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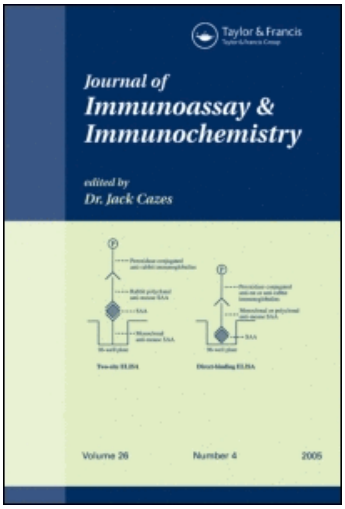

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THE QUANTITATION OF COUPLED BEAD ANTIBODY BY ENZYME-LINKED IMMUNOSORBENT ASSAY

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ABSTRACT

Quantitation of antibody coupled to a derivatized polystyrene bead through a bifunctional cross linker can be accomplished by a competitive enzyme-linked immunosorbent assay (ELISA) method. This sensitive method is less subject to interference than other protein assay methods such as bicinchoninic acid (BCA) or Lowry. The competitive ELISA method consists of incubating the coupled bead with a (20/80) weight ratio of goat anti mouse kappa alkaline phosphatase/goat anti mouse kappa (GAMKAP/GAMK) for 1.5 hours at 37° C, washing, adding p-nitrophenyl phosphate (PNPP) substrate, and reading the absorbance at 405/450 nm. A standard curve is established with radiolabeled antibody beads for microgram quantitation. (KEYWORDS: Enzyme-linked immunosorbent assay, goat anti mouse kappa alkaline phosphatase, goat anti mouse kappa)

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INTRODUCTION

The coupling or passive absorption of antibody to a solid support is of widespread use in the area of medical diagnostics (1,2). Convenient and sensitive detection methods are often needed to quantitate protein, antibody or enzymes bound to a solid support. A simple method for quantitating immobilized antibody is to measure the removal of protein from the absorbing or coupling solution by measuring absorbance at 280 nm or via a protein assay such as the Lowry (3) or BCA (4). The sensitivity of detection for these methods is low since only a few µg/mL of the protein are removed from the solution.

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Our method describes the quantitation of antibody coupled to a derivatized polystyrene bead by using alkaline phosphatase labeled goat anti mouse polyclonal antibody specific for the kappa chain. The enzyme-labeled GAMKAP is diluted with un-labeled GAMK to a (20/80) ratio to obtain maximum assay sensitivity. The labeled GAMKAP and the un-labeled GAMK compete for the kappa chains of the mouse antibody coupled to the bead. This ELISA assay can be adapted for nanogram range detection(5,6) as compared to the microgram detection range of the BCA protein assay (4,7).

MATERIALS AND METHODS

Reagents

Goat anti-mouse kappa, goat anti-mouse kappa alkaline phosphatase at 1 mg/mL (Southern Biotech), derivatized-

polystyrene beads (5/16"), 0.2mM p-nitrophenyl phosphate (pNPP), quench: 0.13 M EDTA, 0.18 M potassium phosphate, 0.15 % Triton X-100, pH 9.0. Dilution buffer for the conjugate is a phosphate buffered saline Tween solution (PBST): 0.5 % Tween-20, pH 7.4. The wash is a phosphate buffered saline/Tween-20 solution (PBST) : 0.1 % Tween-20, pH 7.4.

Bead ELISA Procedure

IgG mouse monoclonal antibody coupled beads are incubated in triplicate in plastic Sarstedt tubes with 0.25 mL of a (20/80) mixture of GAMKAP/GAMK at 5 µg/mL in PBST (0.5 % Tween-20/phosphate buffered saline) for 1.5 hours at 37 °C in a water bath shaker. Beads are washed 3X with (0.1% Tween-20/0.1M PBS, pH 7.4), and incubated with 0.2 mL of p-nitrophenyl phosphate substrate for 5 minutes. The reaction is stopped with 1.5 mL of quench and absorbances are read at 405 nm/450 nm.

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RESULTS

Bead ELISA Optimization

A series of experiments were conducted to optimize the sensitivity of detection for the bead ELISA. The optimized experimental parameters were the GAMKAP/GAMK concentration including ratio and volume, assay binding time, temperature and substrate dilution.

Determination of the Optimal GAMKAP/GAMK Ratio

Beads lots coupled with 0.2 µg and 1.25 µg of a mouse IgG_1 anti Human Choriogonadotropin (HCG) $F(ab')_2$, as determined by radiolabeling, were used as controls for the determination of the GAMKAP/GAMK concentration that gave the greatest signal difference. The following mixtures of GAMKAP to GAMK (10/90), (20/80), (30/70) were tested at a 10 µg/mL total concentration. To keep the 405 absorbance reading on scale, the (100/0) ratio was tested at 2 µg/mL, this gave the lowest signal difference because the enzyme level was still too concentrated causing some saturation of the 405 signal. The (20/80) ratio of GAMKAP/GAMK had the greatest difference in signal (Fig 1).

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Determination of the Optimal GAMKAP/GAMK Concentration

The (20/80) ratio of GAMKAP/GAMK was evaluated at the following total concentrations: 5, 10, 20, 40 µg/mL using anti-PSA antibody coupled beads at 0.2 and 1.25 µg per beads. The 5 µg/mL total concentration gave the maximum signal difference between the 0.2 µg and 1.25 µg beads (Fig. 2). Concentrations of the (20/80) GAMKAP/GAMK ratio below 5µg/mL may result in greater signal differences; however, the lower concentrations cause a decrease in the signal to noise ratio for the 0.2 µg bead.

Determination of the Optimal Time and Temperature for GAMKAP/GAMK Binding

The 0.2 and 1.25 µg anti-PSA antibody coupled beads were incubated with 0.25 mL of a (20/80) ratio of

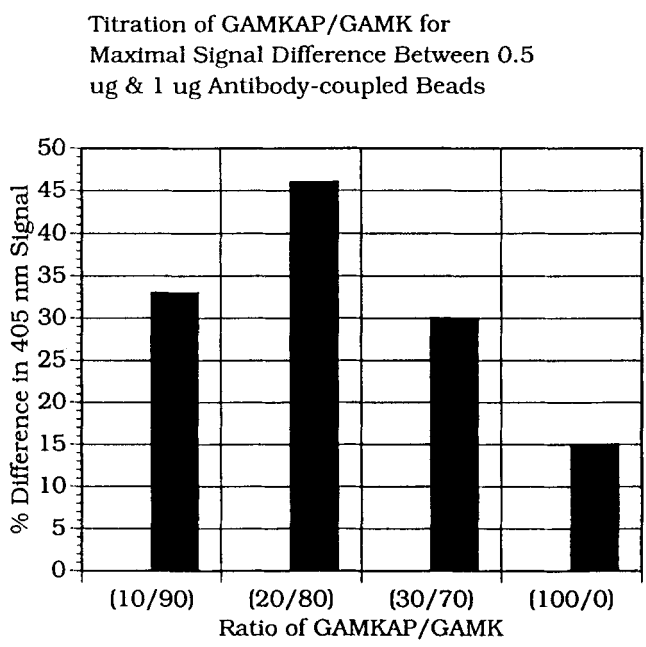

FIGURE 1

A triplicate set with 1.0 mL/bead of a 10 µg/mL solution of GAMKAP/GAMK in PBST tested at the above ratios. The 100% GAMKAP is at a concentration of 2 µg/mL. Beads were incubated for 1.5 hrs at 37° C in a water bath shaker. The 0.5 µg radiolabeled bead had 10,000 cpm and the 1 µg radiolabeled beads had 20,000 cpm of ^{125}I anti-HCG $F(ab')_2$.

GAMKAP/GAMK at 5 µg/mL total concentration for the 1, 1.5, 2, and 3 hours at room temperature and 37 °C to determine the optimal incubation time. A temperature of 37 °C and a binding time of 1.5 to 2 hours gave the best signal. GAMKAP/GAMK (10µg/mL) binding to the beads was compared at 0.25, 0.5 and 1.0 mL per bead. No significant signal differences were observed at these volumes. The 0.25 mL was

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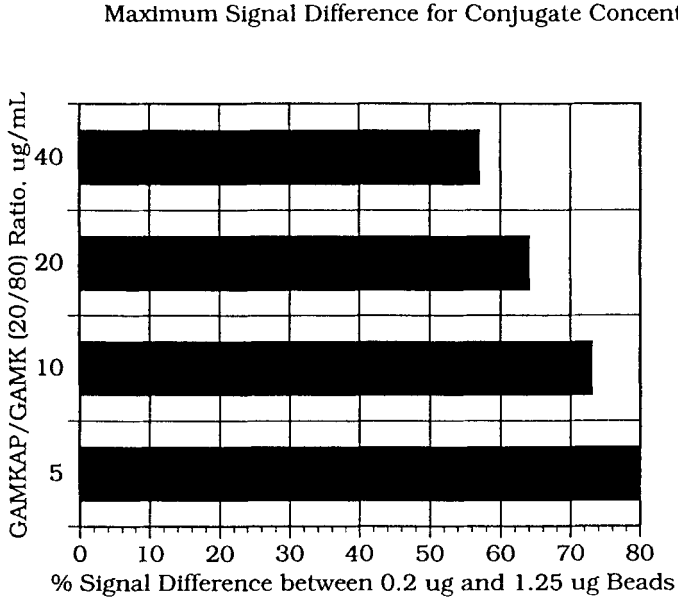

FIGURE 2

GAMKAP/GAMK at 5 µg/mL gave the optimal signal difference between the 1.25µg and 0.2 µg beads at OD_{405}.

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chosen because it is the smallest volume that can cover the bead.

The Correlation of Absorbance Signal and Cpms of Bead Antibody

A standard curve was generated by coupling antibody (91 % pure anti HCG by SECHPLC) to beads. Antibody per bead was 0.62 µg, 0.83 µg, 1.14 µg and 1.46 µg as determined by radiolabeling. Results indicate a linear correlation coefficient

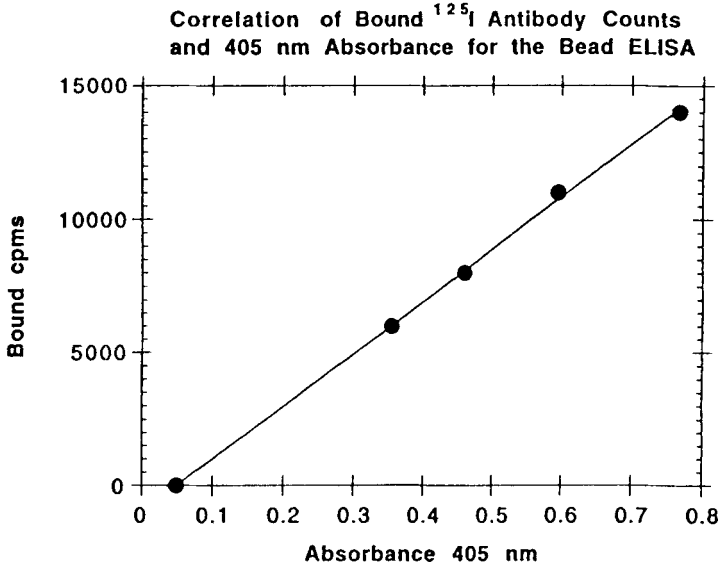

FIGURE 3

Standard Curve with Radiolabeled Beads
Beads were coupled with ^{125}I anti-HCG F(ab')$_2$. Standard curve consists of 0.62 µg, 0.83 µg, 1.14 µg and 1.46 µg F(ab')$_2$ per bead.

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of 0.999 between bead antibody cpms and the 405 signal (Fig. 3).

DISCUSSION

This bead ELISA assay can determine micrograms of bound bead antibody by reference to a standard curve generated with radiolabeled beads. The method is efficient for relative bead bound antibody determination based on a maximum coupled control bead. The ratio of the maximum to minimum

coupled beads can be used as an overall assay control to ensure assay consistency. This assay can be used in conjunction with an activity assay to determine antibody purity, functionality or loss of activity due to denaturation. Once a standard curve is established, the bead ELISA assay can be used to quickly screen large batches of beads based on OD_{405} readings for evaluation of the bead antibody coupling process, supports used, crosslinking reagents and process conditions.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; GAMK, goat anti mouse kappy; GAMKAP, goat anti mouse kappa alkaline phosphatase; PNPP, p-nitrophenyl phosphate; BCA, bicinchoninic acid; PBST, phosphate buffered saline Tween solution; HCG, human choriogonadotropin; PSA, prostate specific antigen.

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