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Research paper

Quantitation of monoclonal antibody by capture ELISA based on initial enzyme activity rate

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ABSTRACT

We developed a noncompetitive two-site sandwich ELISA to quantitate monoclonal antibodies in culture supernatant. This assay measures the initial enzyme activity rate during the first minute of the reaction, which ensures linear velocity relative to time and a progress curve slope proportional to analyte concentration. During this period, the enzyme substrate is in large excess relative to the analyte/antibody-enzyme complex, and enzyme catalysis proceeds in steady-state conditions. Analyses of repeatability gave coefficients of variation between 4.4 and 9.7 (interassay) and 4.4 and 6.4 (intra-assay), and analyte detectability ranged from 5.8 to 12 ng/ml. The Z-factor calculated for analyte samples at their end dilution yielded mean values from 0.57 to 0.87, which confirmed assay robustness. This initial velocity-based sandwich ELISA is a simple, sensitive, reproducible method to quantitate bi-epitopic antigens.

1. Introduction

Enzyme-linked immunoassays (ELISA, EIA) are antibody-based analyses used to measure antigen-antibody interactions by an enzyme reaction. ELISA is a simple, flexible, low-cost technology that can be developed in a variety of innovative formats to implement sensitive, specific analytical tools for biomedical, environmental, and basic scientific research (Law et al., 2015; Peruski and Peruski Jr, 2003; Zheng et al., 2006).

Enzyme immunoassays are amenable to different formats based on the type of analyte studied (antigen or antibody), detection technique (competitive vs noncompetitive), or the separation method for bound and free reactants (homo- or heterogeneous) (Butler, 1994; Crowther, 2009). Heterogeneous non-competitive sandwich ELISA is performed in 96-well microtiter plates with anti-analyte-specific antibodies attached to the surface. Solid phase-bound antibodies are subsequently exposed to the analyte-containing sample and, after separation of free and bound reactants, the analyte-captured fraction is quantitated with a second antigen-specific antibody conjugated to a reporter enzyme. After a usually lengthy incubation period, product formation is measured by a single readout, from which the sample titer is derived. The main drawback of this method is that a single measurement of product formation does not ensure that it was obtained within the linear range of the progress curve, which is the condition that guarantees proportionality between enzyme reaction rate and analyte bound fraction, and ensures that enzyme substrate has not been depleted.

An ELISA method that circumvents these shortcomings is the so-called kinetic ELISA (k-ELISA) (Tsang et al., 1980). In this assay, the reaction rate is monitored continuously or at discrete time intervals during the linear phase period, when product formation is directly proportional to analyte concentration, substrate concentration is saturating, and the enzymatic catalysis operates in steady-state conditions (Tsang et al., 1983). k-ELISA has been developed in a variety of formats to quantitate antibodies to parasites (Hancock and Tsang, 1986; Werre et al., 2002), Lewis blood group antigens (Spitalnik et al., 1983), feline coronavirus (Barlough et al., 1983), flu virus glycoproteins (Snyder et al., 1988), and bacterial genera (Winter et al., 1983; Shin et al., 1993; Van Schaik et al., 2003).

To measure antibody concentration in murine serum and culture fluids, ELISAs were developed based on endpoint measurement (Barlough et al., 1983; Colino et al., 1996; Fleming and Pen, 1998; Mushens et al., 1993; Picard et al., 1996). In these assays, the reaction product is determined by a single readout taken after extended incubation, which raises doubts as to the quantitative accuracy of such assays. It has since become customary to follow signal development until sufficient color is observed, usually after a recommended 10-min

Abbreviations: GaM-HRP, Goat anti-mouse IgG-horseradish peroxidase; ShaM, Sheep anti-mouse IgG

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incubation (Crowther, 2009). This potentially inexact practice has not changed with the now-generalized use of mouse IgG ELISA kits; our survey of the suggested procedure for six commonly used commercial mouse IgG kits showed that a single measurement after 10 to 30 min enzyme development is the usual protocol. Most of these kits also leave construction of the calibration curve to the user; if not managed carefully, this can introduce additional uncertainty as to accuracy.

To overcome this limitation, we developed a variant k-ELISA to determine murine monoclonal antibody (mAb) concentration, in which reaction velocity is recorded with a colorimetric reporter molecule during the initial phase of the progress curve. As antibody quantitation is based on comparison of a sample of unknown concentration to the calibration curve, we first determined the linear range of the progress curve, and subsequently measured product formation within the time delimited by this range.

2. Materials and methods

2.1. Reagents and buffers

Materials and reagents used in these experiments included bicinchoninic acid (Thermo Fisher, Rockford, IL, USA), diethylamine (Fluka, Buchs, Switzerland), ELISA 96-well microplates (Maxi Sorp, 442,404; Nunc, Roskilde, DK), Costar high-binding plates (9018; Corning, NY, USA), High-trap Protein G HR and Sepharose CL-4B (both from GE-Healthcare Life Sciences, Chicago, IL, USA), Profinity epoxide resin (Bio-Rad, Madrid, Spain), Tween-20 and ortho-phenylenediamine (OPD) (Sigma, St. Louis, MO, USA). Buffers were PBS (phosphate-buffered saline, pH 7.4), PBST (PBS with 0.05% Tween-20), PBSM blocking buffer (5% non-fat dry milk in PBS with 10 μg/L NaN_3), and 0.1 M glycine-hydrochloride pH 2.5.

2.1.1. Antiglobulin reagents

Sheep anti-mouse IgG (ShaM) was isolated from hyperimmune serum by immunoadsorption (16 h, 4 °C) on a mouse IgG-Sepharose CL-4 column prepared as described (March et al., 1974). After incubation, the column was washed with PBS, eluted with 0.1 M glycine-hydrochloride pH 2.5, and the eluate dialyzed against PBS and filtered (0.45 μm pore size). To eliminate potential crossreactivity with goat IgG, ShaM was batch-incubated (1 h, 4 °C) with Profinity-goat IgG beads, centrifuged (2500 × *g*, 5 min, 4 °C), the supernatant dialyzed against PBS, filtered (0.22 μm pore), quantitated by absorbance ($A_{280\ nm}$ 1.4 = 1 mg/ml), aliquoted, and stored at −20 °C. Horseradish peroxidase-conjugated goat anti-mouse (GaM-HRP; 0.35 mg/ml) IgG γ-chain-specific antibody was from Southern Biotech (human-adsorbed; 1030.05, Birmingham, AL, USA).

2.1.2. Antibody reference standards and analytes (mAb)

Monoclonal antibodies used as IgG standards and analytes were prepared in-house (Monedero et al., 2019). The three mAb IgG standards were SIM 169–3 (IgG1; anti-bovine serum albumin), SIM 378–16 (IgG2b; anti-human C5adesArg), and SIM 65–8 (IgG2a; anti-human IgG); a polyclonal commercial mouse IgG (Charisela Technologies, Menlo Park, CA, USA) was used as IgG reference. SIM 169–3 and SIM 65–8 were isolated from culture supernatants by antigen-specific affinity chromatography, and SIM 378–16 using High-trap Protein G. Purified antibodies were quantitated by absorbance ($E_{1\%,}$ at 280 nm = 1.4) and by bicinchoninic acid plate assay (Smith et al., 1985), and homogeneity determined by 10% SDS-PAGE. To minimize error propagation in the preparation of IgG standards and analyte samples, all dilution series were prepared from master stock solutions, as indicated (Bisswanger, 2011). mAb used as analytes were SIM 247–3 (IgG1; anti-streptococcal enterotoxin B), SIM 256–8 (IgG2a; anti-TSST-1 [toxic shock syndrome toxin]), SIM 253–19 (IgG2b; anti-CT [cholera toxin]), SIM 119–6 (IgG3; anti-*Vibrio cholera* [Inaba strain]), SIM 47–15 (IgG1; anti-TNP [trinitrophenyl epitope]), and SIM169–3 (IgG3; anti-bovine serum albumin).

2.2. Determination of antiglobulin plate-coating time

Plate wells (quadruplicates) were sensitized with 50 μl ShaM at 1 or 5 μg/ml in PBS for 0, 2.5, 5, 10, 15, 20, 30, and 60 min (37 °C). Wells were washed three times with PBST, blocked (1 h, 37 °C) with 100 μl/well PBSM, and washed three times with PBST. mAb SIM47–15 culture supernatant (50 μl/well at 48 μg/ml) was added and incubated (1 h, 37 °C). Wells were washed three times with PBST, 50 μl GaM-HRP was added (diluted 1/500 in PBST) and incubated (1 h, 37 °C). Wells were washed three times with PBST and the enzyme reaction initiated with 70 μl/well HRP substrate (0.4 mg/ml OPD in citrate-phosphate buffer pH 4.8, with 33% H_2O_2 diluted 1/1000). After 60 s, the reaction was terminated by adding 30 μl/well 3 N H_2SO_4, and the product measured by absorbance (A_{492nm}) in a microplate reader (Anthos 2020, Salzburg, Austria). Reaction time was measured with a laboratory stopwatch timer. ELISA plates were incubated in a humidified plastic chamber.

2.3. Optimization of antiglobulin (ShaM) coating concentration

In a 96-well plastic ELISA plate, PBST (75 μl/well) was added to rows A-D of column 1 (control wells), and 75 μl ShaM (serially diluted 1:2 in PBS; from 62 ng/ml to 8 μg/ml) was added to rows A-D of columns 2–12. ShaM (50 μl at 5 μg/ml in PBS) was added to rows E-H of columns 1–12. The plate was sensitized (15 min, 37 °C), washed three times with PBST, and blocked with 100 μl PBSM (1 h, 37 °C). The plate was again washed thrice with PBST, and 75 μl purified mouse IgG (100 ng/ml) were added to rows A-D of columns 2–12, and 75 μl PBST to rows E-H of columns 1–12, and plates were incubated (16 h, 4 °C). Wells in rows E-H were washed with PBST, and 50 μl of the contents of wells in rows A-D transferred to the corresponding wells in rows E-H. The plate was incubated (1 h, 37 °C), washed three times with PBST, followed by 50 μl GaM-HRP (diluted 1/500 in PBST) to each well, and incubated (1 h, 37 °C). Wells were washed three times with PBST, the enzymatic reaction allowed to proceed for 60 s, and the products measured by absorbance at A_{492nm}.

2.4. Optimization of the GaM-HRP reporter dilution

Plate wells were coated with 50 μl ShaM (5 μg/ml in PBS; 15 min, 37 °C), washed three times with PBST, and blocked (1 h, 37 °C) with 100 μl PBSM. After three further washes with PBST, 50 μl mouse IgG at 0, 3, 5, 7, 10, 12, 15, or 20 ng/ml were added respectively to rows A-H, and incubated (1 h, 37 °C). Wells were washed three times with PBST, followed by 50 μl GaM-HRP, diluted 1/100, 1/200, 1/400, 1/800, 1/1000 and 1/2000 in PBST, added respectively to duplicate columns 1–2, 3–4, 5–6, 7–8, 9–10 and 11–12. Wells were incubated (1 h, 37 °C), washed three times with PBST, the reaction initiated, terminated at 60 s, and product formation measured at A_{492nm}.

2.5. Generation of the linear range of the progress curve

Two 96-well ELISA plates were coated (15 min, 37 °C) with ShaM (5 μg/ml), washed three times with PBST, and blocked with 100 μl PBSM (30 min, 37 °C). After three washes with PBST, 50 μl were added to the first plate of a 1–15 ng/ml dilution series of mAb SIM169–3 (IgG_1, standard), in triplicate to wells of columns 1–3 (1 μg), 4–6 (2 μg), 7–9 (4 μg) and 10–12 (6 μg); to the second plate, 50 μl were added to wells of columns 1–3 (8 μg), 4–6 (10 μg), 7–9 (12.5 μg) and 10–12 (15 μg). Plates were incubated (1 h, 37 °C), washed three times with PBST, 50 μl GaM-HRP (diluted 1/500 PBST) were added and incubated (1 h, 37 °C). Plates were washed three times with PBST and the reaction initiated with 70 μl HRP-substrate. The reaction was terminated with 30 μl 3 N H_2SO_4 after 0 s (row A), 10 s (B), 20 s (C), 40 s (D), 60 s (E), 80 s (F), 100 s (G) and 120 s (H) incubation. The product was measured

at A_{492nm}.

2.6. Quantitation of mAb concentration in culture supernatants

ELISA plates were coated with 50 µl ShaM (5 µg/ml in PBS; 15 min, 37 °C), washed three times with PBST, blocked with 100 µl/well PBSM (1 h, 37 °C), and plates were again washed thrice with PBST. To establish the IgG standard curve, 50 µl PBST were added to row A wells of columns 1–12 (background), and 50 µl of a 1 to 15 ng/ml dilution series of IgG standard (mAb SIM169–3), in triplicate, to wells of rows B–H (columns 1–3) of each plate as follows: row B (1 µg/ml), row C (2 µg/ml), row D (4 µg/ml), row E (6 µg/ml), row F (8 µg/ml), row G (10 µg/ml), row H (15 µg/ml).

Four to seven test analyte dilutions were used, depending on the assay. In most cases, we analyzed duplicate test samples of a seven-fold dilution series in rows B–H of columns 4–5, 6–7, 8–9, and 10–11, with 50 µl analyte in order of decreasing dilution (row B, 1/10,000 to row H, 1/3000). Plates were incubated in a humidified plastic chamber (16 h, 4 °C), then washed three times with PBST, after which 50 µl GaM-HRP (diluted 1/500 in PBST) was added to all wells, and incubated (1 h, 37 °C). Plates were washed three times with PBST, the enzyme reaction initiated by adding 70 µl HRP-substrate; after 60 s, the reaction was terminated with 30 µl/well 3 N H_2SO_4. The reaction product was measured by absorbance at 492 nm.

The IgG concentration in culture supernatants was derived from y-axis values ($A_{492\ nm}$) interpolated from a calibration curve with the GraFit 7 program. These values were multiplied by each sample dilution to convert concentration to µg/ml. mAb concentration was expressed as mean ± SD.

2.7. ELISA precision and robustness

Inter-assay variability was determined in eight ELISA assays performed using a seven-dilution series of five mAb supernatants within one week. Intra-assay variability was calculated from four mAb supernatants, each analyzed in four different plates, for a total of 36 measurements per mAb. The Z-factor for the samples was determined as described (Zhang et al., 1999). Figures were prepared and data analyzed using GraFit (version 7) software (Leatherbarrow, 2009).

3. Results

3.1. ELISA plate sensitization with ShaM

ShaM coating time was determined by measuring its adsorption kinetics at two concentrations, 1 and 5 µg/ml. Independently of ShaM concentration, binding reached maximum at 5 min incubation (37 °C; Fig. 1, inset); to ensure equilibrium, the sensitization reaction was extended to 15 min. We then defined optimal coating; plates were sensitized with a series of ShaM concentrations (62 ng/ml to 8 µg/ml), and assessed their capacity to bind IgG (75 µl of 100 ng/ml). Wells coated with ≥1 µg/ml ShaM showed maximum antibody capture. Nevertheless, in wells coated with 1 to 3 µg/ml ShaM, we detected a substantial fraction of free IgG, with ~65% (at 1 µg/ml) to ~20% (at 3 µg/ml); thus, independently of the ShaM sensitizing concentration, free antibody remained. The largest differences between bound and free antibody fractions were observed at ShaM concentrations > 3 µg/ml (Fig. 1). To ensure antiglobulin excess, plates were routinely coated with 5 µg/ml ShaM.

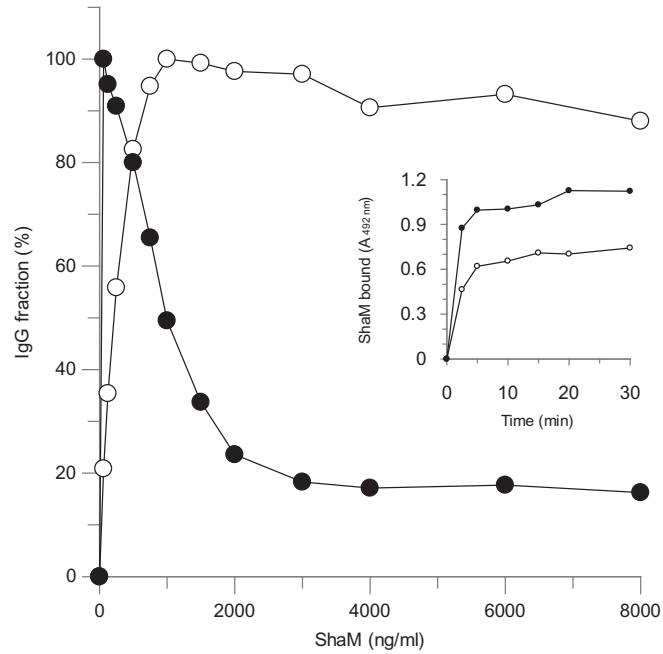

Fig. 1. Determination of IgG-captured fraction in plates sensitized at different ShaM concentrations. Percent fraction of free (●) and bound (○) IgG by ShaM adsorbed to plates (62 ng/ml to 8 µg/ml). Inset: ShaM adsorption kinetics at 1 (○) and 5 µg/ml (●).

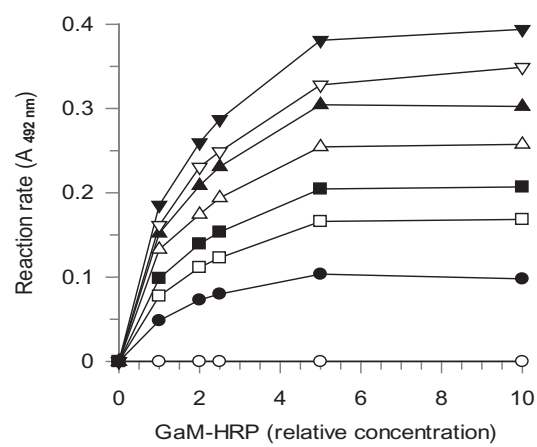

Fig. 2. Optimization of GaM-HRP dilution. ShaM (5 µg/ml)-coated plates were exposed to increasing IgG concentrations (0 to 20 ng/ml); IgG captured was measured by reaction with a GaM-HRP dilution series (1/100 to 1/2000). y-axis: IgG bound ($A_{492\ nm}$) at different IgG concentrations: 0 (○), 3 (●), 5 (□), 7 (■), 10 (Δ), 12 (▲), 15 (▽) and 20 (▼) ng/ml. x-axis: GaM-HRP dilution expressed as relative concentration; 1/2000 = 1, 1/1000 = 2, 1/800 = 2.5, 1/400 = 5, 1/200 = 10.

3.2. Determination of optimal GaM-HRP dilution

To calculate the GaM-HRP dilution that would lead to a molar excess of conjugate relative to analyte concentration, wells coated with 5 µg/ml ShaM were exposed to increasing concentrations of IgG (0 to 20 ng/ml), and bound antibody was measured using a GaM-HRP dilution series (1/100 to 1/2000). Binding to IgG generated a family of progress curves whose course rate was proportional to the IgG amount captured and to GaM-HRP dilution (Fig. 2). We therefore used GaM-HRP at a 1/500 dilution, an excess that also maintained low background signals.

3.3. Determination of the linear range of the progress curve

To build the progress curve, to ShaM-coated wells (5 µg/ml) we added a series of IgG standards (seven concentrations from 0 to 15 ng/ml), in triplicate. ShaM-IgG complexes were then incubated with a molar excess of GaM-HRP, and captured IgG was measured at fixed time intervals for the first 120 s of the reaction. The IgG capture rate

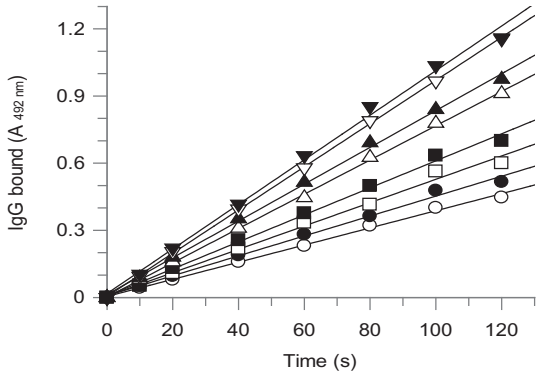

Fig. 3. Progress curve linear range. IgG standard (0 to 15 ng/ml) was added to ShaM (5 μg/ml)-coated plates and incubated. An excess of GaM-HRP was then added, and captured IgG was measured at fixed time intervals (0–120 s). x-axis: time intervals (seconds); y-axis: IgG binding rate ($A_{492\ nm}$) for each IgG concentration added (ng/ml): 1 (○), 2 (●), 4 (□), 6 (■), 8 (Δ), 10 (▲), 12.5 (▽), and 15 (▼).

increased linearly with incubation time from zero to 100 s; by 120 s incubation, the progress curve began to lose linearity. We used 60 s to register enzyme activity, as it fell in the middle of the linear range of the progress curve. When the enzyme signal was robust, we nevertheless found it advisable to measure reaction rate at a shorter incubation time (30–40 s), as proportionality between product formation and analyte concentration was better maintained.

A secondary graph was generated from the same data by plotting reaction velocity (IgG bound/time) against the IgG standard (analyte) concentration, which confirmed reaction linearity (Fig. 4).

3.4. Quantitation of mAb concentration in culture supernatant

A reference curve was built using a concentration series of three purified mAb, SIM 169–3, SIM 378–16 and SIM 65–8 (0, 3, 5, 7, 10, 12, and 15 ng/ml; Fig. 5A). To quantitate antibody, we used a seven-dilution series of five mAb supernatants (1/10,000 to 1/3000), and plotted data as IgG bound vs. relative mAb concentration in the samples. For the relative mAb concentration, we established 1/10,000 = 1, 1/8000 = 1.25, 1/7000 = 1.43, 1/6000 = 1.66, to 1/3000 = 3.33.

Reaction velocity followed a linear course proportional to IgG concentration in the samples (Fig. 5B). Concentrations of four sample mAb were determined by interpolating bound IgG values ($A_{492\ nm}$) from the IgG reference curve. At the highest dilution (1/10,000), mAb

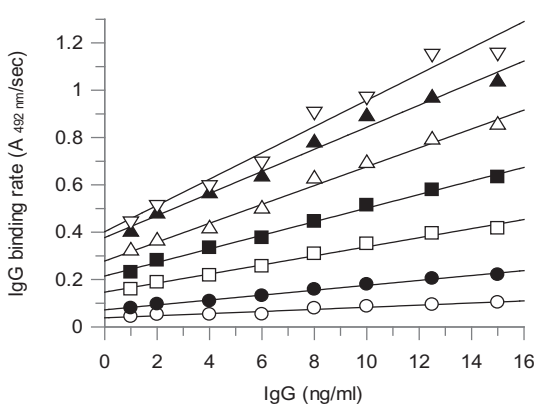

Fig. 4. Linear relationship between ELISA initial velocity (IgG bound/time) and IgG (analyte) concentration. Secondary plot generated from data in Fig. 3, represented as y-axis: reaction velocity (IgG bound at 10 s (○), 20 s (●), 40 s (□), 60 s (■), 80 s (Δ), 100 s (▲) and 120 s (▽) intervals vs. x-axis: IgG concentration.

concentrations were 8.8 ng/ml (SIM 169–3), 8.9 ng/ml (SIM 253–19), 11.8 ng/ml (SIM 256–8) and 13.6 ng/ml (SIM 247–3). Five additional mAb were quantitated in a second ELISA from a standard curve prepared with a polyclonal IgG (Fig. 6A), using a four-dilution series over several ranges, depending on mAb concentration in each supernatant. A plot of analyte bound vs. relative mAb concentration again produced a linear graph (Fig. 6B). mAb concentrations, calculated at a limiting dilution of 1/7000, ranged from 2.2 ng/ml (SIM 253–19) to 6.4 ng/ml (SIM 119–5).

3.5. Precision and assay robustness

Random variation in the ELISA was evaluated in a homogeneous group of mAb supernatants. Inter- and intra-assay coefficient of variation values (CV%) are shown in Table 1. Inter-assay variation ranged from 5.1 to 6.7, and mean variability (CV% ± SD) of three inter-assay studies performed over a 1.5-year period ranged from 4.4 ± 1.9 to 9.7 ± 3.3. Intra-assay variability was determined in a group of four mAb supernatants, with a seven-dilution series for each supernatant and four plates per mAb (36 measurements per mAb dilution); the tests were performed within a one-week period. The intra-assay CV% ranged from 4.4 to 6.4, and the mean Z-factor for the samples analyzed, calculated at the highest dilution, was between 0.57 and 0.88 (Table 1).

4. Discussion

To develop this quantitative ELISA, we first ensured that the reaction rate followed a linear time course, that the velocity gradient was proportional to analyte concentration, and that all reaction components, including capture antiglobulin (ShaM), indicator reagent (GaM-HRP), and enzyme substrate (OPD), were present in molar excess relative to analyte concentration (Murachi, 1981; Tsang et al., 1983).

Optimal plate coating with antiglobulin was determined by measuring IgG capture capacity of wells sensitized with different ShaM concentrations. Coating wells with 5 μg/ml ShaM produced the greatest difference between bound and free analyte fractions (Fig. 1). ShaM supersaturates at this concentration, and is reported to cause antibody multilayer formation (Butler, 2000). Protein adsorption to plastic inactivates ~75% of bound antibodies (Butler et al., 1993), which might explain why a > 2 log ShaM excess relative to IgG concentration was needed for optimal analyte capture.

IgG quantitation by reaction with the antiglobulin conjugate showed 1/500 GaM-HRP to be the highest dilution that accurately detected an IgG sample at 20 ng/ml. Before quantitation, mAb supernatants were normally serially diluted from 2000- to 10,000-fold. A 20 ng/ml IgG sample, pre-diluted 5000-fold, would initially contain 100 μg/ml IgG, which is high for a typical mAb supernatant. At the 0.35 mg/ml GaM-HRP concentration used, and assuming a 1:1 interaction between IgG and GaM-HRP, the GaM-HRP/IgG molecular ratio would be ~35/1 (calculated without considering the bi-paratopic properties of goat antiglobulin or the heterogeneous number of target IgG epitopes, and assuming one HRP molecule per conjugate). These data indicate that a 1/500 GaM-HRP dilution was a sufficient excess relative to the IgG analyte.

Data from two representative assays confirmed the linear course and the proportionality between reaction rate and analyte concentration (Figs. 5, 6). The ELISAs were quantitated using reference curves generated with two different IgG standards. During the initial reaction period, the HRP substrate is in great excess and the reaction velocity is proportional to ShaM saturation by the IgG-GaM-HRP complex; our experiments met these conditions, and the enzymatic reaction followed zero order kinetics.

The quantification limit of this ELISA at a sample dilution of 1/10,000 ranged from 8.8 ng/ml (SIM 169–3) to 13.6 ng/ml (SIM 247–3) (Fig. 5B), and at a 1/7000 dilution from 2.2 ng/ml (SIM 253–19) to 6.4 ng/ml (SIM 119–5) (Fig. 6B); in both cases, assay sensitivity might

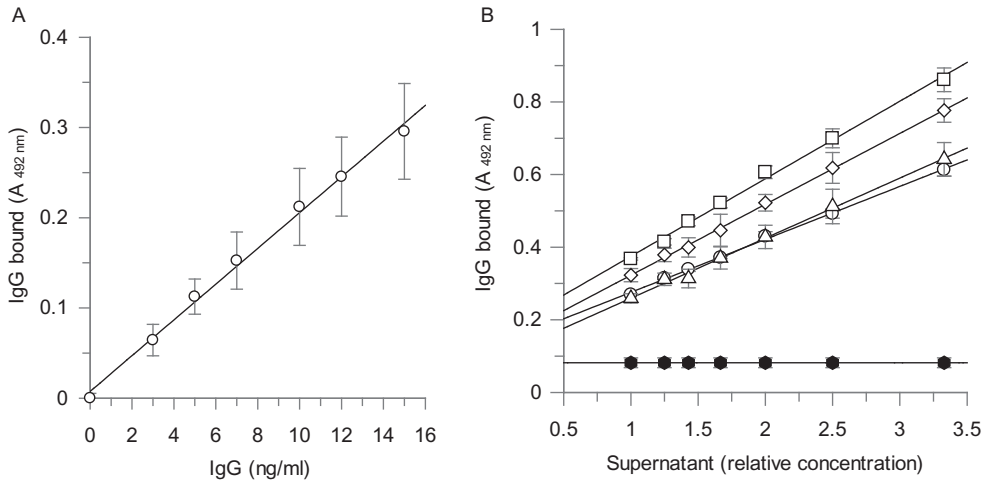

Fig. 5. mAb quantitation by ELISA based on a mAb-generated calibration curve. A. The reference curve indicates IgG bound (y-axis) vs. IgG standard (ng/ml) (x-axis). For the IgG standard, a concentration dilution series (0 to 15 ng/ml) was used for each of three purified mAb: SIM 169–3 (IgG_1), SIM 378–16 (IgG_{2b}), and SIM 65–8 (IgG_{2a}), after which data were pooled and rate data expressed as mean ± SD. B. Quantitation of four mAb samples. y-axis: IgG bound (mAb captured/min); x-axis: relative concentration for diluted mAb samples of SIM 247–3 (□), SIM 256–8 (◊), SIM 253–19 (Δ) and SIM 169–3 (○); background control (●). Relative concentrations were calculated; 1/10,000 = 1, 1/8000 = 1.25, 1/7000 = 1.43, 1/6000 = 1.66, 1/5000 = 2, 1/4000 = 2.5, 1/ 3000 = 3.33. Rate data are expressed as mean ± SD; for background data, mean ± 3 SD. All measurements were taken at 60 s reaction time.

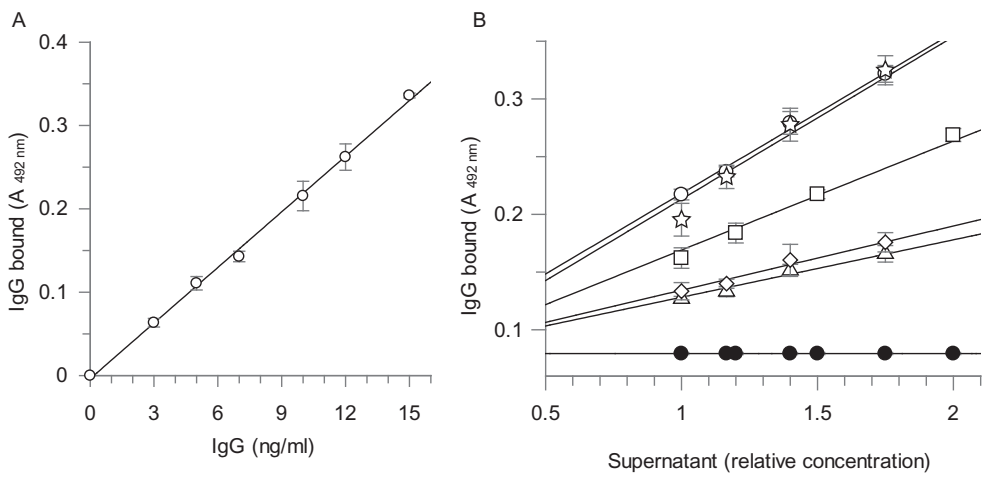

Fig. 6. mAb quantitation by ELISA using a polyclonal IgG calibration curve. A. The reference curve indicates IgG bound (y-axis) vs. IgG standard (ng/ml) (x-axis). For the IgG standard, a concentration dilution series (0 to 15 ng/ml) was used of a purified polyclonal murine IgG; rate values expressed as mean ± SD. B. Quantitation of five mAb samples. y-axis: IgG bound (mAb captured/min); x-axis: relative concentration for diluted mAb samples of SIM 119–5 (○), SIM 247–3 (☆), SIM 256–8 (◊) and SIM 253–19 (Δ) were calculated; 1/7000 = 1, 1/6000 = 1.166, 1/5000 = 1.4, and 1/4000 = 1.75, and for SIM 47–15 (□), 1/12,000 = 1, 1/10,000 = 1.2, 1/8000 = 1.5 and 1/6000 = 2; background control (●). Rate data are expressed as mean ± SD; for background data, mean ± 3 SD. All measurements were taken at 60 s reaction time.

Table 1
ELISA quality data. Inter- and intra-assay precision values for six mAb studied. Interassay variation values are derived from two groups of ELISA assays. Group I (n = 8) assays were performed within a one-week period; values for Group II show the coefficient of variation (CV% ± SD) for pooled data for three independent sets of ELISAs (n = 28–29) performed over a 1.5-year period. Intra-assay variation data were obtained from four mAb supernatants (n = 4) analyzed within a one-week period. ELISA robustness (Z-factor values) was determined from data for 9 assays (Figs. 5, 6), calculated at the highest analyte dilution. nd, not determined.

mAb superntant	Interassay variability I		Interassay variability II		Intra-assay variability		Z-factor	
	(n)	CV%	(n)	Mean (CV% ± SD)	(n)	CV%	ELISA (Fig. 5B)	ELISA (Fig. 6B)
SIM47–15 (IgG1)	8	5.5	nd	nd	4	4.4	nd	0.75
SIM253–19 (IgG2b)	8	5.9	29	6.2 ± 0.5	4	6.4	0.68	0.88
SIM256–8 (IgG2a)	8	6.7	28	7.3 ± 4.3	4	6.0	0.65	0.48
SIM247–3 (IgG1)	8	5.1	29	6.3 ± 1.3	nd	nd	0.92	0.68
SIM169–3 (IgG1)	8	6.1	29	9.7 ± 3.3	nd	nd	0.88	nd
SIM119–6 (IgG3)	nd	nd	29	4.4 ± 1.9	4	5.0	nd	0.90

be improved by further sample dilution. These values are similar to those reported for mouse (Bosworth Jr et al., 1983; Colino et al., 1996; Fleming and Pen, 1998; Mushens et al., 1993; Picard et al., 1996) and commercial IgG kits based on HRP/OPD detection, which probably approach the limits of assay sensitivity using this colorimetric method (Zhang et al., 2014).

Values (CV%) for precision (inter-assay) ranged from 4.4 (SIM119–6) to 9.7 (SIM169–3), and for repeatability (intra-assay) from 4.4 (SIM47–15) to 6.4 (SIM253–19) (Table 1), which fall within the accepted ranges for these parameters (Crowther, 2009; Findlay et al., 2000; Lee et al., 2006). All nine mAb supernatants yielded Z-factor values between 0.48 and 0.90; values in the range 1 > Z > 0.5 are indicative of a high-quality, robust assay (Zhang et al., 1999).

The accuracy of ELISA results depends greatly on the nature and quality of the analyte used to produce the reference curve. At equal concentrations, poly- and monoclonal IgG antibodies can produce standard curves with distinct gradients that generate quantitatively discrepant artifactual results (not shown). Possible causes for slope disparity between polyclonal and mAb IgG reference curves can be due to the different number and heterogeneity of epitopes recognized by

antiglobulin reagents. As suggested by Mushens et al. (1993), a suitable reference curve for mAb quantification could be based on a mAb of the same isotype as the analyte, which would eliminate potential antiglobulin reactivity differences.

5. Conclusions

We describe a non-competitive kinetic ELISA variant to quantitate mAb in culture supernatant. The method is based on the principle that sample measurements within the linear range of initial reaction velocity will yield assay accuracy and precision. Developed to measure mAb concentration, this ELISA format can be used to quantitate any bi-epitopic antigen, provided that analyte-specific antibodies are available, and is adaptable to other types of detection systems.

Declaration of Competing Interest

The authors declare no conflict of interest.

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