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Research paper

A fast and simple dot-immunobinding assay for quantification of mouse immunoglobulins in hybridoma culture supernatants

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Abstract

Mouse monoclonal antibodies of IgG subclasses and IgM class in hybridoma culture supernatants were quantified using a dot-immunobinding assay. Immunoglobulins were bound to nitrocellulose (NC) membrane and, after blocking, the membrane was incubated with anti-mouse antibody conjugated to horseradish peroxidase (HRP). Binding was revealed by incubation with a sensitive chemiluminiscence reagent. Quantitation was achieved by densitometric comparison with standard curves produced by purified monoclonal antibodies of the same subclass or purified antibodies of the same clone as the antibody to be quantified. These quantitative results were compared with those obtained using purified IgG from mouse serum or purified mouse myeloma IgM as standards. The dot-immunobinding assay requires 1 μl of hybridoma culture sample and takes about 1 h in total. Good linearity between the staining intensity and the amount of immobilized immunoglobulins was observed over the range of 0.05–5 ng/spot. The assay is simple, reproducible and can process simultaneously a large number of samples.
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Keywords: Dot-immunobinding assay; Hybridoma culture supernatants; Mouse immunoglobulins; Quantitation

1. Introduction

The widespread use of hybridoma technology for the production of specific monoclonal antibodies has created the need for simple, rapid, specific and sensitive methods for the quantification of mouse antibody production in hybridoma culture supernatants. An accurate determination of immunoglobulin levels in hybridoma culture supernatants is essential for a better understanding of the effect of physical parameters on hybridoma growth and immunoglobulin secretion. This aspect has increased in importance with the widespread use of fermentors for the production of monoclonal antibodies in vitro under controlled culture conditions in media with or without sera. Determination of immunoglobulin concentrations in supernatants is also necessary when establishing reproducible conditions in various immunochemical assays if monoclonal antibodies are available only in the form of culture supernatants. Typical monoclonal antibody levels in supernatants from stationary cell cultures range between 5 and 50 μg/ml, depending on the individual clone and cell

Abbreviations: BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; PBS, phosphate-buffered saline pH 7.2; NC, nitrocellulose.

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density (Goding, 1996). Much higher concentrations are reached in bioreactors.

A variety of methods have been devised for the determination of immunoglobulin concentrations in hybridoma supernatants. Most often, immunoglobulins are measured by a standard sandwich enzyme-linked immunosorbent assay (ELISA) using anti-immunoglobulin antibodies to capture the immunoglobulin to be quantified in a solid-phase system (Fleming and Pen, 1988; Delaunay et al., 1990; Mushens et al., 1993; Gupta and Siber, 1995). Generally, such methods are complex and time-consuming. Since this approach requires high-quality affinity-purified capture antibodies, often specific for immunoglobulin subclasses, the assays are relatively expensive.

The use of nitrocellulose (NC) for immobilization of macromolecules is well documented. Bound proteins can be stained by various staining methods and quantified by densitometry (Nakamura et al., 1985; Li et al., 1989; Dráber, 1991). Nitrocellulose membranes have also been employed as antigen carriers for sensitive screening assays of hybridoma supernatants (Bakkali et al., 1994).

Here, we describe a simple, rapid and cost-effective method for the quantification of mouse immunoglobulins in hybridoma culture media. The method consists of spotting 1-μl samples onto a NC membrane and staining the dots with anti-mouse antibodies conjugated with horseradish peroxidase (HRP). The concentration of immunoglobulins is determined from a calibration curve prepared from purified immunoglobulin.

2. Material and methods

2.1. Material

NC membranes BA 85 (Schleicher and Schuell, Dassel, Germany), Synpor 6 (Synthesia, Prague, Czech Republic) or PVDF membranes Immobilon P (Millipore, Bedford, NA, USA) with 0.45-μm pores were used. Protein G immobilized on agarose and molecular-mass markers for SDS–PAGE were obtained from Sigma-Aldrich (St. Louis, MO, USA). Non-fat dry milk was obtained from Hannaford (Scarborough, ME, USA). SuperSignal WestPico Chemiluminescent reagents were from Pierce (Rockford, IL, USA). Mouse-IgG ELISA kit was bought from Roche Diagnostics (Penzberg, Germany) and autoradiography films X-Omat AR were from Eastman Kodak (Rochester, NY, USA). Flat-bottom 96-well microplates were from Nunc (Roskilde, Denmark; Cat. No. 442404).

2.2. Antibodies

Purified mouse monoclonal antibodies of immunoglobulin subclasses IgG2a (MEM-188), IgG2b (E1–2.5), IgG3 (HL-39) and IgM (VI-01, KN-01, MEM-118) were obtained from EXBIO (Prague, Czech Republic). Monoclonal antibody TU-32 of the IgG1 class (Nováková et al., 1996) was purified from ascitic fluid on protein G (Harlow and Lane, 1999). Purified immunoglobulins were stored in phosphate-buffered saline (PBS). Purified IgG from mouse serum (Cat. No. I-5381) and purified IgM from mouse myeloma (Cat. No. M-3795) were from Sigma-Aldrich. Actual immunoglobulin concentrations were determined spectrophotometrically by applying the following extinction coefficients at 280 nm for 1% (w/v) solutions: IgG1, 13.5; IgM, 12.0 (Harlow and Lane, 1999). Protein solutions (1mg/ml) were diluted 1/10 with PBS or with cell culture medium containing 5–10% (v/v) fetal bovine serum (BioClot, Aidenbach, Germany). Further dilutions were performed with PBS containing 0.2% (w/v) SDS to give final protein concentrations ranging from 1 ng/ml to 50 μg/ml. Prepared dilution rows were dispensed in 5-μl aliquots and stored frozen at − 70 °C. Anti-mouse Ig antibody conjugated with HRP was purchased from Sigma-Aldrich (Cat. No. A-0412) and anti-mouse IgG antibody conjugated with HRP from Promega Biotec (Madison, WI, USA, Cat. No. W402B). Mouse monoclonal antibodies HTF-14 (IgG1), KN-02 (IgM), MA-05 (IgG3), MT-03 (IgG2b), NF-09 (IgG2a), TU-01 (IgG1), TU-16 (IgM) and TU-30 (IgG2b), in the form of spent culture supernatants, were prepared in the Laboratory of Cytoskeleton Biology, Institute of Molecular Genetics (Prague, Czech Republic).

2.3. Quantification of immunoglobulins

The NC membrane was cut to a suitable size, washed in water and blotted to filter paper. The

wetted NC membrane was positioned on top of a 96-well microtiter plate and placed on a light box. The round well boundaries were easily discernible on the NC membrane and provided a guide when applying the samples at regular distances. One-microliter aliquots of immunoglobulin from the prepared dilution row of standards were spotted into the centers of individual circles using an adjustable Eppendorf Research pipette (0.1–2.5 μl). The tested supernatants were diluted with 0.2% (w/v) SDS in PBS over a range from 1/10 to 1/100. Samples were loaded in triplicate. The loaded membrane was washed for 5 min in a large volume of TBST (10 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.05% (v/v) Tween 20) and then blocked for 15 min in 3% (w/v) bovine serum albumin (BSA) in TBST. After blocking, the NC membrane was incubated for 20 min at room temperature with anti-mouse antibody conjugated to HRP. The HRP-conjugated anti-mouse antibodies from Promega and Sigma were diluted 1/10,000 and 1/5000, respectively, with TBST containing 1% (w/v) of non-fat dry milk. After washing in TBST (three times for 3 min each), the bound secondary antibody was visualized by incubation with the chemiluminiscence reagent in accordance with the manufacturer's directions. All washings and incubation steps were done on a horizontal shaker. Exposed autoradiography films were quantified by densitometry using a gel documentation system GDS 7500 (UVP, Upland, CA, USA) and GelBase/GelBlot Pro analysis software (UVP). Alternatively, the chemiluminiscence signal was detected using a Luminescent Image Analyzer LAS-3000 (Fuji Photo Film, Düsseldorf, Germany). As the absorbance density of the spots was related to the amount of loaded immunoglobulin, calibration curves from standards were prepared by plotting the measured absorbance against the amount of immunoglobulin. Concentrations of immunoglobulin in the tested sample were then determined by comparing the corresponding absorbance with the calibration curve.

For comparison, the concentration of immunoglobulins in the tested supernatants was also determined by means of a Mouse-IgG ELISA kit exactly according to the manufacturer's directions. Flat-bottom 96-well microplates were used and the results evaluated with a microplate reader (Tecan-Austria, Groedig, Austria) at 405 nm.

2.4. Gel electrophoresis

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Laemmli (1970). The purified antibodies were diluted 1:1 in a two-times concentrated sample buffer containing 2-mercaptoethanol, the samples were boiled and 10-μl aliquots were loaded into the sample wells.

3. Results and discussion

Purified mouse monoclonal antibodies were used for the preparation of standard curves. The purity of immunoglobulins was verified by SDS–PAGE and exceeded 95% as demonstrated in Fig. 1. The immunostaining of standard amounts of immunoglobulins bound to the NC membrane was consistent, and as little as 1 pg was detectable in the case of antibody of the IgG1 class (Fig. 2). The dilution medium alone did not give any immunostaining. The diameter of the immunostained spots was smaller at low concentrations of immunoglobulin and larger at higher ones. Low concentrations of SDS in the dilution medium for standards and tested samples gave sharper bounderies of the spots. If a single 1-μl aliqot contained

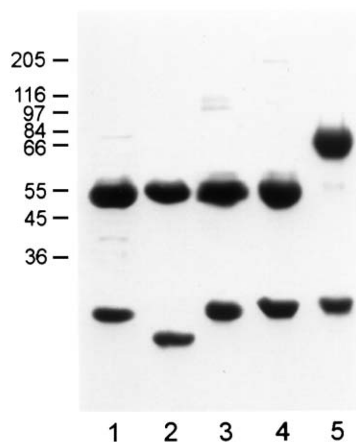

Fig. 1. Electrophoresis of purified mouse monoclonal immunoglobulins used for the preparation of calibration curves. Lane (1) IgG1 (TU-32), lane (2) IgG2a (MEM-188), lane (3) IgG2b (E1–2.5), lane (4) IgG3 (HL-39), lane (5) IgM (VI-01). Proteins were separated on 10% SDS–PAGE and stained by Coomassie Brilliant Blue R 250. Molecular-mass markers (in kDa) are indicated on the left.

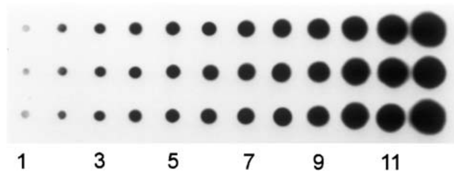

Fig. 2. A series of 1-μl aliquots of purified mouse immunoglobulin IgG1 spotted in triplicate on nitrocellulose and immunostained with anti-mouse IgG antibody conjugated with HRP (Promega). Samples containing 1, 5, 10, 50, 100, 250, 500, 750, 1000, 1500, 1750 and 2000 pg of IgG1 were successively applied at positions 1–12.

more than 2 ng of immunoglobulin, the dots were applied at a greater relative distance to prevent overlaps between adjacent immunostained dots. To eliminate variations in the detection limits of immunoglobulins due to differences in their immobilization on various sheets of the NC membrane or different conditions of X-ray film processing, standards were always applied on the same membrane as the tested samples. The binding capacities of NC membranes BA 85 and Synpor 6 for immunoglobulins of the IgG and IgM classes were much alike, and were comparable with the binding capacity of PVDF membrane Immobilon P. There were no differences in the results obtained by autoradiographic evaluation of immunostained spots and those obtained using the Luminescent Image Analyzer. Because the immobilization step in the dot-immunobinding assay was swift, the entire process could be completed in about 1 h, whereas the standard sandwich ELISA techniques may sometimes require several hours.

The relationship between the absorbance density of immunostained spots and the amount (ranging from 5 pg to 50 ng per spot) of loaded purified mouse immunoglobulins of IgG class is shown in Fig. 3A. There were some differences between the standard curves. While antibodies of subclasses IgG1, IgG2a and IgG2b gave standard curves with similar slopes, absorbance values for IgG3 at the same immunoglobulin concentrations were substantially lower. This was observed with anti-mouse Ig conjugates from both Promega and Sigma. The good linearity between the staining intensity and the amount of immunoglobulin held for the range of 50 pg to 5 ng (Fig. 3B). This corresponds to immunoglobulin concentrations between 50 ng/ml and 5 μg/ml. When purified IgG from mouse serum was used as a standard, the resulting standard curve had a slope resembling that obtained for G1 after staining with HRP conjugates from Promega or Sigma (not shown).

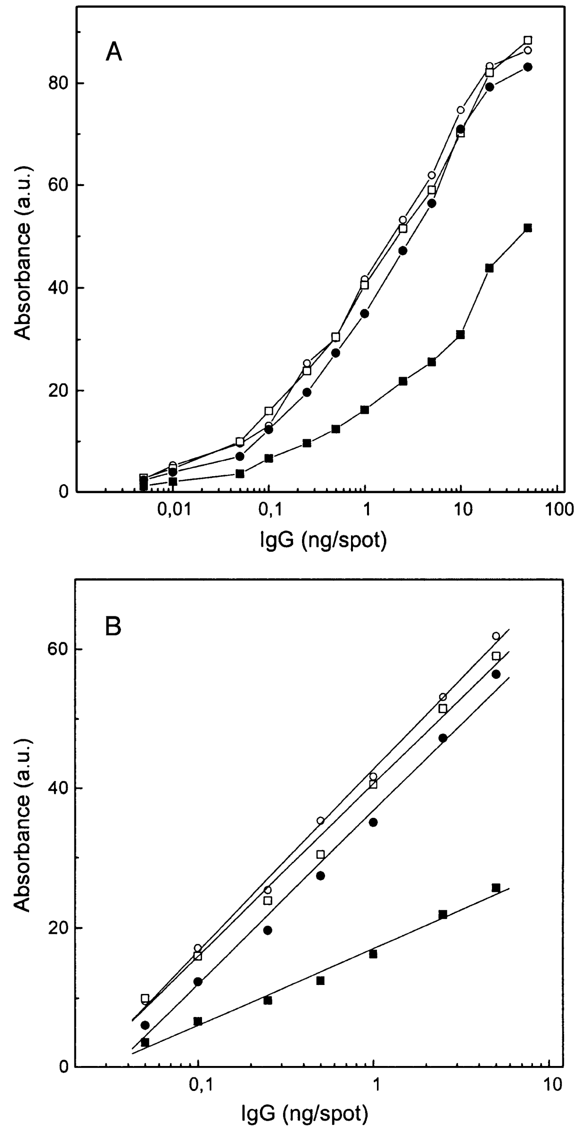

Fig. 3. Relationship between the absorbance (arbitrary units, a.u.) of immunostained spots and the amount of loaded purified mouse immunoglobulins of IgG subclasses detected with anti-mouse IgG antibody conjugated with HRP (Promega). (A) Absorbance of 1-μl spots containing immunoglobulins in the range 5 pg to 50 ng. (B) Absorbance of 1-μl spots containing immunoglobulins in the range 50 pg to 5 ng. IgG1(○), IgG2a (●), IgG2b (□), IgG3 (■). Each point represents the mean of three replicates.

The relationship between the absorbance of immunostained spots and the amount (ranging from 5 pg to 50 ng per spot) of three different purified mouse immunoglobulins of IgM class, detected with anti-mouse Ig antibody conjugated with HRP (Sigma), is shown in Fig. 4A. The purity of IgM antibodies MEM-188 and KN-01 was comparable to that of antibody VI-01 shown in Fig. 1. The good linearity between the staining intensity and the amount of immunoglobulin again held for the range from 50 pg to 5 ng (Fig. 4B). When purified IgM from mouse myeloma was used as a standard, the resulting standard curve had a slope resembling that obtained for purified IgM monoclonal antibodies after staining with anti-mouse HRP conjugate from Sigma (not shown).

As the hybridoma culture supernatants very often contain sera, standard curves for IgG and IgM antibodies were prepared from stock solutions (100 μg/ml) containing either PBS or medium with 5% or 10% fetal calf serum. In all cases the standard curves were very similar. Therefore, the presence of an excess of serum proteins did not interfere with the reproducibility of the assay. Moreover, the secondary antibodies used did not cross-react with fetal calf serum components present in culture media. This specificity thus makes it possible to measure mouse immunoglobulin concentrations in complex samples without first separating the antibody from interfering compounds. It has been shown that monoclonal antibodies in hybridoma culture supernatants effectively adsorb on NC in the presence of excess calf serum proteins (Hořejší and Hilgert, 1983).

Table 1 illustrates the quantification of purified monoclonal antibodies TU-32 (IgG1) and VI-01 (IgM) using as standards both the same purified subtype and purified serum IgG or myeloma IgM. The concentration of purified antibodies was determined by measuring their absorbance at 280 nm. Antibodies were then diluted to concentrations of 50 or 5 μg/ml in medium containing 10% serum, and the samples used for dot-immunobinding assay. Subclass standards appear to be superior for the accuracy of the assay.

A comparison of quantifications by the dot-immunobinding assay using corresponding subtype standards and purified serum IgG or myeloma IgM is shown in Table 2. The only substantial difference

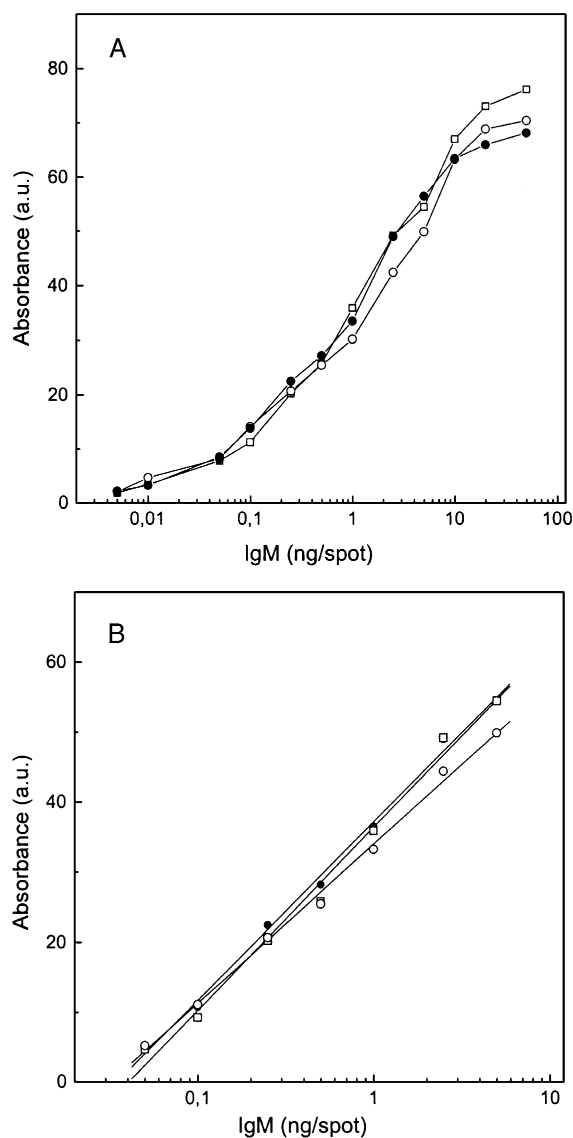

Fig. 4. Relationship between the absorbance (arbitrary units, a.u.) of immunostained spots and the amount of loaded purified mouse immunoglobulins of IgM class detected with anti-mouse Ig antibody conjugated with HRP (Sigma). (A) Absorbance of 1-μl spots containing immunoglobulins in the range 5 pg to 50 ng. (B) Absorbance of spots containing immunoglobulins in the range 50 pg to 5 ng. KN-01 (○), VI-01 (●), MEM-118 (□). Each point represents the mean of three replicates.

concerns the IgG3 subclass. Thus, while it is possible to use purified IgG from mouse serum when measuring the concentrations of IgG1, IgG2a and IgG2b subclasses, quantification of IgG3 antibodies neces-

Table 1
Quantification of purified mouse immunoglobulins using corresponding subtype standards or purified immunoglobulins

Antibody	Subclass	Subclass standard (μg/ml)[a]	Ig standard (μg/ml)[b]	A_{280nm} (μg/ml)
TU-32	IgG1	50 ± 1.5	51 ± 2.0	50.0
		5 ± 0.3	5 ± 0.4	5.0
VI-01	IgM	50 ± 2.1	51 ± 2.3	50.0
		5 ± 0.2	5 ± 0.5	5.0

Anti-mouse Ig antibody conjugated with HRP was from Sigma. Mean ± standard deviations were calculated from three determinations made in triplicate.

[a] Purified mouse monoclonal antibodies TU-32 (IgG1) and VI-01 (IgM) were used as subclass standards.

[b] Purified mouse serum IgG and purified mouse myeloma IgM were used as Ig standards for mouse monoclonal IgG1 and mouse monoclonal IgM, respectively.

sarily require the use of an IgG3 subtype standard. Mouse serum usually contains less IgG3 than other IgG subtypes (Ey et al., 1978), and this could explain the weak reaction with secondary anti-mouse Ig conjugates. Table 2 also presents alternative determinations obtained with a commercial Mouse-IgG ELISA kit. The data demonstrate that both the ELISA and the dot-immunobinding assay provide similar results.

The key element in the dot-immunobinding assay is the selection of proper secondary anti-mouse antibodies conjugated with peroxidase. The two tested anti-mouse antibodies (from Promega and Sigma) differed in their interaction with antibodies of IgM class. While the anti-mouse antibody from Promega reacted weakly, the anti-mouse antibody from Sigma gave a much stronger signal. The discrepancy could be eliminated by using secondary HRP-labelled antibodies specific for the light chains of immunoglobulins (Delaunay et al., 1990). On the other hand, when the calibration curves are based on subclass standards, secondary antibodies that are routinely used for immunoblotting detection can be applied.

As the dot-immunobinding assay uses only 1-μl aliqots, it is applicable to the determination of immunoglobulin concentrations higher than 50 ng/ml. This is less than in standard sandwich ELISAs using anti-immunoglobulin antibodies to capture the immunoglobulin, where the threshold is 1 ng/ml (Fleming and Pen, 1988). However, from a practical point of view, the sensitivity of the dot-immunobinding assay is sufficient for the determination of immunoglobulin concentrations in culture supernatants of established hybridoma cell lines. Since hybridoma supernatants are mostly diluted 1/10–1/100, errors originating from serial dilutions of tested samples were thus suppressed. It is possible to apply this assay to freeze-dried hybridoma culture supernatants containing trehalose after their reconstitution in water (Dráber et al., 1995). Similarly, it can be used with ascitic fluids, though in this case, the concentration of total mouse immunoglobulins is determined. The best application of the assay is in the case when batches of the same antibody are to be evaluated using a standard curve based on the identical purified antibody. Although the dot-immunobinding assay has so far been tested on mouse antibodies of IgG and IgM subclasses, one can anticipate that it could be used for quantification of immunoglobulins of other mouse classes (IgA, IgD, IgE) as well. Compared to the determination of immunoglobulin concentrations by Western blotting of hybridoma supernatants, the dot-immunobinding assay is faster.

Table 2
Quantification of immunoglobulins in hybridoma culture supernatants by dot-immunobinding assay and by ELISA

Antibody	Subclass	Dot-immunobinding assay		ELISA
		Subclass standard (μg/ml)[a]	Ig standard (μg/ml)[b]	Ig standard (μg/ml)[c]
TU-01	IgG1	8 ± 0.5	7 ± 0.4	7 ± 0.3
HTF-14	IgG1	10 ± 0.7	12 ± 1.0	12 ± 0.6
NF-09	IgG2a	8 ± 0.8	9 ± 0.6	8 ± 0.5
MT-03	IgG2b	5 ± 0.5	6 ± 0.6	6 ± 0.3
TU-30	IgG2b	21 ± 1.6	21 ± 1.5	22 ± 0.8
MA-05	IgG3	17 ± 1.1	2 ± 0.2	ND
TU-16	IgM	4 ± 0.3	4 ± 0.4	ND
KN-02	IgM	2 ± 0.2	2 ± 0.2	ND

Anti-mouse Ig antibody conjugated with HRP from Sigma was used for dot-immunobinding assays. Mean ± standard deviations were calculated from three determinations made in triplicate. ND, not determined.

[a] Purified mouse monoclonal antibodies TU-32 (IgG1), MEM-188 (IgG2a), E1–2.5 (IgG2b), HL-39 (IgG3) and VI-01 (IgM) were used as subclass standards.

[b] Purified mouse serum IgG and purified mouse myeloma IgM were used as Ig standards for mouse monoclonal IgG sublasses and mouse monoclonal IgM, respectively.

[c] Purified mouse monoclonal antibody of IgG2a subclass from the Mouse-IgG ELISA kit was used as a standard.

In conclusion, the present assay can be used to quantify mouse immunoglobulins in hybridoma supernatants. It has been developed for the quantification of antibodies in general and does not require immobilization via antigen or affinity purified capture antibodies. Quantification of the chemiluminescent signal is achieved by densitometry of exposed autoradiography films using gel documentation systems, or by luminescent image analyzers. The method is rapid, simple and cost-effective, needs only 1 μl of diluted sample and can easily process simultaneously a large number of samples.

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