Glm(f) Immunoblot Procedure

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Immunoglobulin allotyping can be valuable for discrimination purposes in the forensic laboratory. Current Gm typing procedures include hemagglutination-inhibition (HAI) and enzyme linked immunosorbant assay (ELISA). HAI, the most commonly employed method, has some disadvantages including a high frequency of interferring antibodies as well as being a labor intensive test. The ELISA test method, in microtiter plates, eliminates some of the problems with HAI. A drawback of this system is its technical complexity. To simplify the ELISA technique we have developed a nitrocellulose immunoblot (NIB) procedure.

MATERIALS AND METHODS

Bloodstains were prepared on clean cotton cloth from 208 blood samples (126 Blacks, 30 Caucasian, 31 Amerindian, 21 Mexican-American) drawn for the purpose of parentage testing. Serum/plasma was separated and stored at 4°C. All samples were tested for Glm(f) by HAI during routine paternity testing.

Sixty bloodstains stored at room temperature for 18 months were used as well as 16 bloodstains stored from 2-10 years. The blood samples from which these bloodstains were prepared had been tested for Glm(f) by HAI during routine paternity testing.

For routine paternity testing HAI is performed in V-microtiter plates as follows:

1) 25ul of appropriately diluted antiserum is added to test wells, 25ul of 3% BSA in saline is added to screen wells.
2) 25ul of a 1:20 dilution of serum is added to test wells and screen wells.
3) Plates are incubated at 18°C for 30 minutes.
4) 25ul of 0.2% coated red cells are added to all wells.
5) Plates are incubated at 18°C for 30 minutes.
6) Plates are centrifuged.
7) Plates are set on a 60° slant board for 5-10 minutes.
8) Plates are read by streaming.

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Evaluations made in developing the NIB procedure included membrane type, protein block, dilution of antibodies, and enzyme conjugate.

The NIB procedure used for this study was as follows:

1) Apply 2.5ul sample on nitrocellulose, dry overnight.
2) Block 1 hour in TBS (pH 7.5), 3% Liquid Hipere Gelatin, 5% Tween-20, .1% Hammersten casein.
3) Wash membrane 3 X 5 minutes in wash buffer (1% block solution in TBS).
4) Add to membrane 1:6000 dilution monoclonal anti-Glm(f) (BAM 17 from Seward Laboratory, England) in wash buffer. Agitate gently for 1 hour at room temperature.
5) Wash membrane 3 X 5 minutes in wash buffer.
6) To membrane add 1:12000 dilution of alk phos conjugated goat anti-mouse IgG (Sigma) in wash buffer. Agitate gently for 1 hour at room temperature.
7) Wash membrane 3 X 5 minutes in TBS.
8) Wash membrane X1 in stain buffer (TBS, pH 9.1).
9) To membrane add substrate mixture of NBT/BCIP in ratio 1:3 for 30 minutes; NBT (75mg NBT, .7ml dimthylformamide, .3 ml H_2O) and BCIP (50mg, 1 ml dimethylformamide).
10) Wash membrane briefly in deionized water blot dry.
11) Dry completely before scoring.

Bloodstains were extracted by placing two 4mm threads in 75 ul of 3% BSA for 1 hour at room temperature. Dilutions of 1:2, 1:5, and 1:10 were prepared.

Serum/plasma samples were prepared by making dilutions of 1:100, 1:200, and 1:300 in saline.

Positive and negative controls were run on each membrane in the following dilutions: Serum - 1:100, 1:200, 1:400, 1:800, 1:1000, 1:2000; Stains - Neat, 1:2, 1:5, 1:10, 1:20, 1:40. These controls were assigned a value based on the color development assessed visually on a scale of 0 to 4.

Each of the three dilutions on the unknown tested was assigned a numeric value by two readers after comparison to the controls. The values determined by each reader were added. The results were interpreted as follows: 0-5 - negative; 6-14 inconclusive/ repeat; and 15-24 - positive.

RESULTS

Membranes evaluated for use in the NIB procedure included: Biodyne (nylon), Genatran - 45 (nylon), Zetaprobe (nylon), Immulon (polyvinyl), and S & S nitrocellulose. The nitrocellulose membrane had the clearest background, the least non-specific binding, and was the least expensive. The enzyme

conjugates evaluated include alkaline phosphatase (ALP) and horseradish peroxidae (HRP). ALP substrates, unlike HRP, showed little reactivity with endrogenous enzymes found in serum/plasma.

Table one lists the results on the serum/stain samples. Inconclusive results by HAI include three samples that had a positive antibody screen and two in which duplicate testing did not agree. The NIB inconclusive results include four serum samples which were HAI Glm(f) negative, and five stain samples which were HAI Glm(f) positive.

Table two lists the results on the stored stain samples.

CONCLUSION

The NIB procedure we describe was found to be suitable for both serum/plasma samples and bloodstains. Advantages of testing for Glm(f) by NIB include the following: 1) low set up cost, 2) permanent record of results, 3) greater sensitivity and specificity, 4) minimal technician time/ skills, and 5) no interference with antibodies.

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Table 1

G1m(f) Results
Serum/Stain Samples

	HAI	NIB	
	Serum	Serum	Stain
Positive	90	91	86
Negative	113	113	116
Inconclusive	5	4	6

Table 2

G1m(f) Results
Stored Stains

	Positive	Negative	Inconclusive
App. 18 mo.			
NIB	30	27	3
Serum sample by HAI	32	26	2
2-10 years			
NIB	7	5	4
Serum sample by HAI	11	4	1