

Improvements and Variants of the Multiple Antigen Blot Assay-MABA: An Immunoenzymatic Technique for Simultaneous Antigen and Antibody Screening

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Abstract

This simple, versatile, reliable, reproducible, multipurpose, and inexpensive technique is based on the adhesion of different antigens to a single nitrocellulose strip using, as template, an acrylic device containing 28 parallel channels. The inclusion of channels containing normal human serum improves the quality control of this assay. Antigen-sensitized nitrocellulose strips are cut perpendicularly to the antigen-rows, exposed to immune sera followed by the appropriate conjugate. Positive signals are recorded using chemiluminescent or precipitable colorimetric substrates. This assay allows the simultaneous qualitative demonstration of antigenicity and immunogenicity of antigens obtained as synthetic peptides, recombinant molecules, or crude preparations, with high sensitivity and specificity. Its major value is based on the rapid and simultaneous comparative evaluation of various antigenic preparations allowing the diagnosis of a variety of infectious, allergic, and autoimmune diseases. It can in general be used to detect any type of antibody or circulating antigen. Some improvements and variants of the original technique are included.

Key words Multiple, Blot, Antigen, MABA, Immunochromatographic, Multiscreen enzyme-immunoassay, Immunodiagnosis, Luminescent, Colorimetric

1 Introduction

Three diagnostic techniques comply with the concept of simultaneous multidagnosis: the antigen/antibody microarrays [1], the multiple laser detection beads (Multiplex®) [2] and the multiple antigen blot assay (MABA) [3, 4]. 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 health personnel to be aware of a patient's current or past history of contact with the most

important pathogens, as well as his/her immune status, in order to have a more integral knowledge of each patient for a more rationale medical practice. To this end, MABA can be implemented and adapted for particular populations and institutions, such as blood banks, pediatric diseases, immunocompromised patients, vaccine evaluation, this being a major line in research of our laboratory. It is also a practical, rapid, reproducible, sensitive, and cheap technique for the identification and evaluation of antigens (antigenicity and immunogenicity) and sera. This can be done with crude, chemically synthesized, or recombinant antigens [5–9]. Allergic conditions detecting specific IgE have been successfully evaluated with this technique [10]. Another advantage is that a single MABA assay allows the evaluation of at least 26 different sera against 26 different antigens this being equivalent to 13–14 conventional ELISA plates. The cost of evaluation of each antigen per patient is \$0.006 by MABA versus \$0.129 by conventional ELISA. For this technique, an acrylic device with grooves is needed, so nitrocellulose paper can be sensitized with different antigens. Moreover, this device can be made with the appropriate acrylic plastic by a skilled artisan in various formats depending on the requirements (number and width of channels, etc.).

This technique has been used for other purposes such as the identification and selection of the best candidate antigens [11] as well as the identification of a specific molecule from eluent fractions during a chromatographic separation [12]. Schematic diagram of the whole protocol of MABA is presented in Fig. 28.

2 Materials

The quality of the water is critical. We recommend the use of Type I ultrapure water (18.2 M Ω).

1. Miniblotter® Immunetics Inc, Cambridge, MA, USA or Mini-PROTEAN® Multiscreen Apparatus Bio-Rad.
2. Nitrocellulose Membrane Trans-Blot.
3. Carbonate–bicarbonate buffer, 0.05 M, pH 9.6: 1.59 g sodium carbonate (Na₂CO₃); 2.52 g sodium bicarbonate (NaHCO₃). Adjust pH to 9.6 and complete to a final volume of 1,000 mL with distilled water. Store at 2–8 °C.
4. Phosphate Buffered Saline (PBS)-0.05 % Tween 20 (PBST), pH 7.5; Solution A (0.2 M NaH₂PO₄): 24 g NaH₂PO₄ in 1,000 mL distilled water; solution B (0.2 M Na₂HPO₄): 5.68 g Na₂HPO₄ 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 to 1,000 mL with distilled water and add 0.5 mL of Tween 20. Store at 4 °C.
5. Tris Buffered Saline Buffer (TBS)-0.05 % Tween20; 6.05 g Tris (50 mM); 8.76 g NaCl (150 mM), pH 7.5. Dissolve in 800 mL

of H₂O. Adjust pH to 7.5 with 1 M HCl and make volume up to 1 L with H₂O. Add 0.5 mL Tween20. Store at 4 °C.

6. Blocking solution (5 % nonfat milk in PBST): 5 g nonfat milk in 100 mL PBST. Prepare fresh.
7. Anti-human IgG (Fc specific) horseradish peroxidase antibody produced in goat. 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. Standardization is necessary for each conjugate and each brand. Prepare fresh. Anti-human IgG alkaline phosphatase (AKP) conjugated antibody should be used at approximately 1:25,000. If MABA is performed with AKP conjugate, TBST rather than PBST should be used. Other immunoglobulins (IgM, IgE, IgA, IgG1, etc.) may also be evaluated.
8. SuperSignal® (West Pico Chemiluminescent Peroxidase Substrate, Pierce, USA,). The two solutions should be mixed 1:1. Prepare fresh.
9. TMB membrane peroxidase substrate. This substrate contains 3,3', 5,5'- tetramethylbenzidine in acidic buffer and is a very sensitive chromogenic substrate for peroxidase detection, developing a deep blue color in the presence of the horseradish peroxidase conjugates. The substrate is ready to use. Store at 2–8 °C.
10. BCIP/NBT substrate for alkaline phosphatase, ready to use.
11. Acetate buffer 0.05 M, pH 4.8: 110 mL Solution A+15 mL Solution B+distilled water to a final volume of 500 mL. Solution A: Sodium acetate 0.2 M: 5.44 g sodium acetate·3H₂O in 200 mL distilled water; solution B: Acetic acid 0.2 M: 2 mL acetic acid+172 mL distilled water. The buffer may be stored at 2–8 °C for up to 1 month.
12. Sodium metaperiodate (SMP), stock solution 0.02 M in acetate buffer: 85.6 mg SMP in 20 mL acetate buffer. Prepare fresh.
13. 0.05 M sodium borohydride (NaBH₄) in PBS: 0.189 g NaBH₄ in 100 mL PBS. Prepare fresh.
14. Hyperfilm®.

3 Methods

3.1 Luminescent MABA [3, 4]

1. A rectangle of nitrocellulose (NC) paper (7.5×10 cm), is cut (Fig. 1) and labeled with an indicator line along one of the borders 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 the Miniblotter®, with the reference line parallel to channel 1 (Fig. 4). Then, a plastic cushion is placed over the nitrocellulose sheet (Fig. 5).

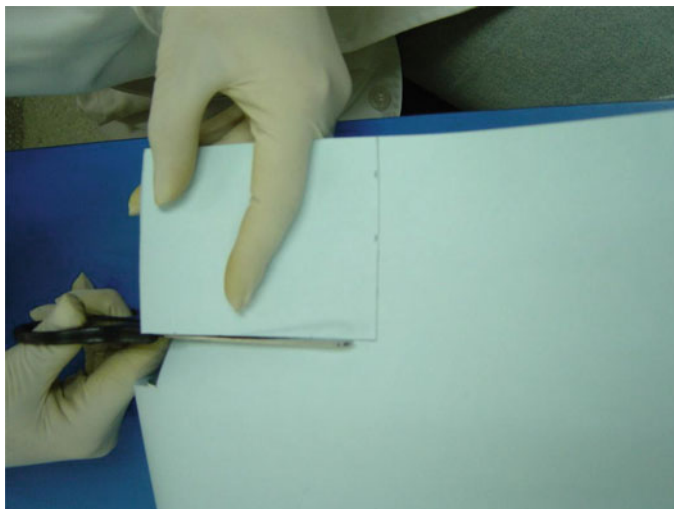


Fig. 1 Cut a rectangle of NC sheet 7.5×10 cm

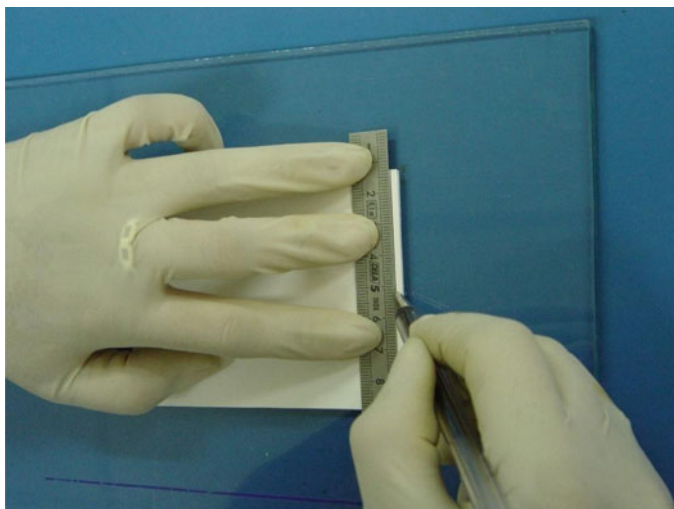


Fig. 2 Reference line in one of the borders

4. The Miniblotter[®] is closed with the lower piece and hand tightened with the two provided screws (Fig. 6). Avoid excessive tightening. The remaining water in the parallel channels is removed with pipette (Fig. 7). The next steps should be carried out in less than 30 min, to prevent drying and possible cracking of the paper.
5. The different antigen preparations (crude, synthetic peptides or recombinant molecules) up to a maximum of 26 can be analyzed on each NC sheet, at a concentration between 10 and 50 $\mu\text{g}/\text{mL}$ in carbonate–bicarbonate buffer, pH 9.6. Inclusion



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



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

of a normal human serum on the membrane of one or two of the parallel lanes is strongly recommended as an internal control, as human serum should always give a positive reaction in the presence of the secondary anti-human antibody. If nonhuman samples are to be assayed (mice, rabbit, etc.), the control row should be sensitized with the corresponding normal serum.

6. To sensitize the NC sheet, 60 μL 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

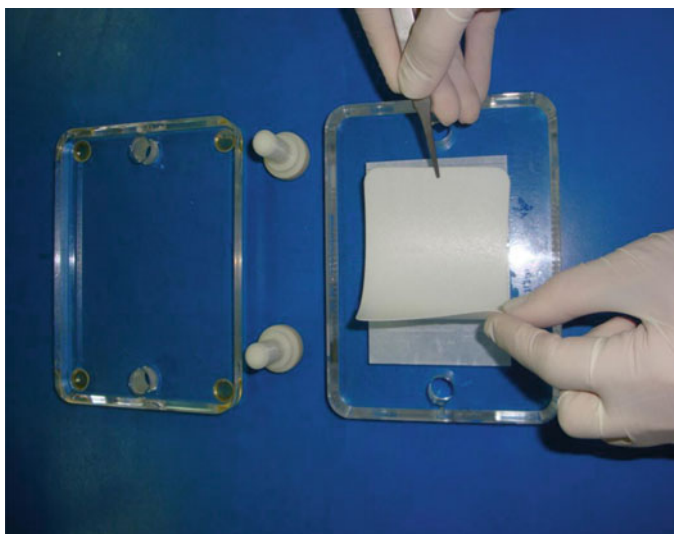


Fig. 5 A plastic cushion is placed over the NC

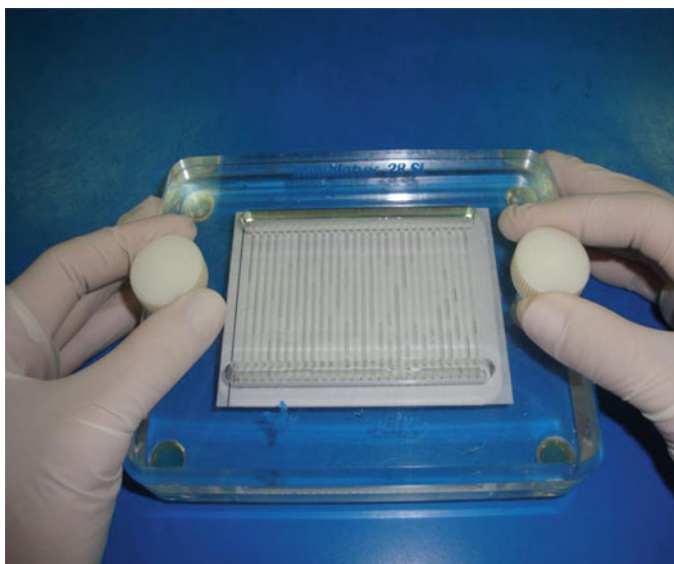


Fig. 6 The Miniblotter® is closed with the lower piece and hand tightened

or horizontal shaker, at room temperature (RT) (Fig. 9). The volume may need to be adjusted depending on the apparatus/size of groove used).

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).

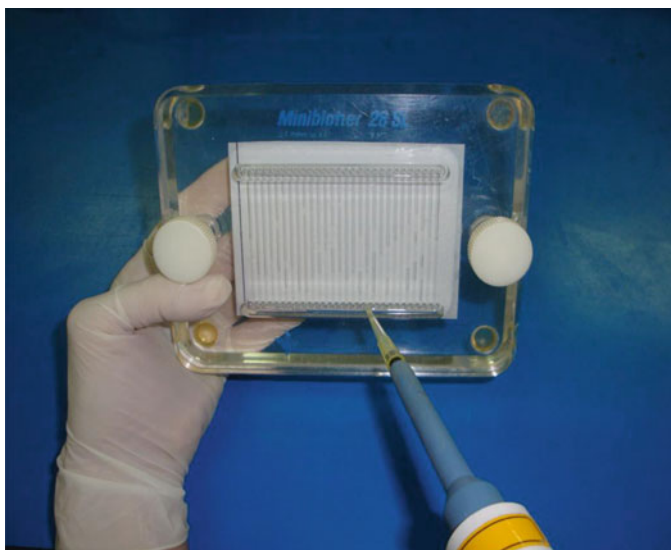


Fig. 7 Remaining water in the channels is removed by aspiration

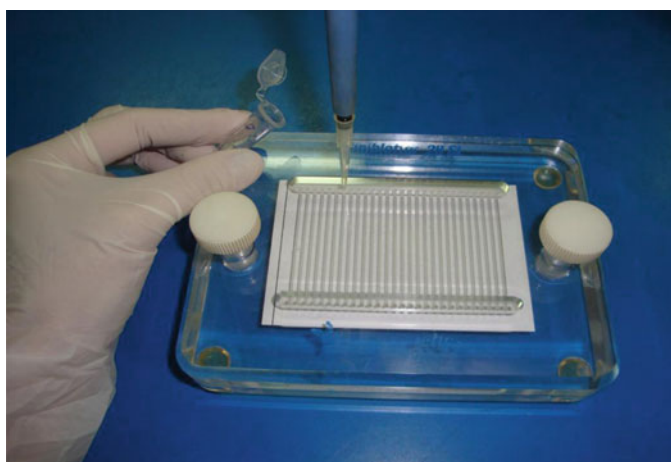


Fig. 8 Sixty microliter of each antigen are introduced into each groove

8. Then, the Miniblotter[®] is opened and the NC sheet is washed three times in a tray 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 may be processed immediately or stored wrapped in filter paper inside a plastic bag at -20 °C.



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 wash bottle

11. Numbered 2 mm-width-strips are cut perpendicular to the channels and the reference line, with a scalpel (Fig. 13). Each strip will thus contain a row of square spots corresponding to a maximum of 26 different antigens. Shorter strips with less spot rows may be used.
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, 15, and 16) and incubated for 90 min at RT on a shaker (Fig. 17). Positive and negative serum controls and a



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

conjugate control (which consists in a strip without serum) should be included.

13. Strips are washed three to five times depending of the background of each system, for 10 min each, with PBST (Fig. 18), and then incubated for 90 min with the corresponding anti-Ig horseradish peroxidase labeled conjugate (Fig. 19), diluted in blocking solution at RT, on a shaker.
14. The strips are washed three times for 10 min each with PBST, as in Fig. 18.

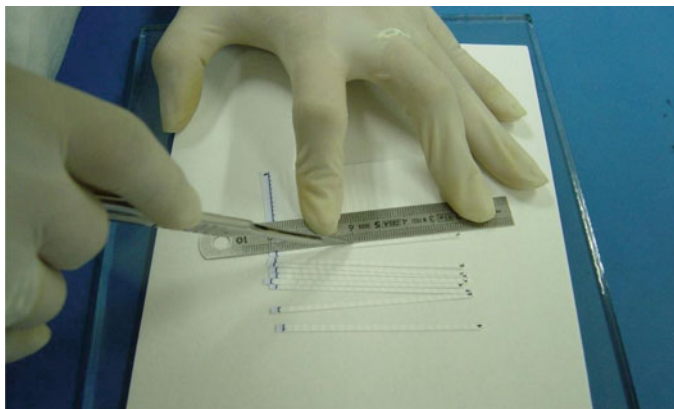


Fig. 13 Numbered 2 mm wide strips are cut perpendicular to the channels and the reference line, with a scalpel

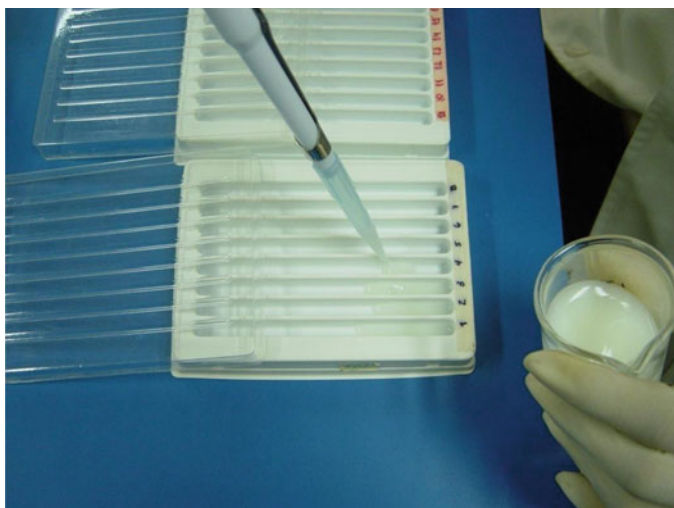


Fig. 14 Add 600 μ L of blocking solution to troughs of an incubation tray

15. All strips are put together in a single tray. When using a chemiluminescent substrate, strips are immersed in approximately 1 mL of SuperSignal[®] solution for at least 1 min (Fig. 20), arranged in parallel onto a glass sheet (Fig. 21) and covered with a plastic wrap (Figs. 22 and 23).
16. Finally, the strips are exposed to Hyperfilm[®], in 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. If a luminescent

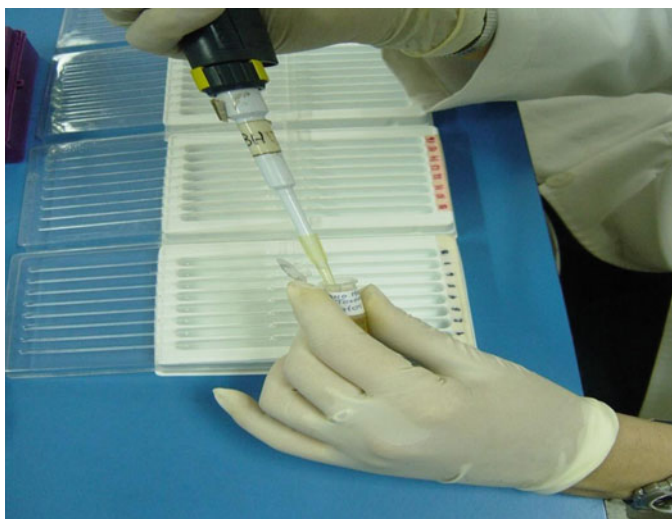


Fig. 15 Add 3–6 μL of sera (dilution 1:100 or 1:200, respectively) in blocking solution

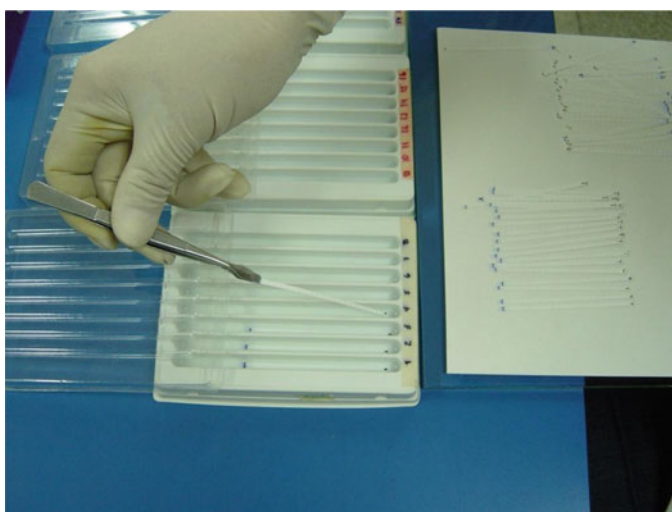


Fig. 16 Strips are immersed individually in the troughs

detection system (ChemiDoc™, BIO-RAD Laboratories, Inc. CA, USA) is available, the equipment registers electronically the luminescent signal. The signal density can also be quantified.

3.2 Colorimetric MABA [4]

The use of colorimetric substrates avoids the need of a dark room or expensive detection equipment (*see* Subheading 3.1, steps 1–12 are the same for luminescent and colorimetric MABA).

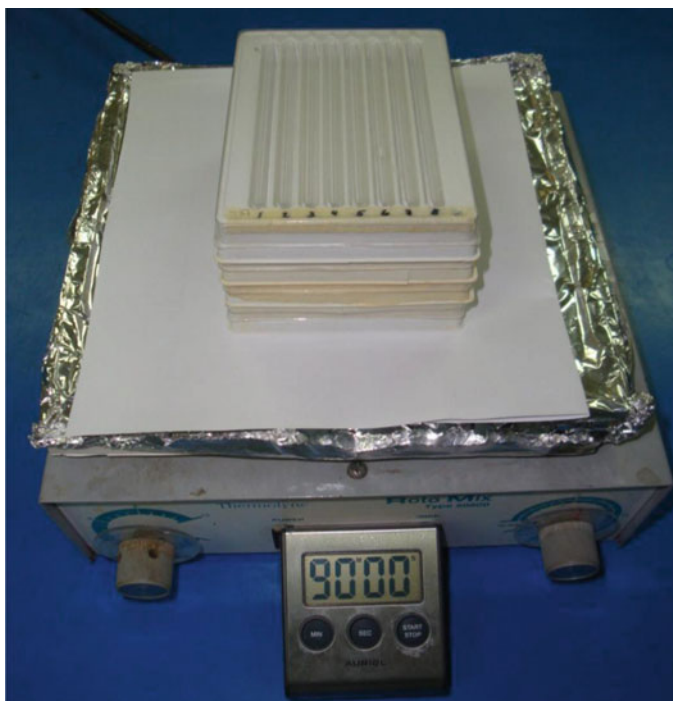


Fig. 17 Incubation for 90 min at RT on a shaker



Fig. 18 Strips are washed three to five times for 10 min each, with PBST on a shaker

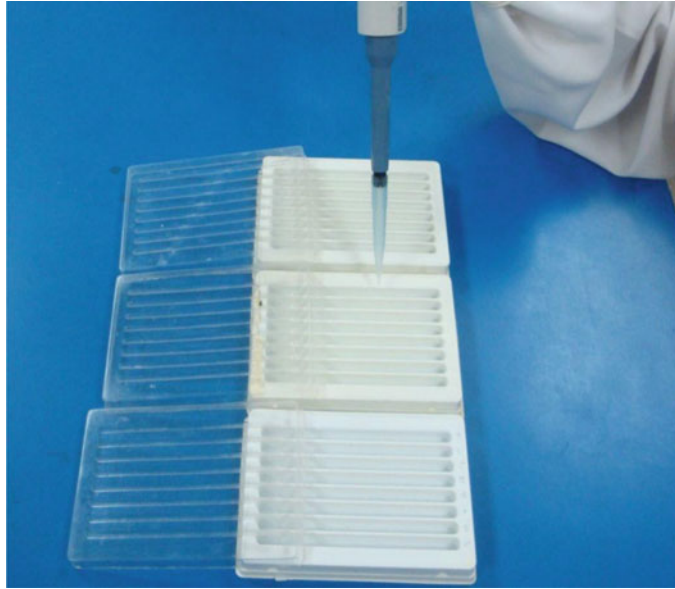


Fig. 19 Incubation for 90 min with the corresponding anti-Ig enzyme-labeled conjugate



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

1. To develop with a precipitable colorimetric substrate, the peroxidase-labeled conjugate should be diluted in blocking solution and incubated for 90 min at room temperature with continuous gentle agitation on a shaker.
2. Wash the strips three times for 10 min with PBST.

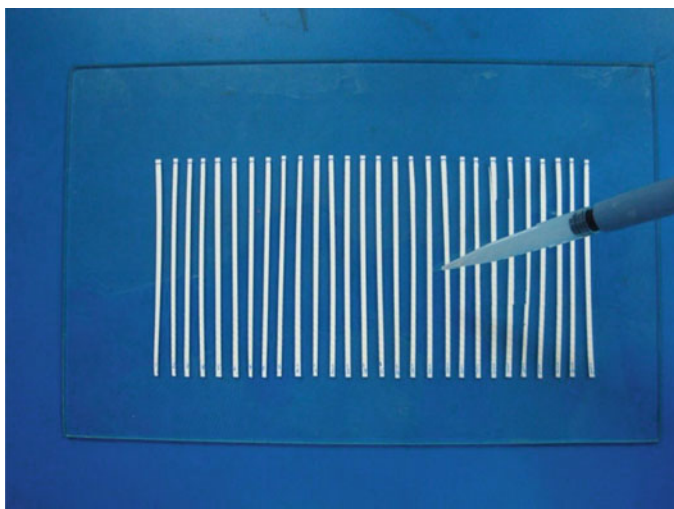


Fig. 21 NC strips are arranged in parallel on a glass sheet



Fig. 22 Strips are covered with a plastic wrap

3. Remove the PBST and immerse strips in TMB[®] peroxidase substrate solution substrate for 5–15 min, or until the desired color is achieved.
4. Stop the reaction by immersing the membrane 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 plastic wrap and store in the dark to minimize fading.

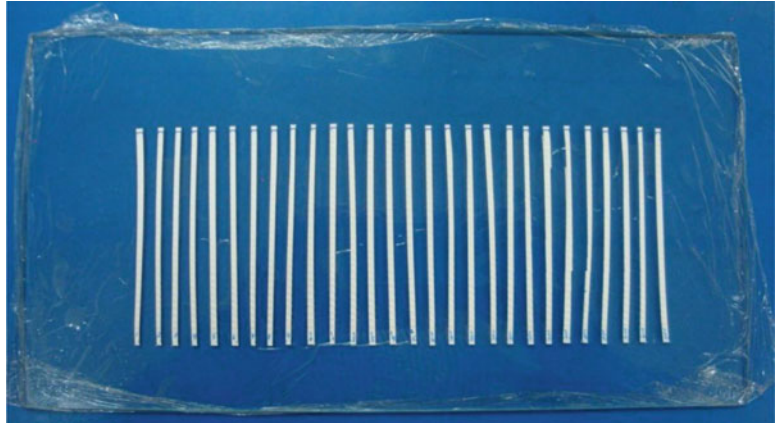


Fig. 23 Strips are exposed to Hyperfilm® in darkness

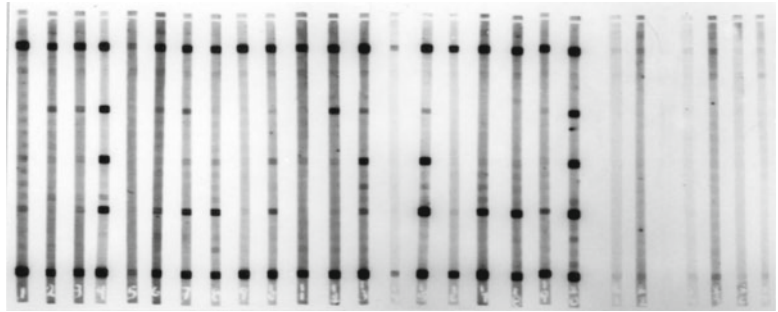


Fig. 24 Representative MABA of antigenicity of crude extracts and synthetic peptides from different infectious agents recognized by patient's sera developed with chemiluminescent substrate

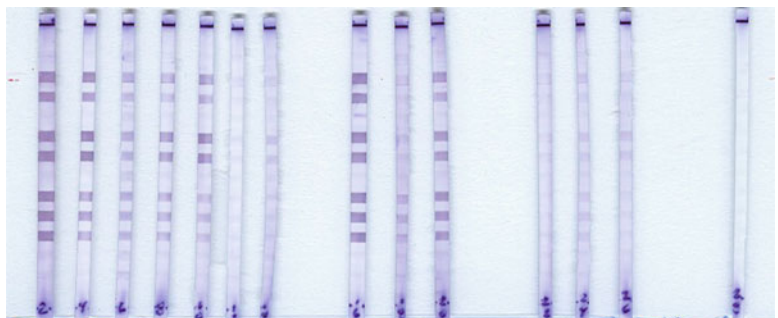


Fig. 25 Representative MABA of antigenicity of crude extracts and synthetic peptides from different infectious agents recognized by patient's sera developed with colorimetric substrate

5. If an AKP conjugate is used, strips should be immersed in enough volume of ready-to-use BCIP/NBT substrate at RT. Wait 5–15 min, or until the desired purple color is achieved and stop the reaction by immersing the membrane in distilled water. For storage, dry the membrane, seal with plastic wrap and store in the dark to minimize fading.

For each antigen and antibody system, all steps must be standardized.

3.3 SMP Treatment in MABA

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

3.4 Cross-MABA

A variation in the technique, the Cross-MABA, avoids cutting, numbering or manipulation of strips (*see Note 2*).

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. Most of these molecules correspond to glycosylated epitopes [13]. In order to minimize this serological cross-reactivity, oxidation with sodium metaperiodate (SMP) has been used to elucidate the role of the carbohydrate portion of antigenic glycoproteins in the reactivity by serology [14, 15]. 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, which are not recognized by the cross-reactive anti-carbohydrate epitope antibodies [14, 15]. The method is as follows: (a) the NC membrane is sensitized and processed as previously described (*see Subheading 3.1, steps 1–10*); (b) the sheet is washed with agitation for 1 min in PBST on a shaker; (c) the membrane is washed with agitation for 3 min in acetate buffer (50 mM) pH 4.8; (d) for the SMP treatment, it is necessary to standardize the SMP concentration incubating with between 1 and 3 mM SMP in acetate buffer for 60 min, in the dark, at RT. Prepare SMP just before use; (e) the sheet is washed with 0.05 M acetate buffer for 3 min; (f) add 0.05 M 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. Bubbles should be apparent. If the reagent is excessively hydrated and does not bubble, the treatment will not work properly; (g) wash for 3 min with PBS

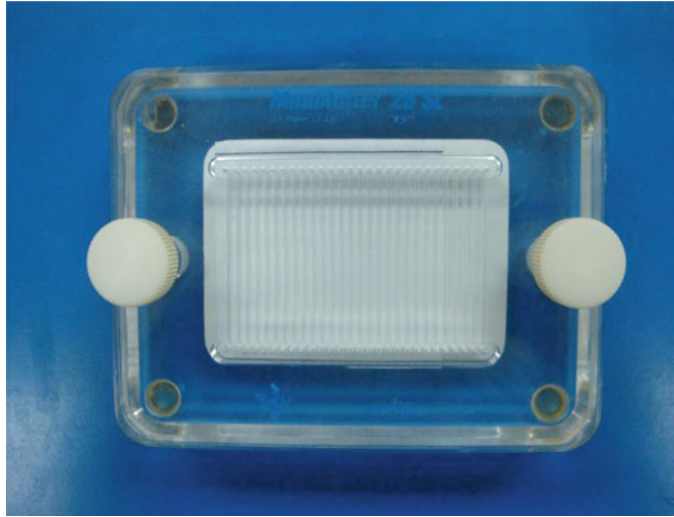


Fig. 26 A square 7.5×7.5 NC membrane is sensitized with antigens. After blocking, NC is placed again inside the Miniblotter[®] device positioned 90° respect to the original position. The reference line should be perpendicular to the channels, aligning perfectly the previous tracks in the paper with the grooves in the apparatus

with agitation; (h) continue the procedure as described previously (*see* Subheading 3.1, steps 11–16).

2. Cross-MABA: This is a simplification of the original technique, since no cutting, numbering or manipulation of strips is necessary. This variant of the technique is as follows: (a) a square 7.5×7.5 NC membrane is sensitized with antigens as described in Subheading 3.1, steps 1–10; (b) After blocking, the wet NC is placed again inside the Miniblotter[®] device positioned at an angle of 90° with respect to the original position, i.e., with the reference line perpendicular to the channels (Fig. 26), aligning perfectly the previous tracks in the paper with the grooves in the apparatus; (c) The Miniblotter[®] is closed tightly with the plastic cushion and water is removed; (d) patient sera are poured into the grooves, avoiding bubbles (1:100 or 1:200 in blocking solution) and incubation proceeds for 90 min; (e) Sera are removed with PBST, the membrane is placed in a tray and three washings are performed continuing the procedure as described previously in Subheading 3.1, steps 13–16. The whole NC paper is incubated with a secondary antibody and with the respective substrate. The resulting image can be analyzed with a grid that helps in the interpretation (Fig. 27). There are two limiting factors with this technique: only 17 antigens can be evaluated and only 16 sera.
3. Schematic diagram of the whole protocol of MABA is represented in Fig. 28.

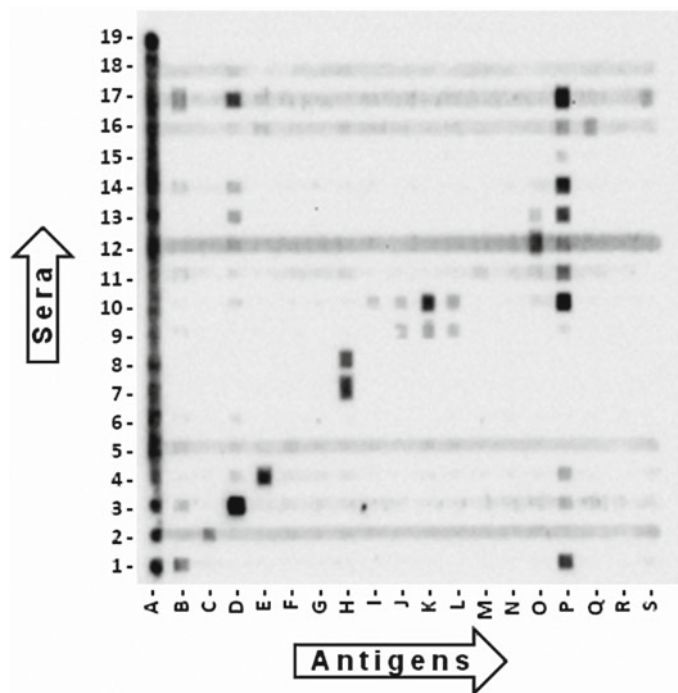


Fig. 27 Representative Cross-MABA

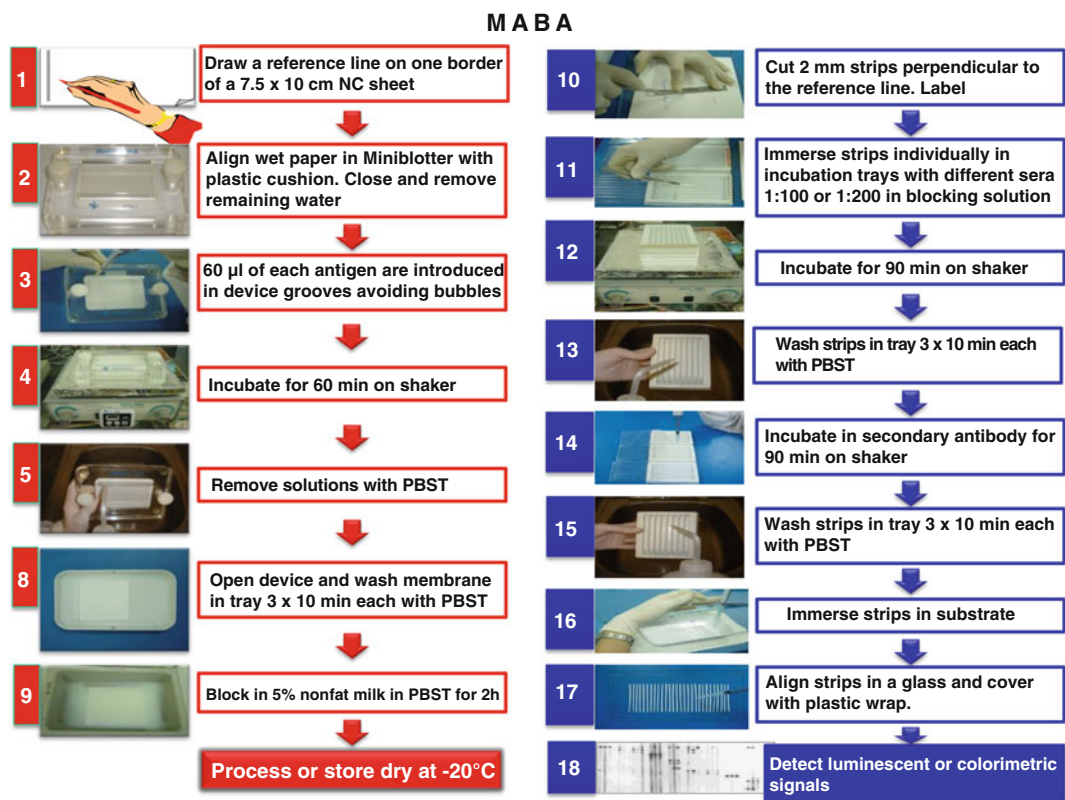


Fig. 28 Schematic diagram of the whole protocol of MABA is represented

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