

# Generation and Characterization of Mouse Hybridomas Secreting Monoclonal Antibodies Specific for Human IgG3

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## Abstract

Mammals express several subclasses of the IgG molecule. In human being there are four homologous IgG subclasses, each of which is structurally unique and has different functions. Quantification of IgG subclasses is fundamental to clinical assessment and diagnosis of many diseases as such assessments depends on the availability of subclass-specific antibodies (Abs), particularly monoclonal antibodies (MAbs). In the present study, we produced and characterized two murine MAbs specific for human IgG3 molecule. These MAbs were obtained by the fusion of myeloma cells with splenocytes from Balb/c mice immunized with heavy chain of a human IgG3 myeloma protein. Fused cells were selected in hypoxanthine, aminopterin and thymidine (HAT) medium and cloned by limiting dilution assay. Ab-secreting cells were screened by enzyme-linked immunosorbent assay (ELISA) and the specificity of secreted MAbs was further analyzed, using a panel of purified myeloma proteins by ELISA and immunoblotting. Two stable hybridomas designated 1F18G7 and 1F18A11 were obtained secreting MAbs specific for Fc fragment of human IgG3. None of these MAbs showed cross-reactivity with other immunoglobulin isotypes derived from human and nine other animals, except 1F18A11 which displayed a weak cross-reactivity with only dog serum. Immunoblotting results indicate that these MAbs react with linear epitope(s) located in the heavy chain of human IgG3 molecules. The affinity constant of 1F18G7 and 1F18A11 MAbs was found to be  $0.81 \times 10^9 \text{ Mol}^{-1}$  and  $0.71 \times 10^9 \text{ Mol}^{-1}$ , respectively, as measured by ELISA. These two MAbs with relatively high affinity can be useful tools for quantification of IgG3 subclass levels in human serum.

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## Introduction

Different IgG subclasses are preferentially produced in response to different antigenic stimuli depending on the nature of the antigen (Ag) and the genetic background of the species<sup>(1-3)</sup>. Thus, while thymus-

dependent protein Ags (TD) elicit predominantly IgG1 and IgG3 subclasses, thymus-independent (TI) polysaccharide Ags tend to induce IgG2 antibody response. The profile of the IgG subclass may also be indicative of the

type of the implicated disease and its severity<sup>(4-8)</sup>. Analysis of the IgG subclass profiles to different antigens of a microorganism, reveals the kind of T helper associated response<sup>(9)</sup> and also is helpful for designing a potential vaccine<sup>(10)</sup>.

In human there are four IgG subclasses, each of which is structurally unique with different functions<sup>(11)</sup>. This is particularly evident concerning their ability to mediate complement activation and to bind Fc $\gamma$  receptors on the surface of cells involved in the immune response<sup>(12-14)</sup>. While there is more than 90% sequence homology between the constant domains of  $\gamma_1$ ,  $\gamma_2$ ,  $\gamma_3$  and  $\gamma_4$  heavy chains, more marked structural differences are present in the corresponding hinge region. IgG3 molecules have an extended hinge region relative to other subclasses<sup>(11)</sup>.

This proline-rich region confers higher flexibility and more susceptibility to proteolytic enzyme degradation to IgG3 molecule compared to other human IgG subclasses. Higher catalytic rate and stronger binding affinity to the Fc $\gamma$  receptors and the C1q complement component are also assumed to be associated to this unique character of IgG3<sup>(15)</sup>. A single miss-sense mutation (L368P) in the CH3 region of the human IgG3 was shown to be associated with impaired secretion of intact and functional Ig<sup>(16)</sup>. Hydrophilicity and accessibility of most of the hinge amino acid residues have made this region highly immunogenic one to which most of the IgG3 specific MAbs have so far been developed.

In the present study two MAbs specific to human IgG3 molecule were produced and characterized.

## Materials and Methods

### Preparation of purified human IgG subclasses

A panel of 27 different purified human IgG myeloma proteins of known IgG subclasses and light chain types was employed in this study. These myeloma proteins, obtained from patients with multiple myeloma, were either purified by diethyl aminoethyl (DEAE) cellulose (Whatmann, UK) chromatography

or by affinity chromatography using Staphylococcal protein A (SPA) or Streptococcal protein G (SPG) Sepharose 4B (Pharmacia, Sweden).

The heavy chain and light chain isotypes and subclasses of myeloma were identified using isotype-specific mouse MAbs including: AF6 (IgM), 8a4 (IgG), 2D7 (IgA), JA11 (IgD), C4 ( $\lambda$ ), 6e1 ( $\kappa$ ), JL512 (IgG1), GOM2 (IgG2), ZG4 (IgG3) and RJ4 (IgG4), kindly provided by Professor R. Jefferis (Department of Immunology, University of Birmingham, UK).

Polyclonal IgG was isolated from normal serum with SPG-Sepharose and polyclonal IgG3 was isolated, as breakthrough fraction, from polyclonal IgG by SPA-Sepharose column. Fc, Fab and F(ab')<sub>2</sub> fragments were prepared from several purified human myeloma proteins of each of the IgG subclasses, by pepsin and papain digestion<sup>(17)</sup>. Digested fragments were isolated by affinity chromatography with SPA-Sepharose column.

### Animal sera

Sera from human and nine animals were obtained prepared from their clotted blood. The animals used in this study were chicken, rabbit, guinea pig, cat, dog, sheep, goat, horse and monkey. The human serum was used as the control.

### Generation and selection of hybridomas

Balb/c mice (8-12 weeks of age) were immunized with four intraperitoneal injections of heavy chain from an IgG3 myeloma protein emulsified in Freund's complete adjuvant (Sigma, U.S.A) (first injection) or incomplete adjuvant (Sigma) (other injections) (50  $\mu$ g every 2 weeks).

Three days after the last injection, spleen cells were fused with SP2/0 myeloma cells (NCBI C129, National Cell Bank of Iran, Pasteur Institute of Iran, Tehran), using polyethyleneglycol (PEG 1500) (Sigma).

Hybridomas were grown in DMEM culture medium (Sigma) containing 20% fetal calf serum (FCS) (Seromed, Germany), penicillin (100 IU/ml) and streptomycin

(100 $\mu\text{g/ml}$ ) and supplemented with hypoxanthine ( $1\times 10^{-4}\text{M}$ ), aminopterin ( $4\times 10^{-7}\text{M}$ ) and thymidine ( $1.6\times 10^{-5}\text{M}$ ) (HAT) (Sigma). Ten to 14 days after fusion, secreting hybrids were identified by ELISA analysis of culture supernatants as described below. Selected antibody producing cultures were cloned by limiting dilution process according to the conventional methods<sup>(18)</sup>. Clones secreting antibody of desired reactivity were expanded in 25 and 75  $\text{cm}^2$  flasks (Nunc, Denmark), harvested and cryo-preserved in 40% fetal calf serum (FCS), 50% RPMI medium and 10% dimethylsulfoxide (DMSO) (Sigma).

#### ***Analysis of specificity of MAbs by indirect ELISA***

Microtiter polystyrene plates (Maxisorp, Nunc, Denmark) were coated with 1-10  $\mu\text{g/ml}$  of purified myeloma IgG subclasses or polyclonal IgG in PBS (0.15 M, pH=7.2). Then 0.05 ml of culture supernatant was added. Appropriate dilution of HRP-conjugated sheep antimouse Ig (prepared in our lab) was subsequently added and the reaction revealed with O-phenylenediamine dihydrochloride (OPD) (Sigma) substrate. Finally, the reaction was stopped with 20% H<sub>2</sub>SO<sub>4</sub> and the optical density (OD) measured by a multiscan ELISA reader (Organon Teknika, Bostel, Belgium) at 492nm.

#### ***Isotype determination of MAbs by capture ELISA***

Goat antimouse IgG1, IgG2a, IgG2b, IgG3, IgA and IgM (Sigma) at 1/1000 dilution, were adsorbed on to the wells of a microtitre ELISA plate (Nunc). Isotype of MAbs in culture supernatants was determined according to the ELISA technique mentioned above.

#### ***Affinity constant determination by ELISA***

We determined the affinity constant ( $K_{\text{aff}}$ ) by ELISA technique as described elsewhere<sup>(19)</sup>. Briefly, ELISA plates (Nunc) precoated with three different concentrations of human IgA2 ([Ag], [Ag'] and [Ag'']) were separately incubated with serial concentrations of each MAb. Sigmoid curves were constructed using

the OD values obtained for different concentrations of each MAb. Three non-overlapping curves were selected for each MAb to calculate the affinity constant. The half maximum OD (OD-50) was assigned for all selected curves from which the corresponding antibody concentrations (Ab, Ab', Ab'') were extrapolated. Accordingly, Ab and Ab' are the measurable total Ab concentrations at OD-50 and OD'-50 for plates coated with Ag and Ag', respectively. The affinity constant was determined using the following equation<sup>(20)</sup>:  $K_{\text{aff}} = (n-1)/2(n[\text{Ab}'t] - [\text{Ab}]t)$  where  $n = [\text{Ag}]/[\text{Ag}']$ .

#### ***Immunoblot analysis of MAbs***

Specificity of MAb was assessed by Immunoblotting technique as described elsewhere<sup>(21)</sup>. Briefly, affinity purified myeloma IgG subclasses, polyclonal IgG and their fragments were electrophoresed under native and denaturing conditions, in 10% polyacrylamide gel (SDS-PAGE) (Sigma) and then transferred to a nitrocellulose membrane (Schleicher & Schuell, Germany).

After blocking with 2.5% skim milk (Merck, Germany), the membrane was incubated with culture supernatants containing MAbs for 1.5 hours at 37°C, followed by HRP-conjugated sheep antimouse Ig. The bands were finally visualized with diaminobenzidine tetrahydrochloride (DAB) (Sigma) substrate.

## **Results**

#### ***Screening and selection of specific hybridomas***

Culture supernatants from growing hybridomas were screened by ELISA using a panel of four IgG myelomas with different subclasses, including the immunogen (heavy chain of IgG3). Representative results obtained for a number of hybridomas with different specificity profiles, including the parental 1F18G7 and 1F18A11 hybridomas are illustrated in Table 1.

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Table 1. Reactivity of representative hybridomas with selected IgG subclasses

Hybridoma	Screening Antigen			
	OD (492 nm)			
	IgG1(MM13)	IgG2(MM12)	IgG3(MM98)	IgG4(MM147)
3F2D8	1.5	1.6	1.9	1.6
6F11E1	1.1	1.2	0.2	1.3
1F18G7	0.1	0.2	1.6	0.2
1F18A8	0.15	0.2	2	0.3
Anti IgG1 (JL512)	1.2	0.27	0.3	0.27
Anti IgG2 (GOM2)	0.4	2	0.3	0.25
Anti IgG3 (ZG4)	0.3	0.3	1.9	0.3
Anti IgG4 (RJ4)	0.3	0.3	0.3	1.6

### Characterization of MAbs

Following cloning and subcloning, culture supernatant from the selected 1F18G7 and 1F18A11 hybridomas was further characterized. Electrophoresis of ascitic fluids revealed a sharp monoclonal band in the  $\gamma$ -globulin region (Figure 1), belonging to IgG1 isotype (Table 2). Specificity of the MAbs was determined, using a panel of purified myeloma proteins, including IgG1 (n=9), IgG2 (n=4), IgG3 (n=7) and IgG4 (n=7) subclasses. Our results demonstrated that both MAbs were specific for isotypic epitope (s) restricted to IgG3 subclass (Figure 2). The MAbs reacted only with Fc, but not Fab fragments of the IgG3 molecule (Figure 3). Immunoblotting studies demonstrated that both of our MAbs recognize sequential epitopes (Figures 4A and 4B) located on human IgG3 heavy chain.

Cross-reactivity studies employing whole sera from a range of animal species indicate that MAb 1F18A8 shows a weak cross-reactivity with only dog serum (Table 3).

### Determination of affinity of MAbs

The ascitic fluids of our MAbs were purified by affinity chromatography using

Table 2. Determination of the isotype of MAbs by ELISA

Isotype	MAbs		
	1F18G7	1F18A8	Control <sup>†</sup>
IgG1	0.7	0.8	0.16
IgG2a	0.1	0.13	0.14
IgG2b	0.12	0.1	0.1
IgG3	0.1	0.15	0.15
IgA	0.14	0.1	0.1
IgM	0.15	0.1	0.16

Control<sup>†</sup>. Culture supernatant from SP2/0 myeloma cells

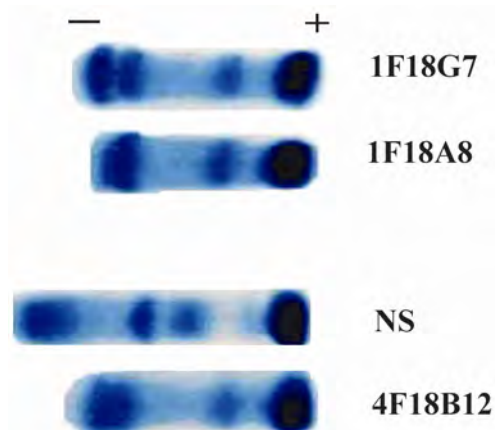


Figure 1. Acetate cellulose electrophoresis of ascitic fluids of 1F18G7 and 1F18A8 MAbs. NS=Normal serum; 4F18B12= Anti nG1m (a) isoallotype MAb employed as a control

Streptococcal protein G column and the affinity constant ( $K_{aff}$ ) was determined by ELISA. Triple serial concentrations of the antigen (IgG3) and MAbs were selected to construct the corresponding curves and extrapolate the  $K_{aff}$  values using the formula given in materials and methods. Representative curves obtained for the MAbs are illustrated in Figure 5 and the calculated average  $K_{aff}$  values are presented in Table 4.

### Discussion

In the present study we produced and characterized two murine MAbs specific for human IgG3 subclass. These MAbs were selected among a large collection of MAbs recognizing different epitopes located on human IgG subclasses. Several MAbs specific to human IgG3 subclass have been produced by other investigators<sup>(22-25)</sup>.

Table 3. Cross-reactivity of MAbs with animal sera

Animal sera	Cross-reactivity%*	
	1F18G7	1F18A8
Monkey	<0.1	<0.1
Rabbit	<0.1	<0.1
Guinea Pig	<0.1	<0.1
Sheep	<0.1	<0.1
Goat	<0.1	<0.1
Cat	<0.1	<0.1
Dog	<0.1	8
Horse	<0.1	<0.1
Hen	<0.1	<0.1

\* % Cross-reactivity is expressed relative to the value obtained for human pooled serum.



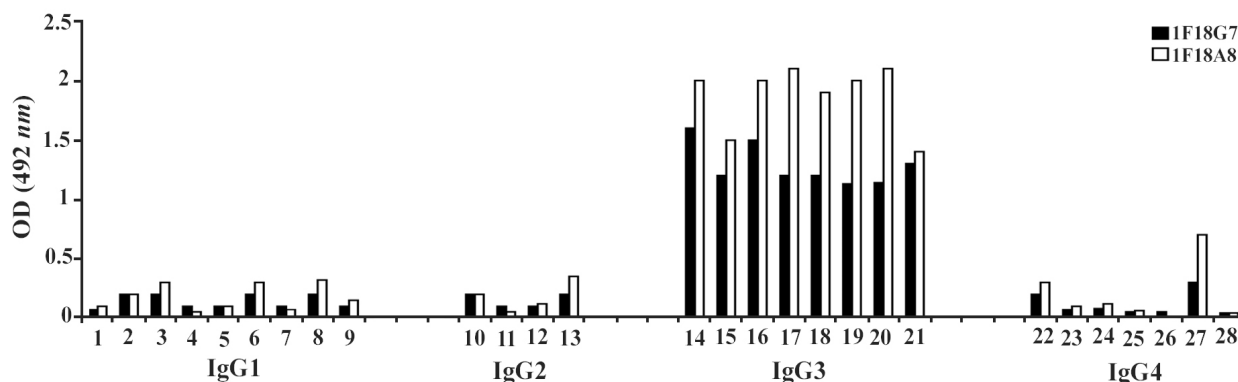


Figure 2. Reactivity of IgG3 specific MAbs with IgG subclasses  
Lanes 1 to 28 represent: MM1, MM11, MM24, MM46, MM50, MM53, MM63, MM65, MM13, MM12, G2 (M), MM62, Camp, MM98, Gale, Hay, Pol, Goe, Ren, Mcw, IgG3 polyclonal, MM147, Rea, Jan, Will, Cart, J. Wil, Ze

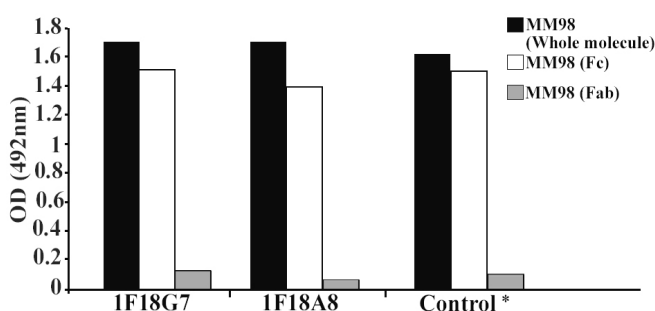


Figure 3. Reactivity of IgG3 specific MAbs with enzymatic fragments of MM98 (IgG3). Control represents a mouse MAbs (HP6050) with anti-IgG3 specificity

These MAbs were shown to be specific for isotypic epitopes of IgG3 subclass, including: B12A8 specific for IgG3<sup>(25)</sup>; HP6047<sup>(22,26)</sup>, HP6050<sup>(22,23,26)</sup>, HP6010, MYSJ33<sup>(22,23)</sup>, BRL1211<sup>(22)</sup>, HP6048 and HP6066<sup>(23)</sup>, with specificity for hinge region; HP6003, HP6004<sup>(22,23)</sup>, HP6005<sup>(23)</sup> and HP60193<sup>(24)</sup> with specificity for Fc region and HP6201<sup>(24)</sup> specific for F(ab')<sub>2</sub> fragments. All the above anti IgG3 MAbs, except MYSJ33 and HP6076 belonged to IgG1 subclass (like our MAbs). MYSJ33

Table 4. Affinity constants of h-IgG3 specific MAbs determined by ELISA

MAbs	Ag (ng/ml)	OD-50*	Ab at OD-50 (ng/ml)	K <sub>aff</sub> (M <sup>-1</sup> )	Average K <sub>aff</sub> (M <sup>-1</sup> )±SD
<b>1F18G7</b>					
	250	0.99	79	0.9×10 <sup>9</sup>	
	125	0.9	81	0.74×10 <sup>9</sup>	0.81×10 <sup>9</sup>
	62.5	0.8	91	0.79×10 <sup>9</sup>	
<b>1F18A8</b>					
	250	0.9	93	0.7×10 <sup>9</sup>	
	125	0.84	100	0.71×10 <sup>9</sup>	0.71×10 <sup>9</sup>
	62.5	0.69	103	0.71×10 <sup>9</sup>	

\*OD-50 represents the half maximum optical density obtained for a given concentration of h-IgG3 heavy chain ([Ag]) and the corresponding MAbs ([Ab]). The affinity constant (K<sub>aff</sub>) for each selected concentration of Ag and Ab was determined using the formula described in the Materials and Methods.

and HP6076 MAbs belonged to IgM and IgG2b isotypes, respectively. The specificity of these MAbs was evaluated with a wide range of assay protocols<sup>(23, 24)</sup>.

A panel of WHO specificity reference reagents (SRR) for IgG subclasses was established<sup>(27)</sup>. In the above studies purified IgG3 paraproteins<sup>(22-24)</sup> or IgG Fc region obtained from human plasma<sup>(25)</sup>, were used for immunization of Balb/C mice while we used a heavy chain of an IgG3 myeloma for immunization.

Thus our IgG3 specific MAbs can be seen useful tools for diagnosis of  $\gamma_3$ -heavy chain diseases as well. In addition we also determined the specificity of our MAbs by immunoblotting under reduced and non-reduced conditions and determined the nature of their epitopes. The most reliable IgG3 specific MAbs produced by other investigators are HP6048, HP6050 and HP6066 which recognize epitopes localized in the hinge region. This specificity may preclude or complicate their application in some conditions; since it has previously been demonstrated that reduction of disulfide bridges in the hinge region destroys these epitopes<sup>(23)</sup>.

Our MAbs, however, recognize linear epitopes on IgG3 heavy chains (under reduced conditions) suggesting stability of the corresponding epitope (s) and their applicability as suitable tools in different assay conditions. IgG3 subclass differs from other subclasses in hinge region and also in amino acid sequences at positions 276 and 291 in

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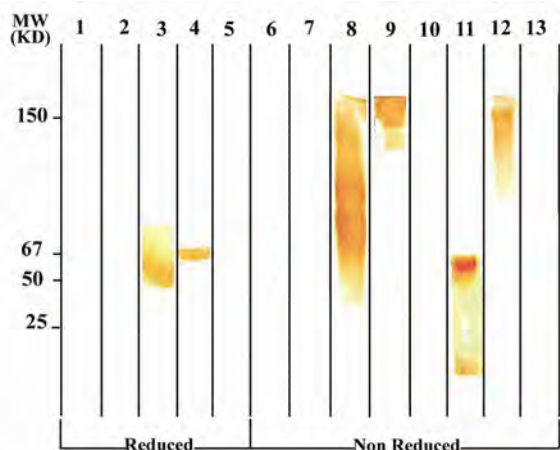


Figure 4A. Immunoblot analysis of 1F18G7 MAb reactivity with IgG subclasses. Lanes 1 to 5 represent reduced forms of MM1 (IgG1), MM12 (IgG2), Hay-ward (Heavy chain of IgG3), MM98 (IgG3) and MM147 (IgG4), respectively. Lanes 6 to 13 represent non reduced forms of MM1, MM12, Hay-ward, MM98 (whole molecule), MM98 (Fab), MM98 (Fc), IgG3 polyclonal and MM147, respectively.

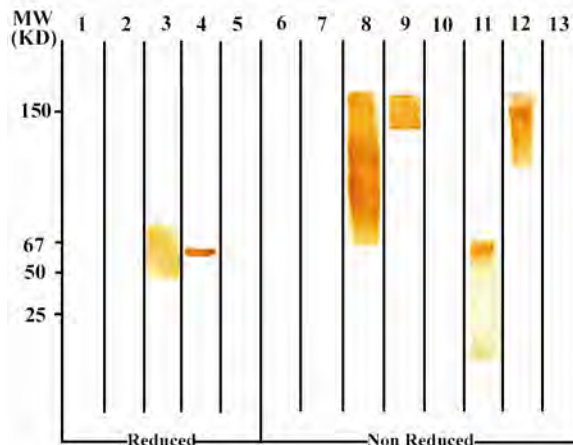


Figure 4B. Immunoblot analysis of 1F18A8 reactivity with IgG subclasses. See footnote to figure 4A.

CH2 domain and positions 392, 422 and 435 in CH3 domain. In IgG1, 2 and 4 subclasses, these amino acids are Asn, Pro, Lys, Val and His, whereas in IgG3 subclass they include Lys, Ile and Arg, respectively<sup>(28)</sup>. It seems that our MAbs recognize epitope(s) located in the CH2 or CH3 domain of human IgG3 subclass. The affinity constant ( $K_{\text{aff}}$ ) of some IgG3 specific MAbs have been determined previously<sup>(24, 29)</sup>.

The  $K_{\text{aff}}$  of HP6003 and HP6004 (anti-IgG3 Fc), HP6010, HP6047 and HP6050 (anti-IgG3 hinge) has been determined by fluorescent sequential-saturation assay<sup>(29)</sup> and found to be  $2.7 \times 10^7$ ,  $2.5 \times 10^7$ ,  $6.5 \times 10^7$ ,  $3.9 \times 10^7$  and  $5 \times 10^7 \text{ Mol}^{-1}$ , respectively. Immunoprecipitation studies have estimated

higher avidity for HP60193, HP60194, HP6201, HP6050 and HP60195 MAbs<sup>(29)</sup>.

Application of different methodologies for affinity determination may explain this discrepancy in part. We measured the affinity constant of our MAbs by an ELISA-based method. The affinity constant of both of our MAbs was found in the order of  $10^8 \text{ Mol}^{-1}$  (Table 4) which is much higher than many of the previously reported IgG3-specific MAbs.

One of our MAbs (1F18A8) showed a weak cross reactivity with dog serum. To the best of our knowledge this is the first report on the cross reactivity of a human IgG3 specific MAb with an animal serum. Canine IgG is composed of four subclasses which are defined as IgG1, IgG2, IgG3 and IgG4<sup>(30, 31)</sup>. Thus weak cross-reactivity of our MAb with dog serum, may suggest reactivity with dog IgG3.

Our MAbs with relatively high affinity

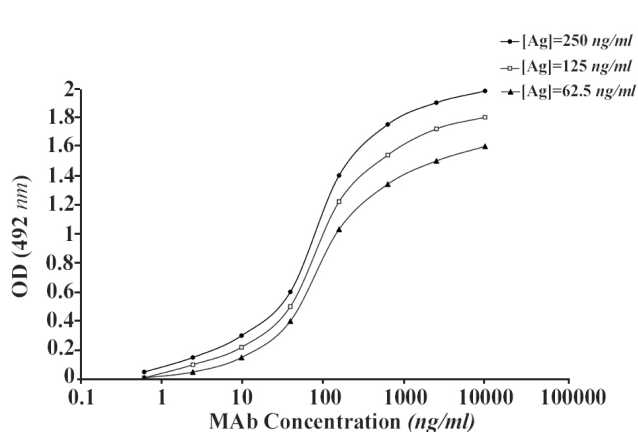


Figure 5A. Representative binding curves employed for extrapolation of affinity constant of 1F18G7 MAb.

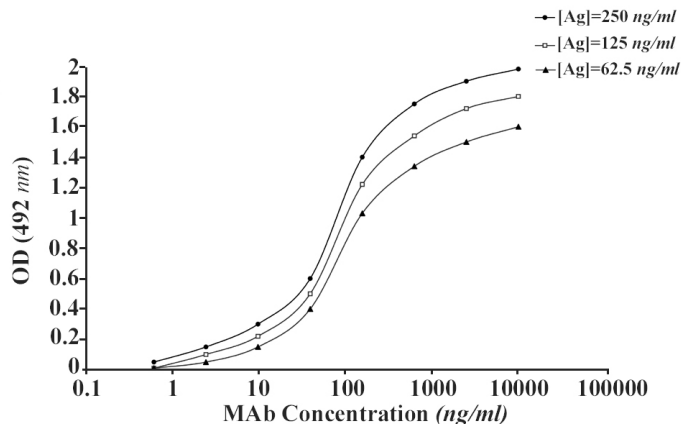


Figure 5B. Representative binding curves employed for extrapolation of affinity constant of 1F18A8 MAb.

for recognizing linear epitopes on IgG3 Fc could be used as suitable tools for quantification of IgG3 subclass in different clinical conditions and also be applied for epitope mapping of the human IgG3 subclass and its structural-functional analysis.

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