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# Mimetic ligand-based affinity purification of immune complexes and immunoconjugates

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**Abstract**

We developed a simple purification method to purify alkaline phosphatase/anti-alkaline phosphatase IgG as immune complexes using mimetic affinity chromatography wherein the antibody was either a monospecific antibody, a bispecific antibody or a commercial polyclonal IgG conjugated with alkaline phosphatase (AP–IgG) covalently. The immune complexes or conjugates were efficiently bound on the mimetic Blue A6XL column and eluted under mild conditions (5–20 m*M* phosphate buffer). A similar strategy of purifying peroxidase/anti-peroxidase antibody complexes was also successfully demonstrated using the mimetic Red 3 column. Mimetic affinity chromatography thus appears to be a simple method to purify the desired monospecific or bispecific antibodies from the respective hybridomas and quadromas. © 1998 Elsevier Science B.V.

*Keywords:* Affinity ligands; Adsorbents; Antibodies; Enzymes

## 1. Introduction

Polyclonal and monoclonal antibodies conjugated with enzymes have been widely used in enzyme immunoassays [1] and some have been used in cancer therapy in combination with certain prodrugs [2]. Enzymes are measurable at very low concentrations due to their ability to convert a chemical substrate into a coloured, fluorescent or luminescent compound. Enzymes such as alkaline phosphatase (AP), and β-galactosidase conjugated with antibodies that are specific to tumor markers can be used to convert a prodrug into an active drug near tumor cells [2]. Chemical conjugation techniques have been used to produce most of the enzyme/antibody conjugates so far and problems encountered during the chemical conjugation procedures include aggregation, non-uniform ratios, inactivation of either molecule and reduced specific activity. Purification of enzyme–antibody conjugates is generally accomplished by gel filtration chromatography [3] and polymer precipitation [4,5]. However, these methods lack the specificity to efficiently remove the free enzyme label, free antibody molecules and inactivated conjugates. Specific affinity chromatography on protein A or protein G [6] or using specific immobilized antigens are also described, but these methods are plagued with problems due to elution with acid or chaotropic ions, which are commonly used [7].

In order to overcome the problems associated with crosslinking an enzyme to a monoclonal antibody by chemical methods, bispecific monoclonal antibodies (bsMabs) with one paratope specific for an enzyme

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and another paratope to an appropriate antigen have been developed [8]. These bsMabs have been prepared for various purposes, such as immunoassays [9], immunohistochemistry [8] and immunotherapy [10]. BsMabs can be developed by chemically crosslinking two different monoclonal antibody molecules [11], fusing two different hybridomas to generate hybrid-hybridomas (quadromas) [8] or by recombinant DNA-based techniques [12]. BsMabs produced by quadromas, however, secrete bispecific monoclonal antibodies as well as parental monoclonal antibodies. We have recently developed a hybridoma secreting monoclonal antibody (P92) against AP and utilizing this, we have also developed several quadromas with anti-tumor specificities in the second arm [e.g., P104: anti-cancer antigen 125 (CA125)×anti-AP; P105: anti-prostate specific antigen (PSA)×anti-AP]. Purification of the desired bsMab, free from both parental monoclonal antibodies, is desirable for applications in immunoassays and immunotherapeutic studies. Methods generally employed to purify bsMabs include ion-exchange chromatography [8] or affinity chromatography on an antigen-immobilized column [13]. The former method lacks specificity and the latter one normally incorporates elution conditions that are harsh to the antibodies purified. Peptide and non-peptide 'biospecific' or 'pseudospecific' mimetic ligands are becoming increasingly popular in the downstream processing of biotech drugs. Dye–ligand affinity chromatography is used in the isolation and purification of various enzymes [14] and non-enzyme proteins [15]. These immobilized dyes selectively bind to target proteins from different origins [15]. One such target protein, AP, is bound by a mimetic blue A6XL matrix in Tricine buffer and subsequently eluted by a phosphate buffer under mild conditions. We chose the Mimetic blue A6XL column to purify bispecific antibodies P105 and P104 as an AP/IgG immune complex from mouse ascites and cell culture supernatants. Mimetic blue A6XL adsorbent was originally used to purify AP from calf intestinal mucosa extracts [14]. In this report, we describe a simple, unique purification method to purify the enzyme/anti-enzyme bispecific antibody immune complexes as well as chemically crosslinked commercial enzyme/antibody conjugates by utilizing dye ligand affinity chromatography, wherein there is no risk of altering either the activity of the enzyme or the binding specificity of the antibody. This method is able to purify bispecific antibody–enzyme immune complexes, enzyme conjugated polyclonal or monoclonal antibodies, while maintaining high specific activity for immunoassays and cancer diagnostics.

## 2. Experimental

### 2.1. Materials

MIMETIC blue AP A6XL adsorbent and Red 1 A6XL were obtained from ProMetic BioSciences (Burtonsvile, MD, USA). Crude AP from calf intestinal mucosa, EC 3.1.3.1 (50 U/mg solid), peroxidase (HRPO) (120 U/mg solid) from horseradish, *p*-nitrophenyl phosphate, ABTS [2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid], molecular weight standards for SDS–PAGE (low molecular mass range), goat anti-mouse IgG (whole molecule), goat anti-rat (whole molecule), rabbit anti-mouse IgG conjugated with AP and Tricine were obtained from Sigma (St. Louis, MO, USA). Dialysis membrane, with a $M_r$ cut-off of 6000–8000 was from Spectrum Medical Industries (Los Angeles, CA, USA). The 96-well enzyme-linked immunosorbent assay (ELISA) plates were from Nunc (Napervill, IL, USA).

### 2.2. Antibodies and cell lines

B87.2 and B27.1 are anti-PSA and anti-CA125 IgG1 monoclonal antibodies, respectively, and were kindly provided by Biomira (Edmonton, Alberta, Canada). YP4 is a rat monoclonal antibody IgG2a against HRPO and has been described elsewhere [8]. P92.3 is a mouse hybridoma secreting monoclonal antibody IgG1 (P92) against calf intestinal AP. B80 and B43 are mouse hybridomas producing anti-PSA and anti-CA125 IgG1, respectively, and were also kindly provided by Biomira. P104 and P105 are two new quadromas that were developed in our laboratory. P105.2R 8.2.1 is a quadroma cell line derived by fusing the P92.3 and B80 hybridomas. P104.1R 3.2.1 is a quadroma cell line derived by fusing the P92.3 and B43 hybridomas. These cell lines were

maintained in RPMI-1640 media supplemented with 2 m*M* L-glutamine, 50 units/ml penicillin and streptomycin as well as 10% (v/v) fetal bovine serum (FBS) (GIBCO BRL, Gaithersburg, MD, USA).

### 2.3. Assays of enzyme activity and proteins

Alkaline phosphatase activities were assayed by measuring the production of the *p*-nitrophenolate anion at 405 nm [14]. Peroxidase activity was assayed by measuring the colorimetric product using ABTS and $H_2O_2$ at 405 nm. Protein concentration was determined by measuring the absorbance at 280 nm using a mass extinction coefficient of 1.35 absorbency units per mg/ml of protein for Mab IgG. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to previously published methods [16] using a Bio-Rad Mini-protein II dual slab cell along with low-molecular-mass standards. The gels were developed with silver stain as described earlier [17] or stained with Coomassie blue R-250 in methanol–glacial acetic acid–water (40:10:50, v/v/v).

### 2.4. Preparation of anti-AP hybridoma and anti AP IgG Mab

BALB/c mice were immunized intraperitoneally with crude AP following a standard immunization protocol. The hybridomas were produced by fusing the spleen cells from immunized mice with the SP2/0 myeloma cell line [18]. The ten positive clones that secrete high amounts of anti-AP IgG in their supernatant were screened as described in this paper. The isotypes of anti-AP subclones were determined with an Isostrip (Boehringer, Mannheim, Germany) kit. The best positive clone (P92.3) was selected based on affinity to AP and high stability in cell culture and was recloned twice by limiting dilution to ensure monoclonality. The clone was then expanded and inoculated into BALB/c mice that had been previously primed with 0.5 ml of Pristine (2,6,10,14-tetramethylpentadecane; Sigma) for ascites production. The anti-AP IgG was purified using ammonium sulfate precipitation followed by DE52 anion-exchange chromatography [8].

### 2.5. Selection of high affinity anti-AP hybridoma

A 96-well ELISA plate was coated with 100 μl of goat anti-mouse IgG (whole molecule), diluted 1:1000, overnight at 4°C. This amount of the solid phase reagent was found to be limiting and, hence, could roughly normalize the varying amount of mouse MAb found in raw culture supernatants at various growth phases. The plate was blocked with 1% skimmed milk in phosphate-buffered saline (PBS; pH 7.4). A 100-μl aliquot of cell culture supernatant collected from different clones was added to each well in duplicate and the plate was incubated at room temperature for 1 h. The plate was then washed three times with PBS containing 0.5% Tween-20 (PBST) and a 100-μl volume of AP at varying concentrations was added and incubated for another 1 h at room temperature, followed by a washing step. In the final step, a 100-μl volume of *p*-nitrophenyl phosphate (1.5 mg/ml) was added. The plate was incubated for 20 min and the optical density at 405 nm was measured.

### 2.6. Preparation of quadroma-producing bsMabs of anti-PSA×anti-AP (P105) and antiCA125×anti-AP (P104)

The development of these two quadromas has been described elsewhere [19]. Briefly, the two hybridomas, B80 and P92.3, were labelled with TRITC and FITC, respectively, fused in a cuvette with a BTX 200 electrofusion apparatus (BTX Inc., San Diego, CA, USA) and sorted by fluorescence-activated cell sorting (FACS) [19]. Cells with both dyes present were seeded into each well of a 96-well cell culture plate. The cells were grown in RPMI 1640 media supplied with 20% FBS and 2 m*M* L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin as well as 10% (v/v) origen (IGEN, MD, USA). After fourteen days, the clones were tested for enriched bsMab activities. The positive clones secreting bsMab anti-PSA×anti-AP were recloned using the limiting dilution technique, clones were then expanded and inoculated into BALB/c mice for ascites production. A P104 quadroma was generated using the same method by fusing hybridomas B43.13 and P92.3.

### 2.7. ELISA assays

#### 2.7.1. Detecting anti-AP IgG alone and anti-AP IgG/AP immune complexes

A 96-well ELISA plate was coated with 100 μl of PBS containing goat anti-mouse IgG (whole molecule), diluted 1:1000 (v/v) and was left overnight at 4°C. The coating solution was discarded and the non-specific binding sites on the plate were then blocked with 5% skimmed milk in PBS. The wells were washed three times with PBS containing 0.1% Tween 20 (PBST) and the column fractions were added at appropriate dilutions and the plates were washed three times after incubation. The anti-AP Mab activity (free) in the goat anti-mouse IgG-coated plate was detected using AP (10 μg/ml) and was washed three times. Subsequently, *p*-nitrophenyl phosphate was used as the chromogen for the color reaction. In order to detect pre-formed AP–anti AP complexes, no additional AP was added in the ELISA procedure while testing the column fractions.

#### 2.7.2. Detecting BsMab anti-PSA×anti-AP (P105) and BsMab P105/AP immune complexes

The coating and blocking procedures are as described in Section 2.7.1. Bispecific antibody activity was detected using a heterogeneous sandwich ELISA [20] as follows: A 25-μl volume of cell culture supernatant or elution fraction was tested together with 50 μl of PSA (260 μg/ml) and 25 μl of AP (40 μg/ml). They were added to a plate that had been coated with 1 mg/100 μl of Mab B87.1. The plate was incubated for 30 min at room temperature, following three washes with PBST. In the presence of the bispecific antibody, anti-PSA×anti-AP, a tetrameric complex will be formed and, upon addition of substrate, bsMAb activity will be detected. On the other hand, for the detection of the AP/P105 immune complex, all of the procedures are the same as for P105 alone, except for the addition of AP to the assay. Thus, only pre-formed immune complexes are detected.

#### 2.7.3. Detecting BsMab anti-CA125×anti-AP (P104) and BsMab P104/AP immune complexes

In order to detect the activity of P104, a B27.1-coated plate (1 mg/100 ml) was used. About 800 U of the CA125 [9] sample were added into the plate and incubated for 3 h at room temperature. Following a washing step, 50 μl of cell culture supernatant or elution fraction (diluted two-fold in Tricine buffer) were added, together with 50 μml of AP (20 μg/ml), and the plate was incubated for 45 min. The final step was the same as described in Section 2.7.2. In addition, the method used to detect the AP/P104 immune complex was the same as that for P104, as mentioned in Section 2.7.2, excluding the addition of AP. The activity of rabbit anti-mouse IgG–AP immunoconjugates in the fractions was detected in a manner similar to that described for the P105–AP immunoassay.

#### 2.7.4. Inhibition assay to estimate contaminating monoclonal antibodies against CA125, in BsMab P104 fractions purified using a DE52 column, and P104–AP fractions, purified using a mimetic blue affinity column

The 96-well ELISA plate prepared for P104 (Section 2.7.3) was used. About 100 μl of solution containing 1000 U/ml of CA125 were added and the plate was incubated for 3 h at room temperature (RT). Following a washing step, 50 μl of sample, together with 50 μl of P104–AP purified from a mimetic blue column, were added and the plate was incubated for 45 min at RT [9]. The rest of the steps were the same as those described in Section 2.7.3 for P104 activity. The presence of more monospecific monoclonal anti-CA125 antibody in the fraction would indicate that inhibition of P104 activity had occurred.

#### 2.7.5. ELISA assays for HRPO–anti HRPO IgG immune complexes and free anti-HRPO IgG

A 96-well ELISA plate was coated with 100 μl of goat anti-rat IgG (whole molecule), diluted 1:1000 (v/v), and incubated overnight at 4°C, blocked and washed as described above. A 100-μl aliquot of each fraction was added to each well in duplicate, and incubated at room temperature for 1 h. This step would bind any YP4 Mab with or without HRPO in its paratope. The pre-formed complexes are easily detected by the HRPO substrates, ABTS (0.5 m*M*) and $H_2O_2$ (3 μ*M*), following incubation for 20 min, and the optical density was measured at 405 nm. The elution profile of free YP4 was obtained by the

addition of HRPO to each fraction and processing as above.

### 2.8. Purification method using a mimetic blue A6XL column

The different crude samples (AP, P92; P92–AP) were dialyzed against three changes of 10 m*M* Tricine–NaOH buffer for 16 h at 4°C. The samples were applied separately at a flow-rate of 3 ml/h to a column containing 2 ml of mimetic blue AP A6XL absorbent pre-equilibrated with the Tricine buffer. The column was washed with the same buffer until the absorbance of the eluate at 280 nm was negligible. The bound proteins were eluted using 10 m*M* Tricine–NaOH containing 5 m*M* potassium phosphate. Fractions, approx. 1 ml in volume, were collected until the absorbance at 280 nm returned to baseline. The column was finally washed with 0.75 *M* potassium chloride and 1 ml fractions were again collected.

### 2.9. Purification of bispecific antibody anti-PSA× anti-AP (P105) and P105–AP immune complex

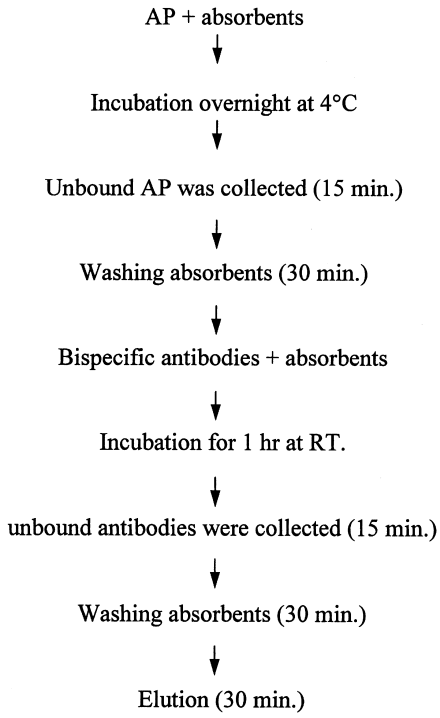
First, 5 ml of crude sample from diluted mouse ascites containing 0.73 mg/ml IgG (anti-AP IgG; anti-PSA IgG; anti-PSA×anti-AP IgG, etc.) were loaded on a 10-ml mimetic blue column at a flow-rate of 3 ml/h and left overnight at 4°C for maximal binding. After all of the unbound fraction was collected, the column was washed with 100 ml of 10 m*M* Tricine buffer. The bound fractions (2 ml) were eluted using a 5 m*M* phosphate–Tricine buffer (pH 8.5), and assayed for P105 activity. In a parallel purification of P105–AP immune complex, 50 mg of AP (20 U/mg) were loaded first on the mimetic blue column. Following the washing step, the same amount of P105 as described above was loaded, eluted and then the fractions were assayed.

### 2.10. Purification of commercial polyclonal IgG conjugated with AP

A 400-μl volume of commercial AP conjugated IgG (about 6300 U of AP) was diluted to 12 ml with Tricine buffer and dialyzed overnight against three changes of Tricine buffer at 4°C. The dialyzed sample was purified on a column containing 10 ml of mimetic blue A6XL absorbent, which had been pre-equilibrated with Tricine buffer as described above.

### 2.11. A simplified method to purify bispecific monoclonal antibody with one paratope against AP

A 50-mg amount of AP (20 U/mg solid) was dissolved in 5 ml of Tricine buffer, pH 8.5, and dialyzed against three changes of the same buffer at 4°C. The AP was incubated in 15 ml of Tricine buffer with 10 ml of mimetic blue absorbent overnight at 4°C. Then, the absorbents were transferred to a 20-ml column, and unbound AP was collected for reusing. The absorbents were washed with 100 ml of the same buffer, and incubated with 15 ml (6 mg of total protein) of bispecific monoclonal antibody that had been fractionated by ammonium sulfate precipitation for 1 h at RT. The absorbents were extensively washed and the bispecific antibody/AP complex was eluted with 40 ml of 20 m*M* phosphate in Tricine buffer (pH 8.5), and the bispecific antibody/AP activity was assayed (see Scheme below).



### 2.12. Purification of P104 using a DE52 column

P104 was fractionated by ammonium sulfate precipitation from cell culture supernatant and was dialyzed against 10 m*M* phosphate buffer. A 15-ml volume of the sample was loaded on a DE52 column (18×1.8 cm) at a flow-rate of 0.5 ml/min and purified as described by Suresh et al. [8].

### 2.13. Chromatography of HRPO, anti-HRPO IgG (YP4) or HRPO–anti HRPO IgG immune complex on a mimetic Red 3 column

A 1-mg amount of HRPO (120 U/mg solid) or YP4 or a mixture containing 1 mg of HRPO and YP4 was dissolved in 20 m*M* sodium acetate buffer (pH 5.0) and dialyzed against three changes of the same buffer for 16 h at 4°C. The samples were applied separately to a 1-ml mimetic Red 3 column at a flow-rate of 10 ml/h. The column was washed with the acetate buffer and 1 ml fractions were collected until the absorbance at 280 nm was negligible. The column was washed subsequently with 20 m*M* phosphate buffer containing 1 *M* NaCl, and the peak fractions were collected.

## 3. Results

### 3.1. Development of primary hybridomas and quadromas

A total of three mice were immunized with AP (50 U/mg) and the mouse with the highest anti-AP IgG activity in the serum was selected for hybridoma fusion with SP2/0 myelomas. The primary screen was performed on goat-anti mouse IgG-coated plates to capture the mouse immunoglobulins from the supernatants and incubate them with AP. Following a washing step, bound AP activity was determined using *p*-nitrophenyl phosphate as a substrate. In all, 100 clones were detected, of which, ten clones were expanded into 24-well plates, rescreened and the best three clones (P92.1R, P92.2R and P92.3R) were recloned twice. All of these were of the IgG1 kappa isotype. Relative affinity analysis of the three clones (Fig. 1) at varying AP concentrations revealed that clone P92.3R was the best and this hybridoma and Mab was used in all subsequent studies. Quadromas secreting bispecific antibody against PSA and AP as well as CA125 and AP were developed as described in Section 2 using electrofusion and FACS. Three positive clones with enriched bsMab anti-PSA×anti-AP were screened out of 33 clones. Meanwhile, one positive clone with anti-CA125×anti-AP activity was screened out of sixteen clones. These clones were recloned and expanded. The P105.3 clone and P104.1 were the best, with stable growth characteristics and these were inoculated into BALB/c mice for ascites production. In addition, 1 l of cell culture supernatant of each cell line was collected.

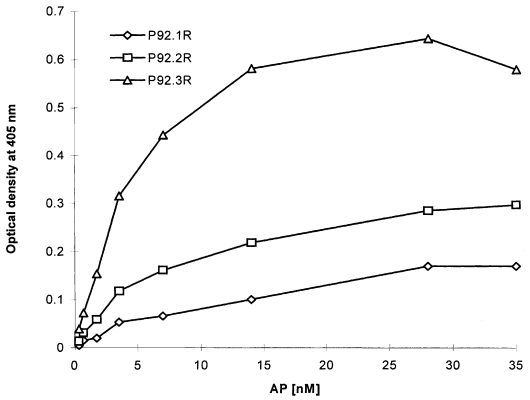



Fig. 1. Selection of the best anti-AP Mab (P92) with high affinity to AP. A limiting amount of goat anti-mouse IgG, immobilized on the plate, was used as the capture. A 100-ml volume of cell culture supernatants from three different subclones was added to the plate and 100 ml of AP (0–35 n*M*) was added to the wells as a tracer. The assay was performed as described in Section 2.

### 3.2. Chromatography of AP or P92 on mimetic blue A6XL column

In order to determine the initial purification parameters, chromatography of commercial crude AP alone and monoclonal antibody P92 alone were performed independently, as described in Section 2. The AP or P92 activity in the fractions was assayed in a 96-well plate, as mentioned previously. The

elution profile [Fig. 2a] demonstrated that the mimetic blue AX6L absorbent selectively and reversibly binds to AP but not anti-AP antibody P92. Mild elution conditions, using tricine buffer containing 5 m*M* phosphate, effectively elute most of the AP bound to the column.

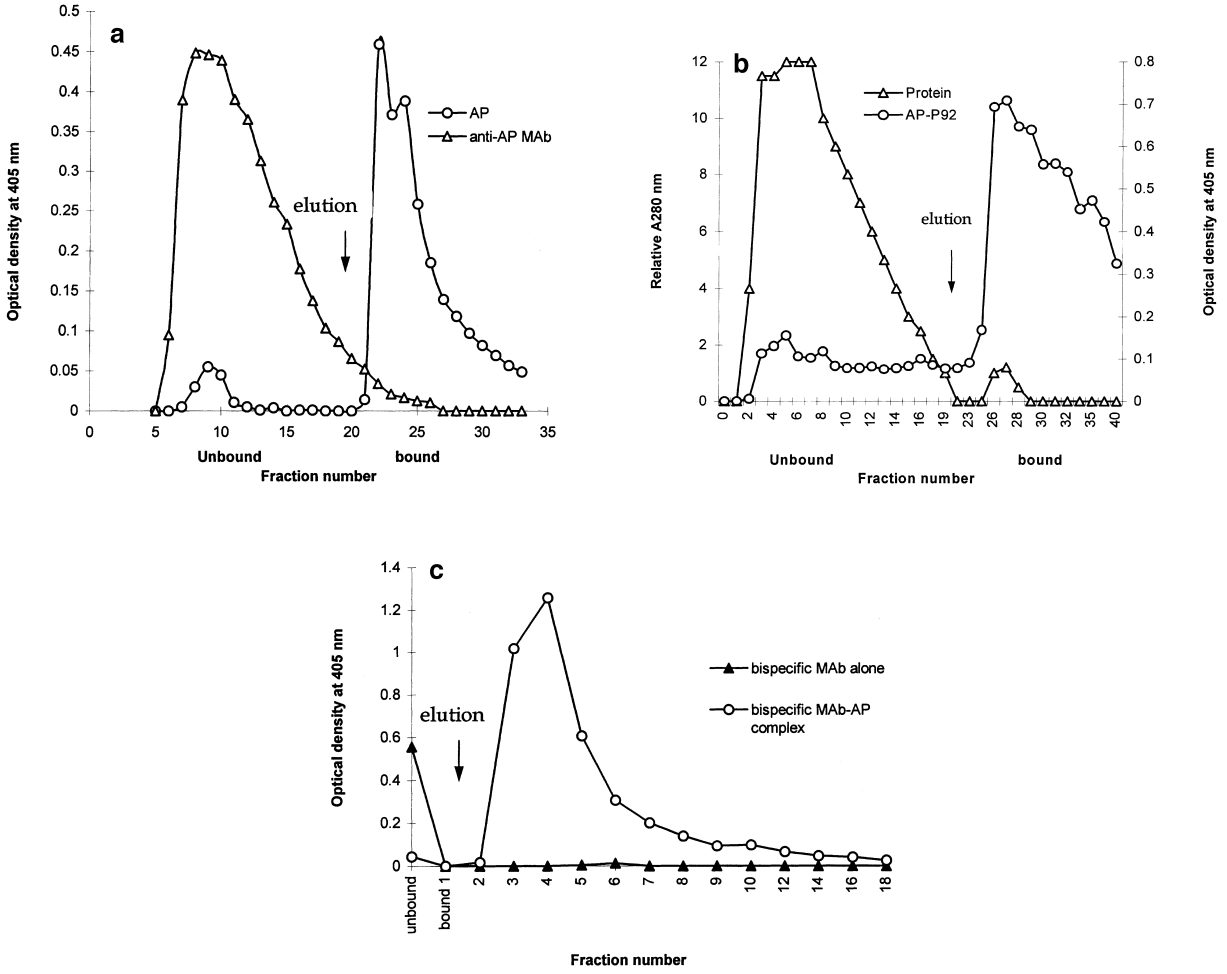



Fig. 2. (a) Chromatography of a commercial crude alkaline phosphatase alone and of mouse anti-AP IgG (P92) alone on a mimetic blue A6XL column. Either 1 mg of crude AP (50 U/mg solid) or 1.5 mg of P92 (DE52 column purified) in 10 m*M* Tricine–NaOH buffer, pH 8.5, was applied at a flow-rate of 3 ml/h to a column containing 2 ml of mimetic blue A6XL absorbent that had been pre-equilibrated with the same buffer. The column was washed at the points indicated as follows: (unbound) 10 m*M* Tricine–NaOH buffer, pH 8.5, (bound) 5 m*M* $KH_2PO_4$ in 10 m*M* Tricine buffer. Fractions were analyzed for AP activity and anti-AP IgG activity (OD, 405 nm). (b) Chromatography of AP/anti-AP IgG immune complexes on a mimetic blue A6XL column. A 2-ml sample containing a pre-incubated mixture of 2 mg of crude AP (100 U) and 1.2 mg of P92, to form antigen–antibody complexes, was purified using the same procedures as described for AP alone. The column was washed at the points indicated, as follows: (unbound) 10 m*M* Tricine–NaOH buffer, pH 8.5, (bound) 5 m*M* $KH_2PO_4$ in 10 m*M* Tricine buffer. Fractions were analyzed for AP/anti-AP immune complexes (OD, 405 nm) and protein (relative absorbance at 280 nm). (c) Chromatography of bispecific anti-PSA×AP IgG alone and of AP/anti PSA×AP IgG immune complexes on a mimetic blue A6XL column. The samples were loaded on a 10-ml mimetic blue column separately at a flow-rate 4 ml/h, overnight at 4°C. After all of the unbound fractions were collected, the column was washed with 100 ml of 10 m*M* Tricine buffer. The bound fractions of P105 alone or of AP/anti-AP IgG immune complexes were eluted using 5 m*M* phosphate–Tricine buffer (pH 8.5), collecting 2 ml fractions and assaying for AP/anti-AP IgG as well as AP/P105 activities.

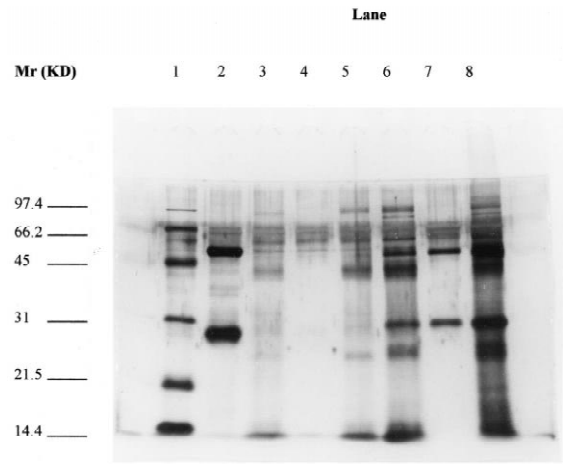



Fig. 3. SDS–PAGE analysis of the AP and AP–P92 immune complex purified using a mimetic blue A6XL column. Samples were electrophoresed on 12% SDS pacrylamide gels and then the proteins were visualized by silver staining. Lane 1, low molecular mass markers for SDS–PAGE; lane 2, mouse IgG heavy (50 kDa) and light (25 kDa) chains; lane 3, unbound fractions from the chromatography of crude AP; lane 4, bound fractions from the chromatography of crude AP; lane 5, unpurified AP; lane 6, unbound fractions from the purification of AP–P92 immune complex; lane 7, bound fractions from the purification of AP–P92 immune complex; lane 8, unpurified AP–P92 mixture.

### *3.3. Purification of AP anti-AP IgG immune complexes (AP/P92)*

A similar experiment was performed to purify AP/anti-AP IgG complexes formed by mixing 2 mg of crude AP with 1.2 mg of P92.3. The elution profile of AP/P92 immune complexes [Fig. 2b] indicated that the elution buffer also eluted AP/P92 complexes selectively and, more importantly, it did not dissociate the complexes. The results from SDS–PAGE showed that the majority of contaminants were removed and three very clear bands were seen, which represented AP, Mab heavy and light chains (lane 5 in Fig. 3).

### *3.4. Chromatography of bispecific antibody P105 and AP/P105 immune complex*

The chromatography of P105 alone or of the P105/AP complex was performed on the mimetic blue A6XL column as described in Section 2. The elution profiles of P105 and AP/P105 immune complexes (Fig. 2c) showed that this mimetic blue A6XL absorbent also binds bispecific antibody/AP immune complexes tethered by the enzyme bound with one of the antibody arms but not to P105 bispecific antibody in the absence of AP bound to it. Monoclonal antibody against PSA and other protein contaminants was not bound. It is pertinent to note that the P105 quadroma also secretes monospecific anti-AP antibody, which would be co-eluted with the bispecific antibody. This may not interfere in a bsMab-based PSA assay and only requires a higher amount of free AP to be added to saturate both Mabs. There was no background problem in our ELISA assays due to the presence of the monospecific anti-AP Mabs with the enzyme bound to the paratopes. This simple method allowed us to purify the required Mab in one step with near quantitative yield under mild conditions.

### *3.5. Purification of polyclonal IgG conjugated with AP*

We also investigated if the mimetic blue A6XL column could be used to purify the widely used chemically linked polyclonal–AP conjugates or qualify the purity of commercially available conjugates. Purification of commercial polyclonal IgG conjugated with AP was performed on a 10-ml mimetic blue column as mentioned earlier. Fractions (2 ml) were collected and the protein, AP and conjugate activity in each fraction were assayed. The elution profile of AP conjugates showed that this blue dye absorbent bound to AP conjugates in a quantitatively similar manner to that for AP alone [Fig. 4a]. However, the results from SDS–PAGE showed that AP–IgG conjugates are heterogeneous in size, since there were multiple bands on the top of lane 5 in Fig. 3.

### *3.6. Simplified purification of bispecific monoclonal antibody with one paratope against AP*

In this experiment, we tried to optimize and simplify the purification procedure for bsMAbs. In order to allow maximal binding of AP on the mimetic blue absorbents, we incubated AP with absorbents overnight at 4°C in a batch mode, in a

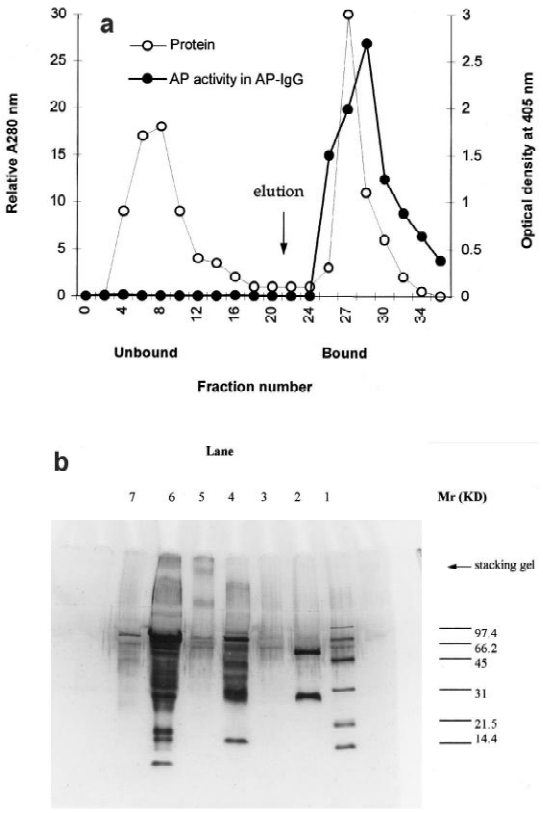



Fig. 4. (a) Chromatography of commercial polyclonal IgG conjugated with AP on a mimetic blue A6XI column. A 400-ml volume of polyclonal anti-mouse IgG conjugated with AP (about 1000 U) in 10 m*M* Tricine–NaOH buffer, pH 8.5 (12 ml), was applied at a flow-rate of 3 ml/h to a column containing 10 ml of mimetic blue A6XL absorbent that had been pre-equilibrated with the same buffer. The column was washed at the points indicated, as follows: (1) unbound: 10 m*M* Tricine–NaOH buffer, pH 8.5; (2) bound: 5 m*M* $KH_2PO_4$ in 10 m*M* Tricine buffer. Fractions (2 ml) were analyzed for AP activity and AP–IgG conjugates (OD 405 nm). (b) SDS–PAGE analysis of fractions collected from the chromatography of AP–IgG conjugates utilizing the mimetic blue A6XL column. The electrophoresis was performed on a 12% SDS–PAGE and the proteins were visualized by silver staining. Lane 1, Low-molecular-mass markers for SDS–PAGE; lane 2, mouse IgG heavy and light chains; lane 3, AP purified from the mimetic blue A6XL column; lane 4, unbound fractions; lane 5, bound fractions; lane 6, fractions washed with 0.75 *M* KCl; lane 7, unpurified AP–IgG conjugates.

flask with shaking. After transferring to a column, excessive unbound AP, as well as other contaminants, was collected and assayed for AP activity. This unbound fraction with high AP activity can be reused directly. The absorbents were washed extensively with ten volumes of Tricine buffer, a step that took about 30 min. A 45 min incubation of P105 with the absorbents was carried out at RT, and the unbound fraction was collected and assayed for P105 activity. If high activity of P105 was discovered in the unbound fraction, it was reused. In this way, both AP and crude BsMab could be recycled. After a washing step, the absorbents were transferred back to the column and the BsMab–AP immune complex was eluted with 20 m*M* phosphate in Tricine buffer, and twenty fractions were collected in 30 min. The elution profiles of P105–AP using both short and long protocols were similar (data not shown).

### *3.7. Comparison of the purification of P104–AP with ion-exchange chromatography (DE52 column) and with mimetic affinity chromatography (A6XL blue column)*

To demonstrate the usefulness of the mimetic blue affinity column for purifying the BsMab with one arm bound to AP, we compared this new method with a DE52 column, a previously described method to purify BsMab [8]. Equal amounts of P104 were purified by both methods. All of the fractions from both purification methods were assayed for P104, monoclonal antibody anti-CA125 activity [Fig. 5a,b]. Elution profiles of P104 and Mab anti-CA125 from a DE52 column showed that P104 was contaminated by Mab anti-CA125 in most of the fractions. In contrast, the majority of MAb anti-CA125 was recovered in the unbound fraction and there was very little inhibition of P104 activity in the fractions eluted from the mimetic blue affinity column, indicating that Mab anti-CA125 was separated from the main peak of P104.

### *3.8. Chromatography of HRPO, anti-HRPO IgG (YP4) and HRPO/anti-HRPO IgG immune complex on a mimetic Red 3 column*

In a similar set of experiments, we investigated if the mimetic Red column, developed to purify HRPO, could be used to purify peroxidase immunoconjugates. The elution profiles of the HRPO and

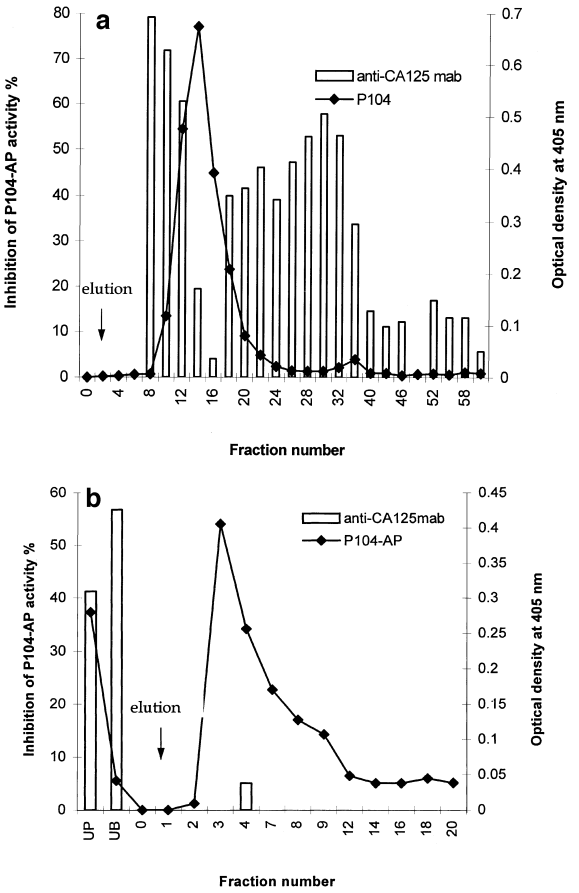



Fig. 5. (a) Elution profile of BsMab P104 and anti-CA125 Mab from a DE52 column. A 15-ml sample containing P104 was loaded on the DE52 column at a flow-rate of 0.5 ml/min. After removing unbound protein, P104 was eluted using 150 ml each of 10–100 m*M* phosphate buffer, pH 7.5. The percentage inhibition was proportional to the amount of anti-CA125 Mab in each fraction. (b) Elution profile of BsMab P104 and anti-CA125 Mab from mimetic blue affinity absorbents using the short purification protocol. UP, unpurified P104; UB, unbound fraction.

anti-HRPO IgG (data not shown) confirmed that the mimetic RED 3 absorbent binds strongly to the other contaminant proteins, and HRPO is in the unbound fraction. This is based on a negative purification principle, unlike the purification of AP on a mimetic blue column. The elution profile of YP4 displayed that these absorbents have certain non-specific binding to YP4 alone. However, unlike the free anti-HRPO IgG, where most of the protein was bound, the elution profile of HRPO/YP4 immune complexes showed the same pattern as that for HRPO alone. HRPO and most of the HRPO/YP4 immune complexes did not bind to the mimetic RED 3 column.

## 4. Discussion

Dye–ligand affinity chromatography has been used to purify many proteins [14,15], because of its ''pseudo-specific'' binding to different proteins in place of the natural biological ligands, in a selective and reversible manner. The purification of certain proteins can be easily achieved using this matrix and conventional low-pressure chromatography [15]. The purification of a desired protein can be achieved in one of two ways. One of them is by a positive mode, where the target protein is bound to an absorbent but contaminants are washed off from the absorbent bed as unbound proteins. The bound proteins are subsequently eluted with a suitable eluent containing solutes that compete with the dye–protein interaction. The other is negative, wherein the contaminants are bound and the target protein passes through the adsorbent bed along with the washing buffer as unbound material. In our experiments, we chose one example of each. The positive mode purification of the AP/anti-AP IgG immune complexes was accomplished with a mimetic blue A6XL absorbent and the negative mode purification of HRPO/anti-HRPO IgG immune complexes was performed using the mimetic RED 3 absorbent. The mimetic blue A6XL absorbent consists of a blue chromophore linked to a functional phosphoric acid group and has high affinity for calf intestinal AP [14].

We attempted to purify bispecific monoclonal antibodies that had one paratope to AP as AP/anti-AP IgG immune complex using this column. The purification of bispecific monoclonal antibodies produced by hybrid-hybridomas is a crucial step in the ELISA applications of bispecific antibody probes. The specific activity and sensitivity of the bispecific antibody-based immunoassay are effected by the amounts of monospecific monoclonal antibody contaminants. Utilizing a mimetic blue A6XL absorbent, those contaminants that do not bind to AP can be separated from those anti-AP bispecific or monospecific antibodies. We successfully purified monoclonal antibody anti-AP IgG and bispecific antibody

anti-AP×anti-PSA as well as BsMab anti-CA125× anti-AP as enzyme-bound immune complexes. In addition, we purified and qualified a commercial polyclonal AP–IgG immunoconjugate by the same chromatography technique. The elution profiles of AP and P92 as well as those of P105 and P104 showed that the blue absorbents have a high affinity with AP but a low affinity with either monoclonal antibody P92 or bispecific antibody alone. The elution profile of AP alone [Fig. 2a] demonstrated that most of the AP was bound to the column tightly and eluted under mild elution conditions, such as 5–20 m*M* phosphate in Tricine buffer. The phosphate ions bring about desorption of the AP molecules bound to functional phosphoric acid groups by competition. In contrast, most of the P92, P105 or anti-CA125 Mabs did not bind to the column and was washed off as unbound fraction [Fig. 2a, Fig. 2c, Fig. 5b]. The results from SDS–PAGE (Fig. 3) indicated that the impurities were removed effectively from the crude AP. Moreover, the elution profiles of AP–P92 and AP–P105 immune complexes, as well as of AP–polyclonal IgG chemically crosslinked immunoconjugates, demonstrated the expected results. The uncomplexed P92 or P105 and other contaminated proteins were mostly washed off as unbound fractions, and only the AP and AP–P92 or AP–P105 immune complexes or AP–IgG immunoconjugate molecules were eluted by the phosphate buffer [Fig. 2a–c, Fig. 4a]. The unbound fractions contained lots of contaminant protein and much less AP/anti-AP IgG immune complex or AP–IgG conjugates [Fig. 2c, Fig. 4a]. The SDS–PAGE showed that, in the affinity method, the majority of contaminants (lane 6) were removed and three very clear bands were seen, which represented AP, Mab heavy and light chains (lane 7 in Fig. 3). Most promising was that this column can be used to purify bsMab with one paratope against AP or the monospecific antibody with two anti-AP paratopes. The second monospecific antibody, without the paratope to AP, was removed as unbound fraction [Fig. 5b]. This resulted in the high specific activity and sensitivity of the bsMab-based heterogeneous immunoassay for PSA or CA125.

The elution profiles of BsMab P104 and anti-CA125 Mab from purification on the mimetic blue column gave further proof of the general applicability of this method, wherein contaminants such as Mab anti-CA125 were resolved from the P104–AP immune complex. Higher sensitivity of the BsMab–AP-based ELISA for detecting CA125 would be achieved with high specific activity immunoprobes. Assuming that each BsMab–AP complex binds to one CA125 epitope, each AP molecule bound to a BsMab will react with its own substrate to generate a signal. The intensity of the signal is proportional to the concentration of CA125. This holds promise for the development of an ultrasensitive ELISA to detect very limited amounts of CA125 or PSA by utilizing the purified P104–AP or P105–AP immune complexes. Compared with using chemically coupled AP–IgG immunoconjugates, this AP/bsMab would allow us to develop an immunoassay with a higher sensitivity and reproducibility, due to a uniform 1:1 ratio of bsMAb and AP. In contrast, the results of the SDS–PAGE showed that chemically crosslinked AP–IgG conjugates are heterogeneous, with a ladder-like resolution. Since there were multiple bands on the top of lane 5 in Fig. 4b, we interpreted them to be due to random crosslinks of AP–IgG heavy chain and AP–IgG light chain with a number of AP molecules and IgG molecules. The ELISA assay demonstrated that there was very little AP enzyme activity in the unbound fractions (data not shown), but the results from SDS–PAGE indicated that there were bands corresponding to subunits of IgG and AP molecules in these unbound fractions. This might be due to a fraction of the conjugate that had lost AP enzyme activity, presumably during the chemical conjugation procedures. Thus, the mimetic blue column could be a great advantage for isolating any functional AP/IgG immunoconjugates made in a laboratory or commercial AP/IgG conjugates with the AP activity from the fraction of conjugates without the enzyme activity.

Ion-exchange chromatography has been used to purify BsMabs [8,9]. However, comparing the two purification methods, the mimetic affinity method is simple and effective. Purification with DE52 lacks specificity, moreover, a large volume of eluent with a gradient concentration of salt is needed (300 ml) and a large number of fractions are collected and assayed. More importantly, some bsMabs are not able to be separated from other contaminants, which will reduce the specificity and the sensitivity of the

bsMab assay. In addition, we achieved purification in a shorter period with simple procedures that can also be used without the need for high pressure. The elution profile showed that most of the bsMab–AP immune complex was recovered in 30 ml of elution buffer. Moreover, either enzyme or bsMab collected in the unbound fraction can be reused without being dialyzed.

The elution profile of the purification for HRPO/anti-HRPO IgG immune complexes showed what could be expected for a ''negative'' mode of purification. Most of the contaminant proteins bound to the mimetic Red 3 column, and the HRPO/anti-HRPO IgG immune complexes were collected in the unbound fractions (Fig. 5), removing most, but not all, of the impurities from the immune complex. This purified immune complex could be used directly in an immunoassay, as alluded to above. The purification was performed at 4°C and, hence, minimized the risk of damaging the activities of the enzymes and complexes. Under reducing conditions, the SDS–PAGE (12%) showed that most of the contaminants were removed from the complexes. Conceptually, the positive mode is likely to yield better purity of the immunoconjugates, due to specific binding and elution steps. In the negative mode, it is also likely that some other proteins could also be unbound along with HRPO. Nevertheless, the results demonstrated that both methods are suitable for the purification of AP immunoconjugates; AP/bsMab immune complexes and HRPO immunoconjugates, as well as HRPO/bsMab immune complexes.

## 5. Conclusion

Mimetic ligand-based affinity purification is a unique method for purifying AP/BsMab immune complex, AP–antibody conjugates and HRPO/bsMab immune complex as well as HRPO–antibody conjugates, and results in a higher specific activity. The non-covalent AP–P92 and AP–P105 immune complexes are more homogeneous than the AP–IgG conjugates obtained by chemical methods of cross-linking. The purified AP/anti-AP bispecific antibody immune complex could be directly used for in vitro diagnostics, wherein the second arm of the bispecific antibody binds specifically to a target, such as a tumor marker.

## 6. List of abbreviations

| | |
|---|---|
| A280nm | absorbance at 280 nm |
| ABTS | 2,2′-azino-di[3-ethyl-benzthiazoline sulfonate] |
| AP | alkaline phosphatase |
| BsMab | bispecific monoclonal antibody |
| ELISA | enzyme-linked immunosorbent assay |
| HRPO | horseradish peroxidase |
| IgG | immunoglobulin G |
| Mab | monoclonal antibody |
| P92 | monoclonal antibody against alkaline phosphatase |
| P104 | bispecific monoclonal antibody anti-CA125×anti-AP |
| P105 | bispecific monoclonal antibody anti-PSA×anti-AP |
| PBS | phosphate-buffered saline, pH 7.4 |
| PBST | 0.05% Tween-20 in PBS |
| PSA | prostate-specific antigen |
| CA125 | cancer antigen 125 |
| RT | room temperature |
| SDS–PAGE | sodium dodecyl sulphate–polyacrylamide gel electrophoresis |
| YP4 | rat monoclonal antibody against HRPO |

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