covered with a plastic wrap.

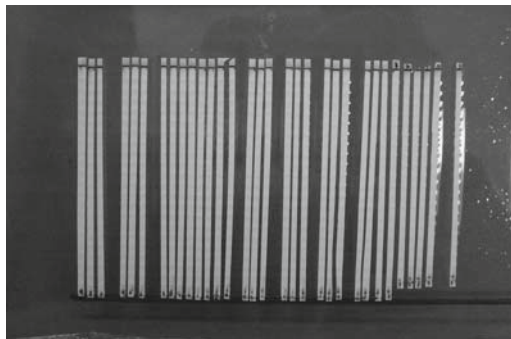


Fig. 23. Strips are exposed to Hyperfilm® under darkness.

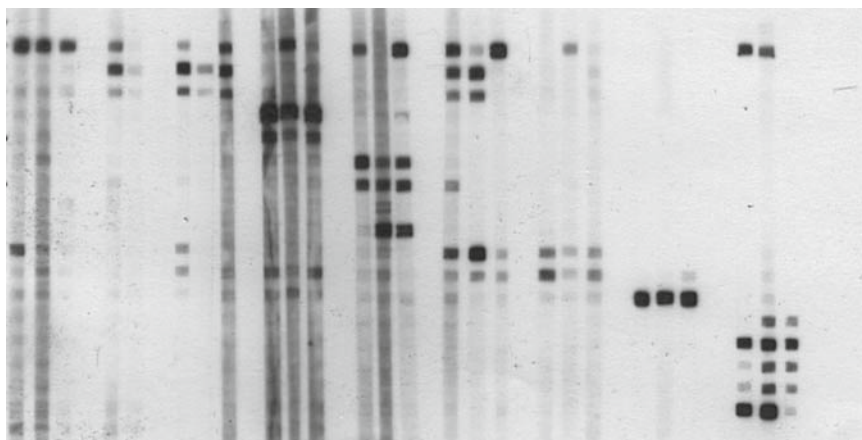


Fig. 24. Representative MABA of antigenicity of crude extracts and synthetic peptides from different infectious agents recognized by patient's sera developed with chemiluminiscent substrate.

Inc. CA, USA), the equipment directly registers electronically the luminescent signal.

17. In case of nonspecific reactivities due to cross-reacting crude antigens, treatment of the antigen preparation with sodium metaperiodate is recommended (*see Note 1*).

3.2. Colorimetric MABA

(*See Subheading 3.1, steps 1–12* are the same for luminescent and colorimetric MABA)

1. To develop with a precipitable colorimetric substrate, the peroxidase-labeled conjugate should be diluted in PBS/0.3%Tween-20 without milk and incubated for 90 min at RT with continuous gentle agitation on a shaker.
2. Wash the strips four times for 10 min each with PBS-0.3%-Tween-20.
3. Remove the PBS and incubate in the TMB[®] membrane peroxidase substrate solution previously warmed at RT for 5–15 min, or until desired color is achieved.
4. Stop reaction by immersing the NC in distilled water for 20–30 s. The reaction should be stopped before background color becomes too intense (**Fig. 25**). For storage, dry the membrane, seal with clear plastic and store in the dark to minimize fading.

4. Notes

1. MABA-SMP: Certain crude antigens have limitations because of false-positive results due to immunological cross-reactions that are frequent within certain organisms such as helminths.

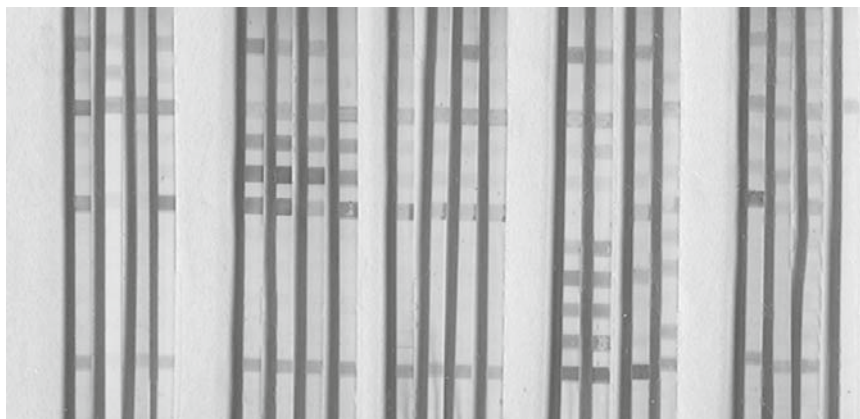


Fig. 25. Representative MABA of antigenicity of crude extracts and synthetic peptides from different infectious agents recognized by patient's sera developed with colorimetric substrate.

Most of these molecules correspond to glycosylated epitopes (10). To minimize this serological cross-reactivity, oxidation with sodium metaperiodate (SMP) has been used to elucidate the role of carbohydrate portion of antigenic glycoproteins in the reactivity by serology (11, 12). After treatment of crude antigens with SMP, the hydroxyl groups of sugars are oxidized to aldehydes under acidic pH. In the presence of a reducing agent such as sodium borohydride (NaBH_4), aldehydes become alcohols, being not recognized by the cross-reactive anti-carbohydrate epitope antibodies (11, 12). The method is as follows: (a) NC membrane is sensitized and processed as previously described (*see Subheading 3.1, steps 1–10*); (b) NC sheet is washed under agitation 1 min in PBST on a shaker; (c) Membrane is washed under agitation 3 min in acetate buffer (50 mM) pH 4.5; (d) For SMP treatment it is necessary to standardize the SMP concentration (1–3 mM) in acetate buffer for 60 min, in the dark, at RT and prepare it just before use; (e) A brief washing is made with 50 mM acetate buffer for 3 min; (f) Add 50 mM sodium borohydride (NaBH_4) in PBS and incubate for 30 min at RT, with continuous gentle agitation on a shaker. The reagent should be prepared just before use. It should be bubbling and if the reagent is excessively hydrated and does not bubble, treatment will not work appropriately; (g) Wash 3 min with PBS under agitation; (h) Continue the procedure as it has been described previously (*see Subheading 3.1, steps 11–16*).

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