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# Direct Antigen Capture by Soluble scFv Antibodies

*A Method for Detection, Characterization, and Determination of Affinity*

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## Abstract

For ease of detection, soluble forms of phage-displayed scFv antibodies are usually expressed with a tag, e.g., c-myc or His (Histidine). The binding is then assayed by a monoclonal antibody to the tag. In this article, we describe the use of biotinylated antigen for detecting soluble scFv antibodies without utilizing the peptide tag detection system. The scFv antibodies were against the oncoplacental antigen heat-stable alkaline phosphatase (HSAP). The method essentially consisted of either reverse Western or antigen capture enzyme-linked immunosorbent assay (ELISA). In the reverse Western, periplasmic extract was electrophoresed, and binding to biotinylated antigen was detected by the detection system based on streptavidin–horseradish peroxidase. The antigen capture ELISA utilized the binding of periplasmic extract to a polystyrene plate. We have also demonstrated the use of antigen capture ELISA for studying specificity and affinity of the selected clones. Although these techniques have been developed for antibodies to HSAP, they have general utility for phage expression systems without a peptide tag.

**Index Entries:** Recombinant antibody; heat-stable alkaline phosphatase; reverse Western; enzyme-linked immunosorbent assay; affinity; phage display.

## Introduction

The production of antibodies to a wide repertoire of antigens by phage display is becoming an increasingly useful technology because of the antibodies' potential as therapeutic agents. Although displayed antibodies

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have been selected for a large number of antigens *(1)*, their primary requisite as diagnostic or therapeutic agents depends on the availability of the soluble single chain variable (scFv) fragment. To detect the soluble form of such antibodies, these are expressed with a peptide tag, which can then be assayed by monoclonal antibodies (MAbs) *(1–4)*. Peptide tags, usually c-myc or His (Histidine), have been essential components of detection systems for such soluble antibodies.

In this article, we describe the use of biotinylated antigen for detecting soluble scFv antibodies without utilizing the peptide tag detection system. Peptide tags have their advantages and disadvantages. They are useful for the detection and purification of scFv proteins and attaching the protein to solid surfaces such as biosensors and affinity chromatography media. However, the tag could interfere with structural studies and X-ray crystallography, and also affect antigen binding in a manner difficult to predict. Hence, there is a need to develop a simple system for detecting antigen binding without involving a peptide tag that would enable the use of phage display libraries that do not incorporate the same.

Antigen binding was detected by both a reverse Western and an antigen capture enzyme-linked immunosorbent assay (ELISA). This is an adaptation of a system called far Western, described by Lee et al. *(5)*, for detecting protein–protein interaction in the study of transcription factors. The method adopted here not only circumvents the need for a peptide tag, but also helps in the direct detection of antigen–scFv binding. Although the present study describes the detection of antibodies to an oncodevelopmental antigen, heat-stable alkaline phosphatase (HSAP), the approach can be used for detecting the binding of specifically selected scFvs to the target antigen. It also reflects the ability of at least some species of these scFv molecules to retain biological activity in the presence of sodium dodecyl sulfate (SDS). A number of proteins including enzymes also have the ability to retain activity in the presence of SDS, indicating that the active site may not be denatured in the presence of the detergent *(6)*.

We selected phage-displayed scFv antibodies to HSAP from a human synthetic immunoglobulin library, described initially by Nissim et al. *(1)*, which was kindly made available to us by Prof. Greg Winter (MRC, Cambridge). HSAP is an isozyme of alkaline phosphatase normally expressed on the surface of the placenta and in a variety of tumors (e.g., seminoma, lung cancer, colon cancer, ovarian cancer, and choriocarcinoma) *(7–12)*. The HSAP binding phage was detected by magnetic bead phage ELISA, reported by us previously *(13)*.

## Materials and Methods

### *Soluble Expression*

The HSAP (Sigma, St. Louis, MO) binding phage used in this study were distinct clones, as evident from their sequences, which differed from each other. The clones were transformed into a nonsuppressor strain of

bacteria (HB2151, Pharmacia expression module). The periplasmic extract was obtained as described by Marin et al. *(14)*, except that the isopropyl-thiogalactoside used was 2 m*M* and the pellet after induction was resuspended in phosphate-buffered saline (PBS) with 1 m*M* EDTA (1/100 vol of initial culture). After centrifugation the supernatant was filter sterilized and used for further experiments. The batch-to-batch variation was minimized by inducing the cultures when the absorbance was 0.8. The periplasmic extract was fractionated on a 15% SDS-polyacrylamide gel electrophoresis (PAGE) containing 2-mercaptoethanol, according to Laemmli *(15)*, followed by silver staining to check for the presence of the induced recombinant protein. The four clones used in this study, which were in the vector pHEN 1, had unique $V_H$ sequences (data not shown), as determined by dideoxy sequencing of both strands by appropriate primers *(16)*.

### *Biotinylation of HSAP*

Biotinylation of HSAP was carried out according to the manufacturer's instructions in the biotinylation kit (Sigma). After separation of the unbound biotin by a column, the stoichiometry of biotinylation was determined by the 4'-hydroxyazobenzene-2-carboxylic acid (HABA) assay (per the instructions of the manufacturers) (Sigma). Approximately 2–3 mol of biotin bound/mol of HSAP.

### *Reverse Phase Western*

After running the periplasmic extract through a 15% SDS-PAGE, the proteins were electrotransferred onto a nitrocellulose membrane (0.2-mm pores; Schleicher & Schuell). The membrane was blocked with 5% MPBS (5% nonfat dry milk in PBS) for 2 h at room temperature. Then 500 µL (diluted in 5% MPBS) of biotinylated HSAP was added, and this was incubated with shaking for 2 h at room temperature. One milliliter (1:200 dilution in 5% MPBS) of streptavidin-horseradish peroxidase (HRP) conjugate was used to detect the binding of the antigen. After 2 h of incubation at room temperature on a rocking platform and washing twice with PBS followed by 0.01 *M* Tris-HCl (pH 7.6), the blot was developed with 20 mL of diaminobenzidene (DAB) (a filtered solution of DAB: 600 µg/mL made in 0.01 *M* Tris-HCl and 3 mg of cobalt chloride) and 20 µL of $H_2O_2$. After each step the membrane was washed three times with PBS (pH 7.2).

### *Antigen Capture ELISA*

ELISA plates (Nunc, Maxisorp) were coated with 200 µL of periplasmic extract (with a protein concentration of 2 µg/mL) of the HB2151 transformed with the phagemid. The total protein in the lysate was 400 ng/200 µL, and the extract was allowed to dry completely on the well. Periplasmic lysate from untransformed bacteria served as control. After blocking the wells with 300 µL of 2% MPBS, 200 µL (66 µg, diluted in 2% MPBS) of

biotinylated HSAP was added and kept for 2 h at room temperature. This was followed by incubating with 200 µL of a 1:1000 dilution of streptavidin–HRP conjugate (Sigma) made in 2% MPBS for 2 h at room temperature. The reaction was developed by using 200 µL of 0.4 mg/mL *o*-phenylenediamine (OPD) and 0.4 µL/mL of 30% $H_2O_2$ made in citrate-phosphate buffer (0.02 *M* trisodium citrate and 0.05 *M* $Na_2HPO_4$, pH 5.0). This was incubated for 10 min at room temperature and the reaction was stopped with 50 µL of 8 *N* $H_2SO_4$. The absorbance at 490 nm was measured. After every step the plate was washed three times with PBS (pH 7.2).

### *Inhibition ELISA*

The specificity of binding was further confirmed by the ability of unbiotinylated HSAP to inhibit the binding of biotinylated antigen. Unlabeled HSAP of different concentrations ($3.5 \times 10^{-7}$, $3.5 \times 10^{-6}$, $7 \times 10^{-6}$, $1.7 \times 10^{-5}$, $3.5 \times 10^{-5}$, and $7.1 \times 10^{-5}$ *M*) was used to inhibit the binding of a constant concentration of biotinylated HSAP ($4.7 \times 10^{-7}$ *M*). The ELISA was done as in the preceding section, except that the competition between the biotinylated and unbiotinylated HSAP binding to scFv was allowed to reach equilibrium, by incubation at 4°C for 15–18 h. The same system was used to find the specificity of the anti-HSAP-selected scFv antibodies to HSAP, human serum, bovine serum albumin (BSA), and milk proteins.

### *Determination of Crossreactivity by Inhibition ELISA*

Crossreactive binding was determined in a manner similar to the inhibition ELISA by HSAP. Inhibition of binding of the biotinylated HSAP was determined, after incubation with increasing concentrations of the potentially crossreactive molecules. Typically, for BSA, increasing amounts of the antigen were added to the reaction and inhibition of binding of biotinylated HSAP was observed.

### *Affinity*

Affinity was measured by using competitive/inhibition ELISA (modified method of Muller *[17]*). To determine the affinity of antibody by this method, it was necessary to know the molar concentration of biotinylated HSAP giving approx 40–70% binding in the assay, the amount of biotinylated HSAP bound to antibody in the absence of unlabeled HSAP, and the molar concentration of unlabeled HSAP giving 50% inhibition of biotinylated HSAP-scFv antibody binding under equilibrium conditions. Because HSAP used in this study was a purified antigen, its molar concentration after biotinylation could be calculated directly by converting the amount used to molar values.

#### Determination of Molar Concentration of Biotinylated HSAP Giving Approx 40–70% Binding in the Assay

The wells were coated with antigen and blocked as described earlier. To each of these blocked wells 200 µL of different amounts of biotinylated

HSAP in 2% MPBS was added. The plate was washed three times with PBS. Binding of biotinylated antigen was detected by the addition of 200 µL (1:1000 dilution in 2% MPBS) of streptavidin–HRP conjugate. After three washes with PBS, the color was developed using 200 µL of OPD as described previously. After 15 min of incubation at room temperature, the reaction was terminated by the addition of 50 µL of 8 $N$ $H_2SO_4$. The absorbance value at the plateau was taken to be 100% because the binding of the antibody to antigen can never be 100%. The concentration of HSAP where the binding was approx 40–70% and linear was extrapolated from the titration curve.

### Determination of Amount of Biotinylated HSAP Bound to Antibody in Absence and Presence of Unlabeled HSAP and Procedure of Competition ELISA

Two hundred microliters of scFv extract with the expressed antibodies was coated as described for the antigen capture ELISA. To the blocked wells, 200 µL (diluted in 2% MPBS) of biotinylated HSAP giving approx 50% binding to the scFv was allowed to compete with different concentrations ($3.5 \times 10^{-7}$, $3.5 \times 10^{-6}$, $7 \times 10^{-6}$, $1.7 \times 10^{-5}$, $3.5 \times 10^{-5}$, and $7.1 \times 10^{-5}$ $M$) of unbiotinylated HSAP. This was kept at 4°C for 15–18 h and allowed to reach equilibrium. Binding in the absence of unlabeled HSAP was also taken. The inhibition of biotinylated HSAP binding by unbiotinylated HSAP was determined as described in the inhibition ELISA.

The percentage inhibition of biotinylated HSAP–scFv binding by HSAP was calculated by the following formula:

$$\% \text{ Inhibition} = 100\ (Y_{max} - Y)/Y_{max}$$

in which $Y_{max}$ are the absorbance values averaged for wells containing no unlabeled HSAP to give the maximum value, and $Y$ is the absorbance values obtained in the presence of competition with unlabeled HSAP.

Plotting a graph of the percentage of inhibition vs the molar concentration of unlabeled HSAP initially gave the molar concentration of unlabeled HSAP giving 50% inhibition to binding of biotinylated HSAP scFv antibody. Once all these values were obtained, they could be applied to the formula given by Muller *(17)* to obtain the $K_D$ values.

## Results and Discussion

The anti-HSAP scFv antibody migrated in SDS-PAGE with an apparent molecular weight of 29–30 kDa (Fig. 1A,B). Antigen capture blotting revealed that biotinylated antigen bound only to the band corresponding to the expressed fragments (Fig. 2A,B), with no corresponding binding in the control. This also indicated that it bound only to the soluble scFv fragment and ruled out the possibility of the antigen binding nonspecifically to the periplasmic extract. There is also the possibility of increasing the sensitivity of the method by using enhanced chemiluminescent techniques.

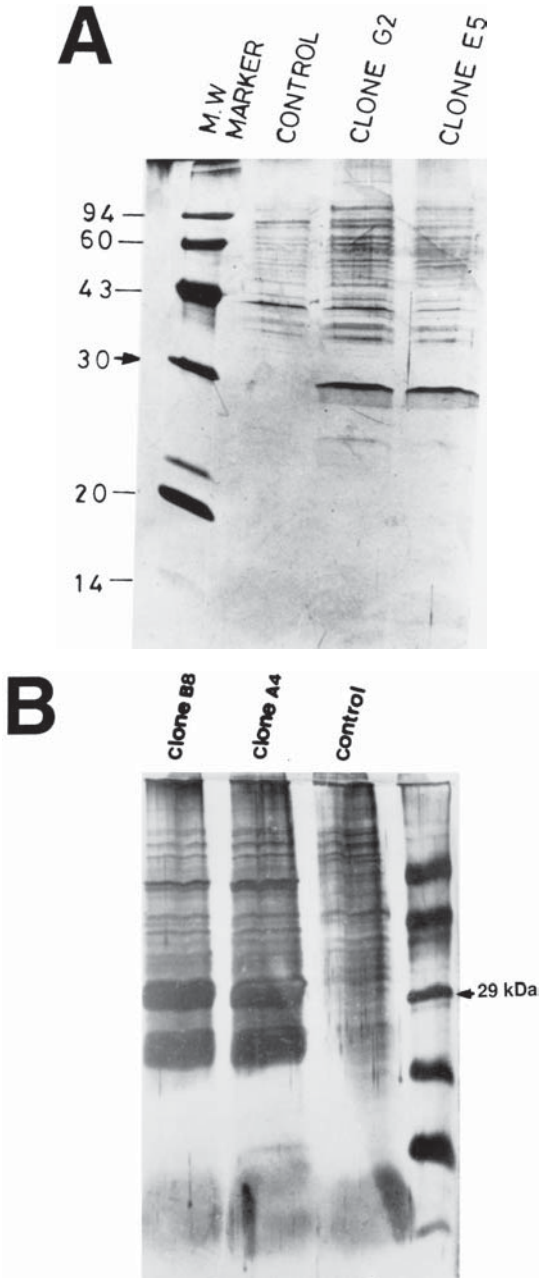


Fig. 1. **(A)** A 15% SDS-PAGE of the periplasmic extract, showing the 29-kDa-expressed scFv from clones G2 and E5 along with untransformed bacteria as the control. **(B)** A 15% SDS-PAGE of the periplasmic extract, showing the 29-kDa-expressed scFv from clones B8 and A4 along with untransformed bacteria as the control.

These observations raised the possibility of the periplasmic extract of transformed scFv-expressing clones being used directly in an antigen capture ELISA (Fig. 3). Appropriate concentration of periplasmic extract coated

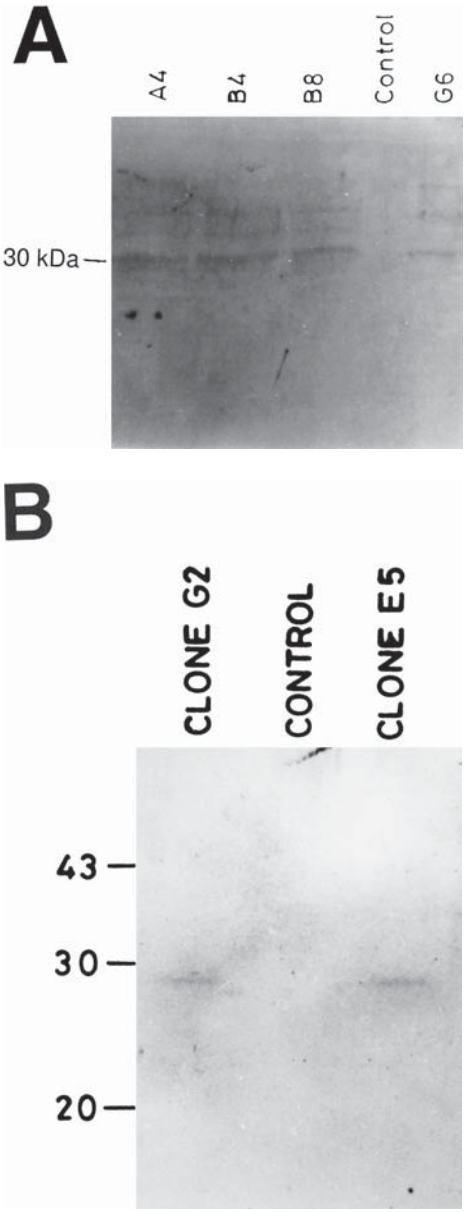


Fig. 2. **(A)** Reverse Western showing the binding of biotinylated HSAP to approx 29- to 30-kDa scFv clones A4, B4, and B8. **(B)** Reverse Western showing the binding of biotinylated HSAP to approx 29- to 30-kDa scFv clones G2 and E5.

on ELISA plates was able to bind biotinylated antigen, which was then detected by streptavidin-HRP conjugate. Unlabeled antigen could compete with the binding by labeled HSAP in a concentration-dependent manner, testifying to the specificity of the detection system (Fig. 4). The binding of scFv to different potentially crossreactive molecules has also been deter-

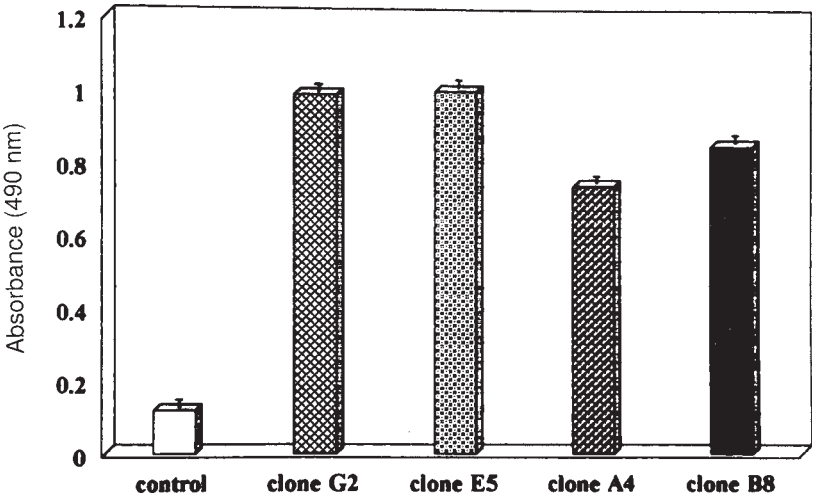


Fig. 3. Antigen capture ELISA of clones G2 and E5.

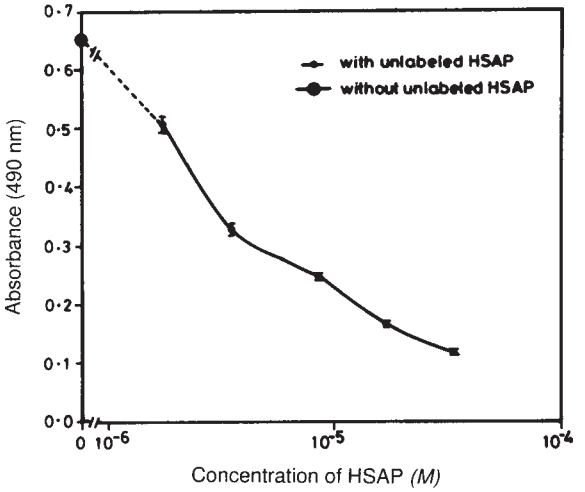


Fig. 4. Inhibition ELISA, showing a concentration-dependent inhibition in the binding of biotinylated HSAP to scFv antibody G2 by unlabeled HSAP.

mined by their ability to inhibit the binding of biotinylated HSAP. Using inhibition ELISA, one of the clones G2 was found to be crossreactive to BSA. The addition of BSA to the reaction could inhibit the binding of biotinylated HSAP in a concentration-dependent manner (Fig. 5). None of the other clones behaved in a similar manner. Similar approaches could also be used for epitope mapping, by using competing peptides, antigenic fragments, and so on. The crossreactivity of the clone G2 to BSA could probably be a consequence of a property of the Nissim library because all the scFvs from the library have a common $V_L$ sequence that originated from an antibody to BSA.

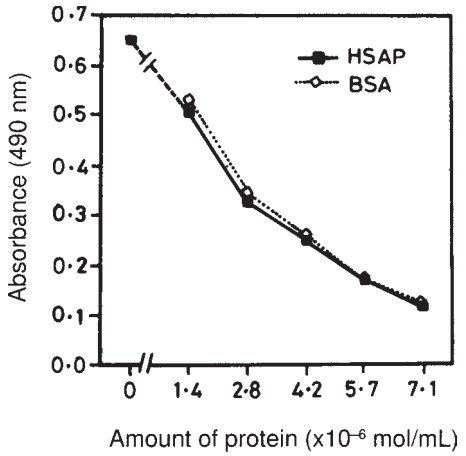


Fig. 5. Inhibition of binding of biotinylated HSAP to the clone G2 by BSA. BSA inhibited the binding of HSAP to G2 in a concentration-dependent manner, similar to the inhibition by HSAP, indicating crossreactive binding. (—■—), Absorbance after incubation with HSAP; (···◇···), absorbance after incubation with BSA; (■), the absorbance of the clone without preincubation. Error bars represent the standard deviation (SD) of four replicates for each point.

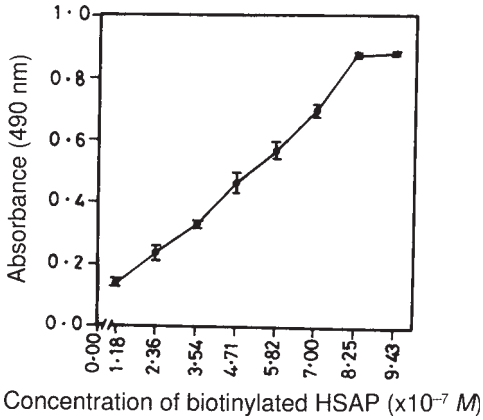


Fig. 6. Absorbance at 490 nm obtained on adding increasing amounts of biotinylated HSAP to ELISA plates coated with periplasmic extract from clone A4. The concentration of biotinylated HSAP showing 50% of the maximum absorbance has been determined to be $5.14 \times 10^{-7}$ *M*. Error bars represent the SD of four replicates for each point.

One of the requirements for determining affinity by this method was to identify the amount of biotinylated HSAP giving approx 40–70% of the maximum binding to the periplasmic extract-coated wells. This was done by adding increasing amounts of labeled HSAP to coated ELISA wells (Fig. 6) to determine maximum antigen binding. At a point corresponding to approx 50% of the maximum binding, competition experiments with unbiotinylated HSAP were performed, and affinity was determined as

described in Materials and Methods. The affinity of the soluble scFv fragments was determined to be in the micromolar range: 0.61, 0.84, and 0.72 μ*M* for clones E5, A4, and B8, respectively. This is probably owing to the differences in the $V_H$ sequences of the clones (unpublished data). Differences in the relative affinities and sequences would be particularly important for comparing two or more scFv clones that recognize the same epitope on HSAP. However, epitope mapping has not yet been done for the clones under study.

Affinity is the ratio of off and on rates of the binding reaction. Ideally, it should be determined for binding in solutions. However, there is published work on ELISA estimation of relative affinities in crude antibody preparations such as hybridoma supernatant and recombinant antibodies. Kazemier et al. *(18)* demonstrated a clear difference in the proportion of active and inactive scFv present in the periplasm of *Escherichia coli* and emphasized the need for measuring active antibody concentrations to obtain an accurate measurement of affinity. Because, in the method described herein, the readout is based on the binding of the antigen to the immobilized active scFv fragment alone, variation in the relative amount of inactive to inactive scFv is less of a problem. However, in the method described here it is important to control the conditions and stoichiometry of biotinylation of the antigen. This would be easier with a soluble purified antigen as compared to controlling the expression and purification of soluble scFvs. Relatively simple methods of assessing the extent of the biotinylation reaction (e.g., by the HABA reaction) are readily available. There is the possibility that biotinylation may alter the binding characteristics of an antigen, especially as compared to iodination. This limitation, however, would be less for large antigens and can be minimized by controlling the biotinylation reaction. But the possibility of altered conformation interfering with the binding of a particular monoclonal phage remains and needs to be considered when interpreting the results. In the case of enzymes such as, HSAP a good indicator of the overall conformation is specific activity. In our study, biotinylation did not alter the specific activity of the enzyme (data not shown). Conformation is also likely to be affected on immobilizing the antigen *(19)*, as may be the case when detecting with antibodies to a peptide tag such as c-myc.

Methods for the estimation and ranking of affinities by ELISA have included noncompetitive methods *(20)*. Although such methods are useful for rapid determination of relative affinity, for more accurate estimations, competitive assays are preferred *(19)*. Other ELISA methods for determining the affinities for MAbs in solution include those in which the proportion of antibody that remains unsaturated at equilibrium is determined *(21)*. In addition, Hardy et al. *(22)* have developed a method in which, after mixing antigens and antibody in solution, aliquots are developed at intervals to determine the amount of free antibody remaining in solution by indirect ELISA. Both these methods have the advantage of measuring the binding in solution and do not require the labeling of either antigen or

antibody. However, the subsequent ELISA step would require a peptide tag for the detector antibody to bind. Although there are certain proteins such as protein L *(23)* that bind to light chains, including the lambda light chain used in the Nissim *(1)* library with which this study was conducted, it would not be possible to generalize the same for all libraries. Also, there is the possibility of protein L interfering with the antigen binding by scFv.

We have used an assay method based on the binding of a molecule in solution (biotinylated antigen) to an immobilized scFv extract. It has been observed *(20)* that no straightforward thermodynamic theory describes the equilibrium in heterogeneous phase systems. However, surface plasmon resonance systems utilizing binding in heterogeneous phases are being used extensively in recombinant antibody research. A solid phase for immobilization of reactants complicates the determination of affinity by slowing down the association and dissociation rate and causing surface effects such as antigen density–dependent steric hindrance, bivalent antibody binding, and lateral cooperative interactions *(24)*. Discussing the problems of heterogeneous phase binding with BIAcore instruments, Nieba et al. *(25)* cautioned against the use of on and off rates for the determination of binding constants without controlling for the rebinding and avidity effects. It has been observed that apparently "cooperative" binding can result from high chemical on rates when the immobilized phase is highly saturated *(26)*. However, this has not happened in our case, as shown from the near-linear nature of the plot depicting the increase in binding on adding increasing amounts of biotinylated antigen (Fig. 6). The conditions for the calculation of affinity by the method in this article require a linear portion of the binding and dissociation curve, which can be achieved by adjusting the protein concentration for coating the ELISA plates. Hence, the qualifiers that need to be addressed in this relatively inexpensive method are not more than those of machines using the plasmon resonance technique.

The results testify to the general applicability of the antigen capture ELISA and reverse Western for detecting and characterizing antigen-binding properties of scFv molecules without utilizing a peptide tag. These methods are simple, use unpurified samples, and do not use expensive complex instrumentation to obtain affinity estimates. This work is for anti-HSAP antibodies. However, the validity of these methods for clones of different sequences indicates a general applicability, especially for soluble, purified antigens. Information regarding binding characteristics and affinity, along with sequence data, would be useful for producing hypotheses about the structural basis of immune recognition.

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