Allotyping By Immunoblot Using Polyclonal Antisera

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INTRODUCTION

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 interfering 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 the ELISA system is technical complexity. To simplify the ELISA technique we have developed a nitrocellulose immunoblot (NIB) procedure. This procedure was initially developed for use with monoclonal antibodies (Weston 1988). Since limited reagents were available, we wanted to determine if polyclonal antibodies could be utilized.

MATERIALS AND METHODS

Samples used for this study included the following: two year old bloodstains and corresponding serum/plasma samples (33 Black, 2 Caucasian, 15 Amerindian, 10 Mexican-American); bloodstains stored at room temperature for 18 months, and bloodstains stored from 4-12 years. Unless otherwise noted all samples were stored at -22°C. All bloodstains were prepared on clean cotton cloth from samples drawn for the purpose of parentage testing. The blood samples from which all bloodstains were prepared had been allotyped by HAI during routine paternity testing.

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

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

Three reagents were evaluated including anti-G3m(b_o) from Allotype (R-61), anti G3m(g) from Allotype (R-68), and anti-G3m(b_o) from the Netherlands Red Cross (3955). Evaluation included specificity and appropriate dilutions for samples and reagents.

The NIB procedure used for this study was as follows:

1. Apply 2 ul sample on nitrocellulose, dry overnight.
2. Block 1 hour in TBS (pH 7.5), 3% Liquid Hipure Gelatin, 5% Tween-20, .1% Hammarstein casein.
3. Wash membrane 10 minutes in wash buffer (1% block solution in TSB).
4. To membrane add 1:250 dilution polyclonal anti-Gm reagent in wash buffer. Rotate gently for 1 hr. at room temp.
5. Wash membrane 2 x 5 minutes in wash buffer.
6. To membrane add 1:5000 dilution of alk. phos. conjugated goat anti-rabbit (whole molecule IgG) in wash buffer. Rotate gently for 1 hour at room temp.
7. Wash membrane 3 x 5 minutes in TBS.

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NIB Procedure (continued)

8. Wash membrane in stain buffer (TBS, pH 9.1).
9. To membrane add substrate mixture of NBT/BCIP in ratio of 1:3 for 30 minutes. NBT (75 mg NBT, 0.7 ml dimethylformamide, 0.3 ml H_20) and BCIP (50 mg, 1 ml dimethylformamide).
10. Wash membrane briefly in deionized water, blot dry.
11. Dry completely before interpreting.

Bloodstains were extracted by placing six 4mm threads in 50 ul of deionized H_2O for 1 hour at room temperature. Doubling dilutions up to 1:64 were prepared. Serum/plasma samples were prepared by making dilutions of 1:250, 1:500, 1:1000, and 1:2000.

If staining occurred in any dilution, with the exception of the strongest, the test was interpreted as positive for the corresponding antigen. If staining occurred only in the strongest dilution the test was considered negative. If there was no staining in the strongest dilution the test was considered inconclusive.

Homozygous and heterozygous positive controls were used as well as a negative control.

RESULTS

Anti-G3m(b_o) polyclonal reagents were found unsuitable because of lack of specificity or sensitivity. Table One lists the results using anti-G3m(g) on frozen serum/stain samples. All stains show concordance between HAI and NIB for G3m(g) positive samples. On stains stored at room temperature (Table Two) no false positive were observed, however, on many samples results were inconclusive. This was not seen with stains stored for 4 - 12 years at -22°.

CONCLUSIONS

Polyclonal antisera for NIB must be carefully selected and are not as easy to use as monoclonal reagents. Use of this method required appropriate controls and antisera dilutions. Inconclusive results may occur if there is failure to elute the stain. Advantages of testing for GM by NIB with polyclonal reagents include the following: low set-up cost, permanent record of results, high sensitivity, minimal technician time and skills, no interfering antibodies, uncomplicated procedure, and small sample size.

Table One

G3m(g) Results

Serum/Stain Samples

	HAI	NIB	
	Serum	*Serum*	*Stain*
Positive	30	30	30
Negative	28	26	27
Inconclusive	2	4	3

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Table Two

Gm3(g) Results

Stored Stains

	Positive	Negative	Inconclusive
App. 18 mo.			
NIB*	6	16	8
Serum sample by HAI**	10	19	1
4–12 years			
NIB*	7	9	0
Serum sample by HAI**	8	8	0

*Stain stored at room temperature
**Test on fresh sample

REFERENCE

Weston-Kirkegaard S, Souhrada J, Copouls B, Dykes, DD, Polesky HF (1988) G1m(f) immunoblot procedure. In: Mayr WR, ed. Advances in forensic haemogenetics 2. Springer-Verlag, Berlin Heidelberg New York, p 466.

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