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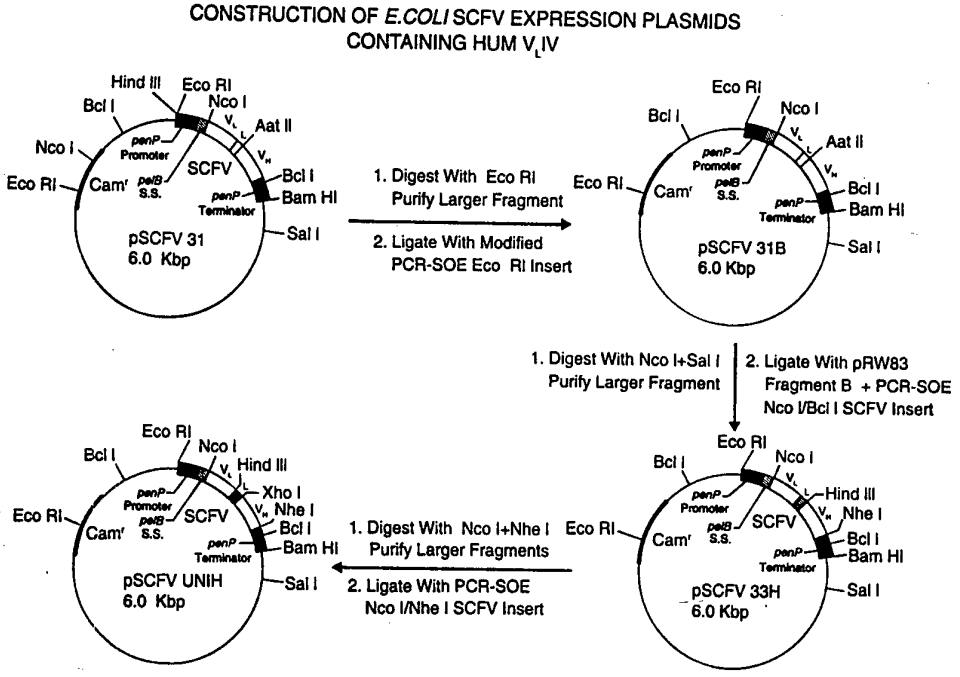

(57) Abstract

The present invention discloses novel proteins which are dimers and multimers of single chain antibodies. The single chain antibodies contain polypeptides of two domains derived from the variable domains of antibodies which are joined by a peptide linker. The dimers and multimers are formed by non-covalent linking of the single chain polypeptides. Uses include all those appropriate for monclonal and polyclonal antibodies and fragments thereof, including use as a bispecific antigen-binding molecule.

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DIMER AND MULTIMER FORMS OF SINGLE CHAIN POLYPEPTIDES

This invention relates to the field of molecular biology and production of single chain polypeptides.

The basic structure of an antibody is a tetramer composed of two identical heterodimers each consisting of a light and a heavy chain. The antibody molecule is divided into variable (V) domains that are responsible for binding specificity and constant (C) domains that carry out various effector functions. The V domain is constructed of one V homology unit from both the light and heavy chains, designated V_L and V_H respectively. The variable region can be further subdivided into framework regions (FR) and complementarity determining regions (CDR), also designated as hypervariable regions. The FR maintain the structural integrity of the variable region domain. The CDR are the polypeptide segments within the variable region that mediate binding to the antigen. As the antibody contains two heterodimers, it has two antigen binding sites (divalent).

By using current techniques in molecular biology, it is possible to clone polypeptide chains. The more complex the molecules, e.g., the multichain immune receptors of the Ig superfamily, the more difficult they are to produce in a single foreign host. Genes coding for each chain of a complex molecule can be cloned and expressed in separate hosts but the aggregation and refolding of the resultant polypeptides into a biologically active entity is difficult to achieve. Multiple chains expressed by multiple genes within the same host have an advantage in aggregating and refolding into native structure but their expression in stoichiometric amounts is difficult to regulate. Thus, neither approach has proven to be efficient.

Since it is the variable regions of light and heavy chain antibodies that interact with an antigen, single chain polypeptides have been created with one V_L and one V_H joined by a peptide linker (U.S. Patent 4,946,778). This single chain fragment (Fv) is univalent and is one of the smallest structures necessary for antigen binding activity having all 6 CDR regions of a V_L-V_H combination.

The use of the functional smaller polypeptides which maintain an antigen recognition site are also advantageous when used for therapeutic purposes in mammals in that the smaller molecules are capable of rapidly localizing in the target tissue, such as single chain antibodies localizing in cancerous tissue. However, while being able to penetrate the desired tissue, polypeptides with a molecular weight of less than about 50,000 daltons have the disadvantage of being retained within the glomerulus of the kidney This is particularly disadvantageous when the polypeptide is bound with a carrier, such as a radioisotope or toxin, as the isotope or toxin will also accumulate in the kidneys.

Single chain polypeptides also suffer from the disadvantage of having only one binding site, thereby reducing their avidity.

It would therefore be advantageous to obtain constructions of the single-chain antibodies which retain their antigen or ligand recognition properties, are of sufficient molecular weight to enhance retention at the target site with reduced retention in the kidney, and have a multiplicity of binding sites to enhance the avidity of the polypeptide. In addition, it would be beneficial if the single chain immunoglobulin could be rendered bispecific to allow for recognition of different epitopes on the target tissue (or lymphocyte) or to allow for antibody-based recruitment of other immune effector functions (i.e., complement proteins, cytotoxic lymphocytes, etc.).

Surprisingly it has been found that single chain antibodies will non-covalently associate to form dimers and multimers. In one embodiment, the invention is a protein comprising a dimer or multimer of single chain polypeptides wherein the polypeptides are non-covalently linked and each polypeptide comprises (a) a first polypeptide comprising an antigen binding portion of a variable domain from an immunoglobulin; (b) a second polypeptide comprising an antigen binding portion of a variable domain from an immunoglobulin; and (c) a peptide linker linking the first and second polypeptides (a) and (b) into a single chain polypeptide having affinity for an antigen.

In another embodiment, the invention is a protein comprising a dimer or multimer of single-chain polypeptides wherein the polypeptides are non-covalently linked and each polypeptide comprises (a) a first polypeptide comprising an antigen binding portion of a variable domain from an immunoglobulin; and (b) a second polypeptide comprising an antigen binding portion of a variable domain of an immunoglobulin wherein polypeptides (a) and (b) are joined by a peptide bond.

In another aspect the present invention includes the aforementioned dimer or multimers conjugated to an imaging marker or therapeutic agent. The invention also includes a composition comprising the dimer or multimer conjugated to an imaging marker or therapeutic agent in a pharmaceutically acceptable carrier.

The invention is also directed to a method for *in vitro* or *in vivo* diagnostics of cancer which comprises administering to an animal containing a tumor expressing a recognizable marker, a pharmaceutically effective amount of a composition containing a dimer or multimer of single chain polypeptides wherein the first and second polypeptide are specific for the recognizable marker.

In another embodiment, the invention is a method for intraoperative therapy which comprises (a) administering a pharmaceutically effective amount of the aforementioned composition containing di- or multimers of a single chain antibody against a recognizable marker to a patient containing a tumor expressing this recognizable marker, whereby the tumor is localized and (b) excising the localized tumor.

In still another embodiment, the invention concerns a process for preparing and expressing the aforementioned dimer and multimers of single chain polypeptides. Such an exemplary process comprises:

(i) providing a genetic sequence coding for a single chain polypeptide;

(ii) transforming a host cell with the genetic sequence;

(iii) expressing the genetic sequence in the host cell; and

(iv) recovery of single chain polypeptides which have non-covalently linked to form dimers and multimers.

Brief Description of the Drawings

Figure 1 shows the digestion of pRW83 to produce vectors used in the construction of plasmid pSCFV31.

Figure 2 shows the construction of plasmid pSCFV31.

Figure 3 illustrates the nucleotide sequence and amino acid sequence of SVFV1.

Figures 4 shows the construction of plasmid pSCFV UNIH.

Figure 5 illustrates the nucleotide sequence and amino acid sequence of Hum4 V_L gene Cla I-Hind III segment in pRL1001.

Figure 6A illustrates the plasmid pSCFV VHH and plasmid pSCFV UHM. (B) and (C) illustrate a schematic representation of a single chain Fv monomer and single chain Fv dimer, respectively. The darkened areas represent the complementarity determining regions.

Figure 7 illustrates the nucleotide sequence and amino acid sequence of a Human Subgroup 4 variable light chain ($H4V_L$) and a murine variable having chain single chain Fv immunoglobulin fragment.

Figure 8 illustrates the DNA sequence and amino acid sequence of CC49 SCFV species with a pI of 8.1(A), 5.8(B) and 5.2(C).

Figure 9 shows the results of a cation exchange chromatograph activity, SDS-PAGE and Western Blot of *E. coli* pSCFV UHM8.1 periplasmic fraction.

Figure 10 shows the results of an anion exchange chromatograph, SDS-PAGE and Western Blot of Pool 2 (Figure 9) of *E coli* pSCFV UHM8.1 periplasmic fraction.

Figure 11 shows the results of a gel filtration chromatograph SDS-PAGE and isoelectric focusing (IEF) from fraction 1 of the anionic exchange (Figure 10).

Figure 12 shows the results of a gel filtration chromatograph, activity SDS-PAGE and Western Blot of *E. coli* pSCFV UHM5.2 periplasmic fraction.

Figure 13 illustrates the results of a CC49 antibody competition based on moles of antibody binding sites.

Figure 14 illustrates the results of a competition ELISA read one hour after addition of substrate.

Figure 15 shows the construction of plasmids pP41, pP42, pP421, pP422, pP431 and pP432.

Figure 16 illustrates the DNA sequence and amino acid sequence of CC49 SCFV in pPY21(A) and pPY22(B).

Figure 17 shows the relative activity of CC49 SCFV species obtained from *Pichia* pP431 and pP432 clones.

Figure 18 shows the results of gel filtration and SDS-PAGE analysis of *Pichia* CC49 scFv species having a pI of 5.2.

Figure 19 shows the results of an anion exchange chromatography and SDS-PAGE of *Pichia* CC49 scFv species having a pI of 5.2.

Figure 20 shows the results of an isoelectric focusing and Western Blot of *Pichia* scFv species having a pI of 5.2.

Figure 21 illustrates the results of a competition ELISA based on (A) moles of antibody sites and (B) moles of antibody.

Figure 22 shows the construction of plasmids pATDFLAG and pSC49FLAG.

Figure 23 illustrates the nucleotide sequences and amino acid sequences in plasmid pATDOFLAG.

Figure 24 illustrates the nucleotide sequences and amino acid sequences in plasmid pSC49FLAG.

Figure 25 shows the results of an M2 affinity chromatograph (A), analytical gel filtration (B), SDS-PAGE (C) and Western Blot of H4L 49HF scFv's.

Figure 26 illustrates the results of binding of H4L49HF single chain species by TAG ELISA (A, B and C) or BSM ELISA (D).

Figure 27 illustrates the results of a competition assay based on moles of antibody binding sites of H4L49F single chain species.

Figure 28 illustrates the results of a competition assay based on moles of H4L49F single chain species.

Nucleic acids, amino acids, peptides, protective groups, active groups and such, when abbreviated, are abbreviated according to the IUPAC IUB (Commission on Biological Nomenclature) or the practice in the fields concerned.

As used herein, the term "non-covalently linked" means a polypeptide that will be separated on the basis of its molecular weight by size exclusion chromatography when done under non-reducing or reducing conditions, but will dissociate into two or more polypeptides when subjected to denaturing conditions such as heating and sodium dodecyl sulfate.

The term "single chain polypeptide" as used herein means a polypeptide from two original peptides linked by a peptide linker where the two original peptides are domains from an antibody and is capable of binding an antigenic determinant or receptor. "Domain" is a segment of protein that assumes a discrete function, such as antigen binding or antigen recognition.

A "dimer" is two single chain polypeptides which are non-covalently linked and the term "multimer" means an even number of dimers which are non-covalently linked. "Vector" means a DNA element used as a vehicle for cloning a fragment of foreign DNA.

The single chain polypeptides which form dimers and multimers in the present invention can be obtained from any member immunoglobulin (Ig) family, including IgG, IgM, IgE, IgD and IgA. The two peptides in the single chain are generally selected from a light and heavy chain variable region of an antibody. Alternatively, it may be desirable to produce dimers and multimers of single polypeptides where the two original peptides are derived from the same domain (e.g., V_L-linker-V_L or V_H-linker-V_H). The present invention also includes the formation of dimers and multimers wherein one single chain polypeptide is directed against one antigen and the second or additional polypeptides are directed against a different antigen.

The dimers and multimers of the present invention formed by domains of the antibody are advantageous for therapeutic purposes in mammals as they retain their antigenic recognition properties, contain multiple binding sites and are of larger molecular weight than the single chain monomers, thereby reducing their retention in the kidneys.

Isolation of an Appropriate DNA Sequence

To prepare a vector containing the DNA sequence for an antigen binding portion of an Ig superfamily protein, a source of the genes encoding for these regions will be required. The appropriate DNA sequence can be obtained from published sources or can be obtained by standard procedures known in the art. For example, Kabat et al., Sequences of Proteins of Immunological Interest 4th ed., (1991), published by The U.S. Department of Health and Human Services, discloses sequences of most of the Ab variable regions which have been described to date. WO 90/04410 published May 3, 1990 discloses a DNA sequence for variable regions of antibodies against tumor associated antigen (TAG).

When the genetic sequence is unknown, it is generally possible to utilize cDNA sequences obtained from mRNA by reverse transcriptase mediated synthesis as a source of DNA to clone into a vector. For antibodies, the source of mRNA can be obtained from a wide range of hybridomas. See, for example, the catalogue *ATCC Cell Lines and Hybridomas*, American Type Culture Collection, 20309 Parklawn Drive, Rockville Md., USA (1990). Hybridomas secreting monoclonal antibodies reactive with a wide variety of antigens are listed therein, are available from the collection, and usable in the present invention. These cell lines and others of similar nature can be utilized as a source of mRNA coding for the variable region or to obtain protein to determine amino acid sequence from the monoclonal antibody itself. The specificity of the antibody to be engineered will be determined by the original selection process. The class of antibody can be determined by criteria known to those skilled in the art.

Variable regions of antibodies can also be derived by immunizing an appropriate vertebrate, normally a domestic animal, and most conveniently a mouse. The immunogen will

be the antigen of interest, or where a hapten, an antigenic conjugate of the hapten to an antigen such as keyhole limpet hemocyanin (KLH). The immunization may be carried out conventionally with one or more repeated injections of the immunogen into the host mammal, normally at two to three week intervals. Usually three days after the last challenge, the spleen is removed and dissociated into single cells to be used for cell fusion to provide hybridomas from which mRNA can readily be obtained by standard procedures known in the art.

From cultured lymphocytes or hybridomas, mRNA can be obtained which in turn is utilized to produce cDNA. From the pool of cDNA, the desired cDNA can be amplified using such techniques as the polymerase chain reaction (PCR) or PCR with splicing by overlap extension (PCR-SOE). PCR in essence involves exponentially amplifying DNA in vitro using sequence specified 5' and 3' oligonucleotides. PCR is described in Mullins et al., *Meth. Enz.*, 155, 335-350 (1987); and *PCR Technology*, Erlich (ed.) (1989).

When an antibody of interest is obtained, and only its amino acid sequence is known, it is possible to reverse translate the sequence.

To form the single chain polypeptides which form dimers and multimers it is generally necessary to have a suitable peptide linker which links the first and second polypeptides. Suitable linkers for joining the polypeptides are those which allow the separate polypeptide chains to fold into a single polypeptide chain which will have a three dimensional structure very similar to the original structure made of two polypeptide chains, and allows non-covalent linkage of single chains to form dimers and multimers of the single polypeptide chain. The linker must thus be (1) sufficiently large to span the distance between the two polypeptides; (2) sufficiently flexible to allow the association of the two polypeptides; and (3) relatively hydrophilic, being on the water-accessible surface of the molecule. Obtaining a three dimensional structure similar to the original antibody molecule allows the resulting single chain polypeptide, dimer or multimer to recognize and react with the initial antigen. Generally, a linker which allows separate polypeptide chains to fold into a single polypeptide chain with a three dimensional structure similar to the original structure made of two polypeptide chains should also allow formation of dimer and multimers. Linkers having the desired properties can be obtained by the method disclosed in U.S. Patent 4,946,778, the disclosure of which is hereby incorporated by reference. From the polypeptide sequences generated by the methods described in the 4,946,778, genetic sequences coding for the polypeptide can be obtained.

It is also necessary that the linker peptide be attached to the peptides such that the binding of the linker to the individual polypeptides does not interfere with the binding capacity of the antigen recognition site.

A preferred linker is the helical linker designated 205 as disclosed in Pantoliano et al. *Biochem.*, 30, 10117-10125 (1991).

Another preferred linker is 205C, which has the amino acid sequence of Leu Ser Ala Asp Asp Ala Lys Lys Asp Ala Ala Ala Lys Lys Asp Asp Ala Lys Lys Asp Asp Ala Lys Lys Asp Leu.

The linker is generally about 10 to about 50 amino acid residues. Preferably the linker is about 10 to about 30 amino acid residues. More preferably the linker is about 12 to about 30 amino acid residues. Most preferred is a linker of about 15 to about 25 amino acid residues.

In another embodiment, the invention comprises two variable domains joined by a peptide bond without an intervening linker polypeptide. Examples include V_L-V_L, V_H-V_H, V_L-V_H and V_H-V_L single chain polypeptides. Such constructs would maximize inter-polypeptide chain interactions to form multimers while minimizing the potential for intra-polypeptide chain interactions to form monomers.

Expression vehicles for production of the molecules of the invention include plasmids or other vectors. In general, such vectors contain replicon and control sequences which are derived from species compatible with a host cell. The vector ordinarily carries a replicon site, as well as specific genes which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is readily transformed using pBR322 [Bolivar et al., *Gene*, 2, 95- (1977), or Sambrook et al., *Molecular* Cloning, Cold Spring Harbor Press, New York, 2nd Ed. (1989)], a plasmid derived from an *E. coli* species which contains genes for ampicillin and tetracycline resistance, and thus provides an easy means for identifying transformed cells. Expression vectors compatible with procaryotic cells are well known in the art and are available from commercial sources, e.g., pBR325 from Gibco Biological Research Laboratories. Typical of vector plasmids suitable for procaryotic cells are pUC8, pUC9, pBR322 and PBR329 available from Biorad Laboratories (Richmond, CA) and pPL and pKK222 available from Pharmacia-LKB (Piscataway, NJ), and the Bluesript SK/KS vectors from Stratagene (LaJolla, CA).

Plasmids suitable for eukaryotic microbes may also be used. *S. cerevisiae*, or common baker's yeast, is the most commonly used among eukaryotic microorganisms although a number of other strains, such as *Pichia pastoris*, are available. Cultures of cells derived from multicellular organisms such as Sp2/0 or Chinese Hamster Ovary (CHO), which are available from the ATCC, may also be used as hosts. Typical of vector plasmids suitable for eukaryotic cells are pSV2neo and pSV2gpt (ATCC); pSVL and pKSV-10 (Pharmacia), pBPV-1/pML2d (International Biotechnology, Inc.).

The use of viral expression vectors to express the genes for polypeptides of the present invention is also contemplated. As used herein, the term "viral expression vector" refers to a DNA molecule that includes a promoter sequence derived from the long terminal repeat (LTR) region of a viral genome. Exemplary phage include λ phage and fd phage (See, Sambrook et al., *supra*; McCafferty et al., *Nature*, 6301, 552-554 (1990).

It is preferred that the expression vectors and the inserts which code for the single chain polypeptides have compatible restriction sites at the insertion junctions and that those

restriction sites are unique to the areas of insertion. Both vector and insert are treated with restriction endonucleases and then ligated by any of a variety of methods such as those described in Sambrook et al., *supra*.

Preferred genetic constructions of vectors for production of dimers and multimers of the present invention are those which contain a constitutively active transcriptional promoter, a region encoding signal peptide which will direct synthesis/secretion of the nascent single chain polypeptide out of the cytoplasm into either an intracellular compartment (in eukaryotes) or the cell exterior (or periplasm in prokaryotes). Preferably, the expression rate is commensurate with the transport, folding and assembly steps to avoid accumulation of the polypeptide as insoluble material. In addition to the replicon and control sequences, additional elements may also be needed for optimal synthesis of single chain polypeptide. These elements may include splice signals, as well as transcription promoter, enhancers, and termination signals.

Vectors which are commercially available can easily be altered to meet the above criteria for a vector. Such alterations are easily performed by those of ordinary skill in the art in light of the available literature and the teachings herein.

Additionally, it is preferred that the cloning vector contain a selectable marker, such as a drug resistance marker or other marker which causes expression of a selectable trait by the host microorganism. Such a drug resistance or other selectable marker is intended in part to facilitate in the selection of transformants. Additionally, the presence of a selectable marker, such as a drug resistance marker, may be of use in keeping contaminating microorganisms from multiplying in the culture medium. In this embodiment, such a pure culture of the transformed host microorganisms would be obtained by culturing the microorganisms under conditions which require the induced phenotype for survival.

Recovery and purification of the dimers and multimers of the present invention can be accomplished using standard techniques known in the art. For example, if the polypeptides are excreted into the culture medium, the polypeptides can be concentrated by ultrafiltration and then separated from monomers by gel filtration. When the polypeptides are transported to the periplasmic space of a host cell, purification can be accomplished by osmotically shocking the cells, and proceeding with ultrafiltration, antigen affinity column chromatography or column chromatography using an ion exchange chromatography and gel filtration. Polypeptides which are insoluble and present as refractile bodies, also called inclusion bodies, can be purified by lysis of the cells, repeated centrifugation and washing to isolate the inclusion bodies, solubilization, such as with guanidine-HCl, and then passage of the solubilized material through a gel filtration column.

While not wishing to be bound by theory, it is believed the dimers and multimers of the present invention form as a result of two discrete physicochemical states (isoforms) of the single chain polypeptide. In one transition state (A) or folding intermediate, only

monomers will be formed. In the second transition state (B) only dimers, or multiples thereof will be formed. The transition states are believed to be related to a conformational or charge equilibrium at a single residue or region in either polypeptide of the single chain polypeptide or even in the linker joining the two. During synthesis, since the single chain polypeptide has not yet assumed a final stable three dimensional structure, the single chain polypeptide is thought to be fairly random in configuration. Single chain polypeptides in this later transition state are capable of forming interchain dimers, a dimer being capable of non-covalently linking to another dimer or multimer.

The activity of the single chain polypeptides can be measured by standard assays known in the art, for example competition assays, enzyme-linked immunosorbant assay (ELISA), and radioimmunoassay (RIA).

USES

The dimers and multimers of the present invention provide unique benefits for use in diagnostic and therapeutics, such as for use in diagnosis or therapy of a variety of diseases. In particular, the dimers and multimers are useful for, but not limited to, *in vivo* and *in vitro* uses in diagnostics, therapy, imaging and biosensors.

Essentially all of the uses for which monoclonal or polyclonal antibodies, or fragments thereof, have been envisioned by the prior art, can be addressed by the multivalent proteins of the present invention. These uses include detectably-labelled forms of the multivalent protein. Types of labels are well-known to those of ordinary skill in the art. They include radiolabelling, chemiluminescent labeling, fluorochromic labelling, and chromophoric labeling. Other uses include imaging the internal structure of an animal (including a human) by administering an effective amount of a labelled form of the multivalent protein and measuring detectable radiation associated with the animal. They also include improved immunoassays, including sandwich immunoassay, competitive immunoassay, and other immunoassays wherein the labelled antibody can be replaced by the multivalent antigen-binding protein of this invention.

The dimers and multimers may be incorporated into a pharmaceutically acceptable carrier. Injectable compositions of the present invention may be either in suspension or solution form. In solution form the complex (or when desired the separate components) is dissolved in a pharmaceutically acceptable carrier. Such carriers comprise a suitable solvent, preservatives such as benzyl alcohol, if needed, and buffers. Useful solvents include, for example, water, aqueous alcohols, glycols, and phosphonate or carbonate esters. Such aqueous solutions generally contain no more than 50 percent of the organic solvent by volume.

Dimers and multimers composed of antigen specific antibody domains are particularly advantageous for use in the diagnosis and/or therapy of diseases, such as cancer, where target antigens are often expressed on the surface of cells. For diagnostic and/or

therapeutic uses, the dimers or multimers can be conjugated with an appropriate imaging or therapeutic agent. Examples of antibodies from which the V_L and V_H domains can be obtained to produce dimers or multimers are CC antibodies, such as CC49, disclosed in published PCT Application WO 89/00692 on January 26, 1989 and published PCT Application WO 90/04410 on May 3, 1990.

Methods for preparing and administering conjugates of the dimers and multimers are accomplished by methods well known or readily determined, as described, for example, in Goldenberg et al., *New England J. Med.*, 298, 1384-1388 (1978); Goldenberg et al.,*Gastroenterol.*, 84, 524-532 (1983); Siccardi et al., *Cancer Res.*, 46, 4817-4822 (1986); Keenan et al., *J. Nucl. Med.*, 25, 1197-1203 (1984); Meares et al., *Anal. Biochem.*, 142, 68-78 (1984). Moreover, suitable dosages will depend on the age and weight of the patient and the therapeutic agent employed and are well known or readily determined.

Conjugates of the dimers and multimers and an imaging marker are administered in a pharmaceutically effective amount for the *in vivo* diagnostic assays, and then detecting the presence of the imaging marker by appropriate detection means. Generally, the dosage should be effective to visualize or detect tumor sites, distinct from normal tissues. Preferably, a one-time dosage will be between 0.1 mg to 200 mg of the conjugate of the dimer or multimer and imaging marker per patient.

Examples of imaging markers which can be conjugated to the dimers and multimers are well known and include substances which can be detected by diagnostic imaging using a gamma scanner or hand held gamma probe, and substances which can be detected by nuclear magnetic resonance imaging using a nuclear magnetic resonance spectrometer

Suitable but not limiting examples of substances which can be detected using a gamma scanner include ^{125}I, ^{131}I, ^{123}I, ^{111}In, ^{105}Rh, ^{153}Sm, ^{67}Cu, ^{67}Ga, ^{166}Ho, ^{177}Lu, ^{186}Re, ^{188}Re and ^{99m}Tc. An example of a substance which can be detected using a nuclear magnetic resonance spectrometer is gadolinium.

A pharmaceutically effective amount of a composition containing dimers and multimers of antibodies is that which should be sufficient to achieve effective binding with the antigens against which the antibodies have specific affinity.

Examples of antibody-therapeutic agent conjugates which can be used in therapy include dimers or multimers of antibodies coupled to radionuclides, such as ^{131}I, ^{90}Y, ^{105}Rh, ^{47}Sc, ^{67}Cu, ^{212}Bi, ^{211}At, ^{67}Ga, ^{125}I, ^{186}Re, ^{188}Re, ^{177}Lu, ^{99m}Tc, ^{153}Sm, ^{123}I and ^{111}In; to drugs such as methotrexate, adriamycin; to biological response modifiers, such as interferon and to toxins, such as ricin.

Methods of preparing and administering conjugates of the dimers and multimers of antibodies and a therapeutic agent are well known or readily determined. The pharmaceutical composition may be administered in a single dose or multiple dosage form.

Moreover, suitable dosages will depend on the age and weight of the patient and the therapeutic agent employed and are well known or readily determined.

Dimers and multimers containing single chain antibodies are particularly suitable for radioimmunoguided surgery (RIGS). In RIGS, an antibody labeled with an imaging marker is injected into a patient having a tumor that expresses a target antigen. The antibody localizes to the tumor and is detected by a hand-held gamma detecting probe (GDP). The tumor is then excised (see, Martin et al. *Amer. J. Surg.*, 156, 386-392 (1988); Martin et al., *Hybridoma*, 5, S97-S108). An exemplary GDP is the Neoprobe™ scanner, commercially available from Neoprobe Corporation, Columbus, OH.

The invention will be further clarified by a consideration of the following examples, which are intended to be purely exemplary of the present invention.

ABBREVIATIONS

BCIP	5-bromo-4-chloro-3-indoyl phosphate
bp	base pair
Bis-Tris propane	(1,3-bis[tris(hydroxymethyl)-methylamino]-propane)
BSA	bovine serum albumin
CDR	Complementarity determining region
ELISA	enzyme linked immunosorbent assay
Fv2	non-covalent single chain Fv dimer
IEF	isoelectric focusing
Kbp	kilo base pair
LB	Luria-Bertani medium
Mab	monoclonal antibody
MES	2-(N-Morpholino)ethane sulfonic acid
MW	molecular weight
NBT	nitro blue tetrazolium chloride
PAG	polyacrylamide gel
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pSCFV	plasmid containing DNA sequence coding for SCFV
RIGS	radioimmunoguided surgery
RIT	radioimmunotherapy
scFv	single chain Fv immunoglobulin fragment
SDS	sodium dodecyl sulfate
TBS	Tris-buffered saline
Tris	(Tris[hydroxymethyl]aminomethane)
TTBS	Tween-20 wash solution
V_H	immunoglobulin heavy chain variable domain
V_L	immunoglobulin light chain variable domain

Antibodies

CC49: A murine monoclonal antibody specific to the human tumor-associated glycoprotein 72 (TAG-72) deposited as ATCC No. HB9459.

CC49 FAB: An antigen binding portion of CC49 consisting of an intact light chain linked to the N-terminal portion of the heavy chain.

ChCC49: Chimeric CC49 has the murine constant regions of the CC49 antibody replaced with the corresponding human constant regions.

CC49 scFv(X): Single chain antibody fragment consisting of two variable domains of CC49 antibody joined by a peptide linker. X refers to the theoretical pI of the scFv (e.g., CC49 scFv8.1).

CC49 Fv2: Two CC49 scFv non-covalently linked to form a dimer. The number after Fv refers to the number of monomer subunits of a given molecule, e.g., CC49 Fv6 refers to the hexamer multimers.

Hum4 V_L or H4VL: Variable domain of the human Subgroup IV kappa light chain.

SCFV UHM(X): SCFV consisting of a CC49 variable light chain and a CC49 variable heavy chain joined by a 25 amino acid linker named UNIHOPE. (X) refers to the theoretical pI of the SCFV which changes as amino acids are added to the C terminus, e.g., scFv UHM8.1; scFv UHM5.2 and scFv UHM5.2.

SCFV UHH: SCFV consisting of a Hum4 V_L and a CC49 variable heavy chain joined by a 25 amino acid linker named UNIHOPE.

EXAMPLES

General Experimental

Procedures for molecular cloning are as those described in Sambrook et al., *Molecular Cloning*, Cold Spring Harbor Press, New York, 2nd Ed. (1989) and Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, New York (1992).

All water used throughout was deionized distilled water

Oligonucleotide Synthesis and Purification

All oligonuclotides (oligos) were synthesized on either a Model 380A or a Model 391 DNA Synthesizer from Applied Biosystems (Foster City, CA) using standard β-cyanoethyl phosphoramidites and synthesis columns. Protecting groups on the product were removed by heating in concentrated ammonium hydroxide at 55°C for 6 to 15 hours. The ammonium hydroxide was removed through evaporation and the crude mixtures were resuspended in 30 to 40 µL of sterile water. After electrophoresis on polyacrylamide-urea gels, the oligos were visualized using short wavelength ultraviolet (UV) light. DNA bands were excised from the gel and eluted into 1 mL of 100 mM Tris-HCl, pH 7.4, 500 mM NaCl, 5 mM EDTA over 2 hours at 65°C. Final purification was achieved by applying the DNA to Sep-Pac™ C-18 columns (Millipore, Bedford, MA) and eluting the bound oligos with 60 percent methanol. The

solution volume was reduced to approximately 50 µL and the DNA concentration was determined by measuring the optical density at 260 nm (OD_{260}).

Bacterial Strains

Both *Eschericia coli* (*E. coli*) RR1 and *E. coli* GM 161 (dam⁻), containing the plasmid pRW83, were obtained from Dr. J. O. Lampen (Rutgers University, New Brunswick, NJ) *E. coli* DH1 was purchased from Invitrogen (San Diego, CA) and competent *E. coli* AG1 was purchased from Stratagene (La Jolla, CA). The plasmid pCGS515 was obtained from Collaborative Research, Inc. (Bedford, MA).

Restriction Enzyme Digests

All restriction enzyme digests were performed using Bethesda Research Laboratories (Gaithersburg, MD), or New England Biolabs, Inc. (Beverly, MA) enzymes and buffers following the manufacturer's recommended procedures. Digested products were separated by polyacrylamide gel electrophoresis (PAGE). The gels were stained with ethidium bromide, the DNA bands were visualized using long wavelength UV light and the DNA bands were then excised. The gel slices were placed In dialysis tubing (Union Carbide Corp., Chicago) containing 5 mM Tris, 2.5 mM acetic acid, 1 mM EDTA, pH 8.0 and eluted using a Max Submarine electrophoresis apparatus (Hoefer Scientific Instruments, CA). Sample volumes were reduced on a Speed Vac Concentrator (Savant Instruments, Inc., NY). The DNA was ethanol precipitated and redissolved in sterile water.

Enzyme Linked Immunosorbent Assay (ELISA)

TAG-72 antigen, prepared substantially as described by Johnson et al, *Can. Res.*, 46, 850-857 (1986), was adsorbed onto the wells of a polyvinyl chloride 96 well microtiter plate (Dynatech Laboratories, Inc., Chantilly, VA) by drying overnight. The plate was blocked with 1 percent BSA in PBS for 1 hour at 31°C and then washed 3 times with 200 µL of PBS, 0.05 percent Tween-20. 25 µL of test antibodies and 25 µL of biotinylated CC49 (1/20,000 dilution of a 1 mg/mL solution) were added to the wells and the plate incubated for 30 minutes at 31°C. The relative amounts of TAG-72 bound to the plate, biotinylated CC49, streptavidin-alkaline phosphatase, and color development times were determined empirically in order not to have excess of either antigen or biotinylated CC49, yet have enough signal to detect competition by scFv. Positive controls were CC49 at 5 µg/mL and CC49 Fab at 10 µg/mL. Negative controls were 1 percent BSA in PBS and/or concentrated LB. Unbound proteins were washed away. 50 µL of a 1:1000 dilution of streptavidin conjugated with alkaline phosphatase (Southern Biotechnology Associates, Inc., Birmingham, AL) were added and the plate was incubated for 30 minutes at 31°C. The plate was washed 3 more times. 50 µL of a para-nitrophenyl-phosphate solution (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) were added and the color reaction was allowed to develop for a minimum of 20 minutes. The relative amount of scFv binding was measured by optical density scanning at 404-450 nm using a microplate reader (Molecular Devices Corporation, Manlo Park, CA). Binding of the scFv

resulted in decreased binding of the biotinylated CC49 with a concomitant decrease in color development.

Amino-Terminal Sequence Analysis

Amino terminal amino acid sequencing was performed by the Edman degradation procedure using an Applied Biosystems model 470A Protein Sequencer (Foster City, CA). Fractions were analyzed with an interface to an Applied Biosystems Model 120A Analyzer (Hewrick, M.W. et al.). Protein samples (about 10 µg each) were prepared by removing PBS present in the sample by 2 cycles of water dilution and concentration using a Centrican 30 device (Amicon).

SDS-PAGE and Western Blotting

Samples for SDS-PAGE analysis (20 µL) were prepared by boiling in a non-reducing sample preparation buffer-Seprasol I (Integrated Separation Systems (ISS), Natick, MA) for 5 minutes and loaded on 10-20 percent gradient polyacrylamide Daiichi Minigels as per the manufacturer's directions (ISS).

Electrophoresis was conducted using a Mini 2-gel apparatus (ISS) at 55 mA per gel at constant current for approximately 75 minutes. Gels were stained in Coomassie Brilliant Blue R-250 (Bio-Rad, Richmond, CA) for at least 1 hour and destained. Molecular weight standards were prestained (Mid Range Kit, Diversified Biotech, Newton Center, MA) and included the following proteins: Phosphorylase b, glutamate dehydrogenase, ovalbumin, lactate dehydrogenase, carbonic amhydrase, B-lactoglobulin and cytochrome C. The corresponding MWs are: 95,500, 55,000, 43,000, 36,000, 29,000, 18,400, and 12,400, respectively.

When Western analyses were conducted, a duplicate gel was also run. After electrophoresis, one of the gels was equilibrated for 15-20 minutes in anode buffer #1 (0.3 M Tris-HCl pH 10.4). An Immobilon-P PVDF (polyvinylidene dichlorine) membrane (Millipone, Bedford, MA) was treated with methanol for 2 seconds, and immersed in water for 2 minutes. The membrane was then equilibrated in anode buffer #1 for 3 minutes. A Milliblot-SDE apparatus (Millipore) was utilized to transfer proteins in the gel to the membrane. A drop of anode buffer #1 was placed in the middle of the anode electrode surface. A sheet of Whatman 3MM filter paper was soaked in anode buffer #1 and smoothly placed on the electrode surface. Another filter paper soaked in anode buffer #2 (25 mM tris pH 10.4) was placed on top of the first one. A sandwich was made by next adding the wetted PVDF membrane, placing the equilibrated gel on top of this and finally adding a sheet of filter paper soaked in cathode buffer (25mM Tris-HCl, pH 9.4 in 40 mM glycine). The screws for the cathode were firmly secured. Transfer was accomplished in 30 minutes using 250 mA constant current (initial voltage ranged from 8-20 volts).

After blotting, the membrane was rinsed briefly in water and placed in a dish with 20 mL blocking solution (1 percent bovine serum albumin (BSA) (Sigma, St. Louis, MO) in Tris-buffered saline (TBS)). TBS was purchased from Pierce Chemical (Rockford, IL) as a

preweighed powder such that when 500 mL water is added, the mixture gives a 25 mM Tris, 0.15 M sodium chloride solution at pH 7.6. The membranes were blocked for a minimum of 1 hour at ambient temperature and then washed 3 times for 5 minutes each using 20 mL 0.5 percent Tween-20 wash solution (TTBS). To prepare the TTBS, 0.5mL of Tween 20 (Sigma) was mixed per liter of TBS. The probe antibody used was 20 mL biotinylated FAID14 solution (10 µg per 20 mL antibody buffer). Antibody buffer was made by adding 1 g BSA per 100 mL of TTBS. After probing for 30-60 minutes at ambient temperature, the membrane was washed 3 times with TTBS, as above.

Next, the membrane was incubated for 30-60 minutes at ambient temperature with 20 mL of a 1:500 dilution in antibody buffer of streptavidin conjugated with alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL). The wash step was again repeated after this, as above. Prior to the color reaction, membranes were washed for 2 minutes in an alkaline carbonate buffer (20 mL). This buffer is 0.1 M sodium bicarbonate, 1 mM MgCl2.H20, pH 9.8. To make up the substrate for alkaline phosphatase, nitroblue tetrazolium (NBT) chloride (50 mg, Sigma) was dissolved in 70 percent dimethylformamide. 5-Bromo--4-chloro-3-indoyl phosphate (BCIP) (25 mg, Sigma) was separately dissolved in 100 percent dimethylformamide. 5-Bromo-4-chloro-3-indoyl phosphate (BCIP) 25 mg, Sigma) was separately dissolved in 100 percent dimethylformamide. These solutions are also commercially available as a Western developing agent sold by Promega. These solutions were stored at 4°C in the dark for up to 3 months. For color development, 120 µL of each were added to the alkaline solution above and allowed to react for 15 minutes before they were washed from the developed membranes with water.

Biotinylated FAID14

FAID14 is a murine anti-idiotypic antibody (IgG2a, K isotype) deposited as ATCC No. CRL 10256 directed against CC49. FAID14 was purified using a Nygene Protein A affinity column (Yonkers, NY). The manufacturer's protocol was followed, except that 0.1 M sodium citrate, pH 3.0 was used as the elution buffer. Fractions were neutralized to pH ~7 using 1.0 M Tris-HCl pH 9.0. The biotinylation reaction was set up as follows. FAID14 (1 mg, 100 µL in water) was mixed with 100 µL of 0.1 M Na_2CO_3 pH 9.6. Biotinyl-ε-amino-caproic acid N-hydroxy succinimide ester (Biotin-X-NHS) (Calbiochem, LaJolla, CA) (2.5 mg) was dissolved in 0.5 mL dimethylsulfoxide. Biotin-X-NHS solution (20 µL) was added to the FAID14 solution and allowed to react at 22°C for 4 h. Excess biotin and impurities were removed by gel filtration, using a Pharmacia Superose 12 HR10/30 column (Piscataway, NJ). At a flow rate of 0.8 mL/min, the biotinylated FAID14 emerged with a peak at 16.8 min. The fractions making up this peak were pooled and stored at 4°C and used to detect the CC49 idiotype as determined by the CC49 V_L and V_H CDRs.

Isoelectric Focusing (IEF) and Western Analysis

Isoelectric points (pI's) were predicted using a computer program called PROTEIN-TITRATE, available through DNASTAR (Madison, WI). Based on amino acid composition with an input sequence, a MW value is given, in addition to the pI. Since Cys residues contribute to the charge, the count was adjusted to 0 for Cys, since they are all involved in disulfide bonds.

Experimentally, pI's were determined using Isogel agarose IEF plates, pH range 3-10 (FMC Bioproducts, Rockland, ME). A Biorad Bio-phoresis horizontal electrophoresis cell was used to run the IEF, following the directions of both manufacturers. The electrophoresis conditions were: 500 volts (limiting), at 20 mA current and 10 W of constant power. Focusing was complete in 90 min. IEF standards were purchased from Biorad; the kit included phycocyanin, β-lactoglobulin B, bovine carbonic anhydrase, human carbonic anhydrase, equine myoglobin, human hemoglobins A and C, 3 lentil lectins and cytochrome C, with pI values of 4.65, 5.10, 6.00, 6.50, 7.00, 7.10 and 7.50, 7.80, 8.00, and 8.20 and 9.60 respectively. Gels were stained and destained according to the directions provided by FMC.

For IEF Westerns, duplicate gels were electrophoresed. Focused proteins in the second gel were transferred to PVDF membranes, as above, using a Biorad Model 483 slab dryer hooked up to a dry ice trap and vacuum pump. A piece of filter paper wetted in water was placed on the drier surface, followed by a prepared PVDF membrane, and then by the gel itself, with its solid support facing up. The plastic cover of the drier was then placed on top of this "sandwich". The drier was operated under vacuum without heat for 20 min to transfer the proteins in the gel to the membrane. Following the transfer, the blotted membrane was blocked and subsequently probed with biotinylated FAID 14 as described above for SDS-PAGE.

Quantitation of CC49 Antibody Species

Purified CC49 antibodies were quantitated by measuring the absorbence of protein dilutions at 280 mm using matching 1.0 mL 1.0 cm pathlength quartz cuvettes (Hellma) and a Perkin-Elmer UV/VIS Spectrophotometer, Model 552A. Molar absorptivities (E_m) were determined for each antibody by using the following formula:

$$E_m = (\text{number Trp}) \times 5{,}500 + (\text{number Tyr}) \times 1{,}340 + (\text{number (Cys)2}) \times 150 + (\text{number Phe}) \times 10$$

The values are based on information given by D. B. Wetlaufer, *Advances in Protein Chemistry*, 17, 375-378).

High Performance Liquid Chromatography

All high performance liquid chromatography (HPLC) was performed using an LKB HPLC system with titanium or teflon tubing throughout. The system consists of the Model 2150 HPLC pump, Model 2152 controller, UV CORD SII Model 2238 detection system set at an absorbance of 276 nm and the Model 2211 SuperRac fraction collector.

EXAMPLE 1 Formation of Single Chain Antibody Dimers in *E. coli*

A. Preparation of pSCFV 31

A vector was prepared from plasmid pRW 83 containing a chloramphenicol resistance (Cam^r) gene for clone selection, and a *penP* gene with a *penP* promoter and terminator (see Mezes et al. (1983), *J. Biol. Chem.*, 258, 11211-11218) and the *pelB* signal sequence [see Lei et al., *J. Bact.*, 169, 4379-4983 (1987)]. The vector designated Fragment A (see Figure 1) was prepared by removal of the *penP* gene with a *Hind* III/*Sal* I digest.

The *penP* promoter and *pelB* signal sequence were obtained by a PCR using pRW 83 as a template and oligonucleotides penP1 and penP2 as primers. The fragment was designated Fragment B (see Figure 1). A *Nco* I enzyme restriction site was introduced at the 3' end of the signal sequence region by the penP2 oligonucleotide.

penP1:

5'-CGATAAGCTTGAATTCCATCACTTCC-3'

penP2:

5'-GGCCATGGCTGGTTGGGCAGCGAGTAATAACAATCCAGCG GCT GCCGTAGGCAATAGGTATTTCATCAAAATCGTCTCCCTCCGTTTGAA-3'

A scFv comprised of a Hum4 V_L, a CC49 V_H, and an 18 amino acid linker (Lys Glu Ser Gly Ser Val Ser Ser Glu Gln Leu Ala Gln Phe Arg Ser Leu Asp) was obtained from pCGS515/SCFV1 by PCR using oligonucleotides penP3 and penP6. This fragment was designated Fragment D (see Figure 2). The complete nucleotide sequence and amino acid sequence of pCGS515/SCFV1 is given in Figure 3. A *Bcl* I site was introduced at the 3' end of the V_H region by the penP6 oligonucleotide.

penP3:

5'-GCTGCCCAACCAGCCATGGCCGACATCGTGATGACCCAGTCTCC-3'

penP6(-):

5'-CTCTTGATCACCAAGTGACTTTATGTAAGATGATGTTTTG ACG GATTCATCGCAATGTTTTTATTTGCCGGAGACGGTGACTGAGGTTCC-3'

Fragments B and D were joined by SOE-PCR (splicing by overlap extention-polymerase chain reaction) using oligonucleotides penP1 and penP6, following the procedures of Horton et al., *Gene*, 77, 61-68 (1989). The new fragment was designated E (See Figure 2).

Fragment C, containing the *penP* termination codon, was isolated by digesting pRW 83 with *Bcl* I and *Sal* I. pRW 83 was isolated from *E. coli* strain GM161, which is DNA methylase minus or *dam*$^-$.

Plasmid pSCFV 31 (see Figure 2) was created with a three part ligation of Fragments A, C, and E.

B. Preparation of pSCFV 31b

The *Nco* I restriction enzyme site within the Cam^r gene and the *Hind* III site located at the 5' end of the *penP* promoter in pSCFV 31 were destroyed through a PCR DNA amplification using oligonucleotides Nco1.1 and Nco1.3(-) to generate an *Eco* RI to *Nco* I fragment and oligonucleotides Nco1.2 and Nco1.4c(-) to generate a *Nco* I to *Eco* RI fragment. These two fragments were joined by PCR-SOE using oligonucleotides Nco1.1 and Nco1.4c(-). The oligonucleotides are set forth below:

Nco1.1:

5'-TCCGGAATTCCGTATGGCAATGA-3'

Nco1.3(-):

5'-CTTGCGTATAATATTTGCCCATCGTGAAAACGGGGGC-3'

Nco1.2:

5'-ATGGGCAAATATTATACGCAAG-3'

Nco1.4c(-):

5'-CACTGAATTCATCGATGATAAGCTGTCAAACATGAG-3'

pSCFV 31 was digested with *Eco* RI and the larger fragment was isolated by polyacrylamide gel electrophoresis. To prevent self ligation, the DNA was dephosphorylated using calf intestinal alkaline phosphatase according to the teachings of Sambrook et al., *supra*.

A two part ligation of the larger pSCFV 31 digested fragment and the PCR-SOE fragment, described above, resulted in the creation of pSCFV 31b (see Figure 4).

C. Preparation of pSCFV 33H

pSCFV 31b was digested with *Nco* I and *Sal* I and a fragment containing the Cam^r gene was isolated.

The Hum4 V_L was obtained by PCR DNA amplification using pCGS515/SCFV1 (See Figure 3) as a template and oligonucleotides 104BH1 and 104BH2(-) as primers.

104BH1:

5'-CAGCCATGGCCGACATCGTGATGACCCAGTCTCCA-3'

104BH2(-):

5'-AAGCTTGCCCCATGCTGCTTTAACGTTAGTTTTATCTGCTGG

AGACAGAGTGCCTTCTGCCTCCACCTTGGTCCCTCCGCCGAAAG-3'

The CC49 V_H was obtained by PCR using p49γ1-2.3 (PCT WO 90/04410) as a template and oligonucleotides 104B3 and 104B4(-) as primers. A *Nhe* I enzyme restriction site was introduced just past the termination codon in the 3' end (before the *Bcl* I site) by oligonucleotide 104B4(-).

104B3:

5'-GTTAAAGCAGCATGGGGCAAGCTTATGACTCAGTTGCAGCAGTCTGACGC-3'

104B4(-):

5'-CTCTTGATCACCAAGTGACTTTATGTAAGATGATGTTTTGACGGATT
CATCGCTAGCTTTTTATTTGCCATAATAAGGGGAGACGGTGACTGAGGTTCC-3'

In the PCR which joined these two fragments using oligonucleotides 104BH1 and 104B4(-) as primers, a coding region for a 22 amino acid linker was formed.

A fragment C (same as above) containing the *penP* termination codon was isolated from pRW 83 digested with *Bcl* I and *Sal* I.

Plasmid pSCFV 33H (see Figure 4) was created with a three part ligation of the vector, fragment C, and the SCFV fragment described above.

D. Preparation of pSCFV UNIH

To create a vector for any SCFV with the desired restriction sites in place, a plasmid designated UNIH was created by digesting pSCFV 33H with *Nco*I and *Nhe*I, and isolating the DNA fragment containing the Cam^r gene.

Hum4 V_L was obtained by PCR DNA amplification using pRL1001 as a template and oligonucleotides UNIH1 and UNIH2(-) as primers. The DNA sequence in pRL1001 coding for the Hum4 V_L is given in Figure 5. Oligonucleotides for the PCR were:

UNIH1:

5'-CAGCCATGGCCGACATTGTGATGTCACAGTCTCC-3'

The *Nco* I site is in bold and the hybridizing sequence is underlined.

UNIH2(-):

5'-GAGGTCCGTAAGATCTGCCTCGCTACCTAGCAAA
AGGTCCTCAAGCTTGATCACCACCTTGGTCCCTCCGC-3'

The *Hind* III site is in bold.

The CC49 V_H was obtained by a PCR using p49γ1-2.3 as a template and oligonucleotides UNI3 and UNI4(-) as primers.

UNI3:

5'-AGCGAGGCAGATCTTACGGACCTCGAGGTTCAGTTGCAGCAGTCTGAC-3'.

The *Xho* I site is in bold and the hybridizing sequence is underlined.

UNI4(-):

5'-CATCGCTAGCTTTTTATGAGGAGACGGTGACTGAGGTTCC-3'.

The *Nhe* I site is in bold and the hybridizing sequence is underlined.

Oligonucleotides UNIH1 and UNI4(-) were used in the PCR-SOE amplification which joined the Hum4 V_L and CC49 VH fragments and formed a coding region for a negatively charged fifteen amino acid linker. The DNA was digested with *Nhe* I and *Nco* I and ligated with the vector fragment from the *Nco* I-*Nhe* I digest of pSCFV 33H. The resultant plasmid was designated pSCFV UNIH (see Figure 4).

With the construction of pSCFV UNIH, a universal vector for any SCFV was created with all the desired restriction enzyme sites in place.

E. Preparation of pSCFV UHH and pSCFV UHM

pSCFV UNIH was digested with *Hind* III/*Xho* I, and the large DNA fragment containing the Cam^r gene, Hum4 V_L and CC49 V_H was isolated.

A fragment coding for a 25 amino acid linker, UNIHOPE, was made by annealing the two oligonucleotides shown below. Annealing conditions were as follows: 10 µg of each oligo were added to Bethesda Research Laboratories (Gaithersburg, MD) enzyme Buffer #2 for a total volume of 53 µL, the mixture was heated to 98°C for one minute, cooled slowly to 40°C and placed at 4°C overnight. Following digestion with *Hind* III/*Xho* I, the linker was electrophoresed through a 6 percent PAG. The DNA bands were excised from the gel and the DNA was electroeluted for five minutes at 200 volts. The DNA was pelleted by ethanol precipitation and dissolved in water. The linker UNIHOPE is based on 205C SCA™ linker (see Whitlow et al., *Methods: A Companion to Methods in Enzymology*, 2, 97-105 (1991), but the first amino acid was changed from serine to leucine and the twenty-fifth amino acid were changed from glycine to leucine, to accommodate the *Hind* III and *Xho* I restriction sites, respectively. The nucleotide sequence encoding the linker UNIHOPE is set forth below:

UNIHOPE (Top Strand):

5'-TATAAAGCTTAGTGCGGACGATGCGAAAAAGGATGCTGCGAAG
AAGGATGACGCTAAGAAAGACGATGCTAAAAAGGACCTCGAGTCTA-3'

UNIHOPE(-) Bottom Strand:

5'TAGACTCGAGGTCCTTTTTAGCATCGTCTTTCTTAGCGT CAT
CCTTCTTCGCAGCATCCTTTTTCGCATCGTCCGCACTAAGCTTTATA-3'

The resulting annealed strand was digested with *Hind* III/*Xho* I and ligated into the vector, thus generating the plasmid pSCFV UHH (shown in Figure 6). Plasmid pSCFV UHH expresses a biologically active, TAG-72 binding SCFV consisting of the Hum4 V_L and CC49 V_H. The expression plasmid utilizes the β-lactamase penP promoter, pectate lyase *pelB* signal sequence and the *penP* terminator region. Different immunoglobulin light chain variable regions can be inserted in the *Nco* I-*Hind* III restriction sites, different SCFV linkers can be inserted in the *Hind* III-*Xho* I sites and different immunoglobulin heavy chain variable regions can be inserted in the *Xho* I *Nhe* I sites.

F. Preparation of pSCFV UNIM and pSCFV UHM

pSCFV UNIM was created in conjunction with pSCFV UNIH. It differs in that the DNA sequence coding for the CC49V_L is incorporated into UNIM as opposed to the DNA sequence coding for the Hum4 V_L. The sequence for murine CC49V_L is disclosed in PCT WO 90/04410, published May 3, 1990, and was produced by PCR using the oligos UNIM1 and UNIM2(-) as primers. The CC49 V_H and the vector fragment were obtained by the manner described above for pSCFV UNIH. The SCFV insert was generated by PCR SOE using oligos UNIM1 and UNI4(-) to join the light and heavy chains and form a coding region for a negatively

charged fifteen amino acid linker. Following *Nhe*I and *Nco*I digestion and purification of the vector and insert, a ligation of the two pieces produced pSCFV UNIM.

UNIM1:

5'CAGCCATGGCCGACATTGTGATGTCACAGTCTCC-3'

UNIM2(-):

5'GAGGTCCGTAAGATCTGCCTCGCTACCTAGCAAAA

GGTCCTCAAGCTTCAGCACCAGCTTGGTCCCAGCAC-3

pSCFV UHM was generated by digesting pSCFV UNIM with *Hind*III and *Xho*I, isolating the vector fragment with the Cam^r gene by 4 percent PAG, and ligating the twenty-five amino acid linker, UNIHOPE, into the vector DNA fragment piece. The procedure was the same as described for pSCFV UHH. pSCFV UHM contains DNA sequence coding for an scFv having the CC49 V_L and CC49 V_H with a computer predicted pI of 8.1. Hence, this plasmid was named pSCFV UHM8.1. Hence, this plasmid was named pSCFV UHM8.1

The plasmid map of pSCFV UHH and pSCFV UHM are illustrated in Figure 6A. For pSCFV UHH, V_L is Hum4 V_L, the linker (L) is UNIHOPE and V_H is CC49 V_H. For the pSCFV UHM series, V_L is CC49 V_H, the linker is UNIHOPE and V_H is CC49V_H. For the pSCFV UHM(X) series, X indicates the pI of the monomer product (pI = 8.1, 5.8 or 5.2) as described herein. Figures 6B and 6C illustrate the monomeric (scFv) and dimeric (Fv2) forms, respectively, of the products. The CDR regions are indicated schematically by the black bands.

The DNA and amino acid sequences of the scFv obtained from pSCFV UHH constructions are given in Figure 7. The UNIHOPE linker sequence is indicated in bold and underlined, while the CDR regions are in bold and italicized. The amino terminus of the mature scFv product is indicated by + 1.

E. coli AG1 (Statagene, La Jolla, CA) cells were transformed with pSCFV UHH and pSCFV UHM8.1 by following the manufacturer's instructions. Competent cells were thawed on ice and gently mixed by hand before 50 µL aliquots were dispensed into 15 mL polypropylene tubes. A fresh dilution of β-mercaptoethanol was added to the tubes for a final concentration of 25 mM. The cells were swirled gently every two minutes for ten minutes. Either 1 µL or 5 µL of the prepared DNA was added to the tubes. The cells and DNA sat on ice for 30 minutes, were heat pulsed in a 42°C water bath for 45 seconds and put back on ice for two minutes. SOC medium (450 µL) (medium containing bacto-tryptone, bacto-yeast extract, NaCl $MgCl_2$ and glucose) was added to each tube and the tubes were incubated at 37°C for one hour with shaking. Either 100 µL or 200 µL of transformed cells were plated on LB agar containing 20 µg/ml chloramphenicol. Plates were incubated at 37°C overnight.

From each transformation, twelve to eighteen bacterial colonies were selected for overnight growth at 37°C in 2 mL of LB/CAM 20. A portion of the overnight culture (1.2 mL) was used for a small scale plasmid preparation following the boiling method adapted from Holmes and Quigley (Sambrook et al, 1989, *supra*). Plasmid DNA was digested with restriction

enzymes and the resultant fragments were size analyzed by agarose gel electrophoresis using ethidium bromide and long wavelength UV for visualization. The remaining 0.8 mL of overnight culture was sonicated (Soniprep 150, MSE Ltd., Sussex), then microcentrifuged for 3 minutes to pellet the debris. The supernatant was reduced in volume by 50 to 80 percent using a Centricon 10 microconcentrator device (Amicon, Beverly, MA) and tested for biological activity against TAG-72.

(G) Construction of pSCFV UHM5.8 and pSCFV UHM5.2

Two Plasmids, pSCFV UHM5.8 and pSCFV UHM5.2 were constructed to express CC49 scFvs and CC49 Fv2s having computer predicted pI's of 5.8 and 5.2, respectively. Extra negatively charged amino acids were added to the carboxyl terminus of the CC49 V_H, by synthesizing two oligonucleotides (oligos) and using them for a polymerase chain reaction (PCR) amplification of the CC49 V_L - linker - CC49 V_H DNA present in pSCFV UHM8.1. These oligos for the 3' end of the target are as follows. For the 5.8 construction, a 58-mercalled SCUHPM 5.8 was made. For the 5.2 construction, a 64-mercalled SCUHPM 5.2 was made.

SCUHPM 5.8

5'-AACA GCTAGC TTT TTA GGA GTC ATA GTC CTC AGG GGA GAC GGT GAC TGA GGT TCC TTG-3'

SCUHPM 5.2

5' - AACA GCTAGC TTT TA CTC ATA CTC TTC AGG GTC TTC AGG GGA GAC GGT GAC TGA GGT TCC TTG - 3'

The nucleotides representing *Nhe* I restriction sites are indicated in bold. These oligos were purified from a 14 percent polyacrylamide gel containing urea (Sambrook et al., *supra*). The 5' oligo for the PCR was a 34 mer designated UNIM1 (sequence given above).

The nucleotides representing an *Nco* I restriction site are indicated in bold. For the PCR's, 100 pmol of the 5'(UNIM1) and 3' (SCUHPM 5.8) oligos were used to generate the CC49 scFv 5.8 DNA product insert, while 100 pmol of each of UNIM1 and SCUHPM 5.2 were used to generate the CC49 scFv 5.2 DNA product insert. The target DNA for the oligos in the PCR reaction (100 uL) was 1.0 ng of pSCFV UHM8.1. The reaction was performed using a programmable thermal controller (MJ Research Inc., Watertown, MA) and Taq polymerase according to the directions provided by Perkin Elmer Cetus (Norwalk, CT). There were 30 cycles consisting of 40 seconds at 94°C, 70 seconds at 45°C and 75 seconds at 72°C. For the final cycle, the temperature was held at 72°C for 2 minutes. The PCR products were analyzed on a 1.0 percent agarose gel containing ethidium bromide and found to be the expected sizes of 788 bp and 794 bp for the 5.8 and 5.2 CC49 scFv constructions, respectively. The DNA was preparatively purified from a 4 percent polyacrylamide gel, digested with *Nco* I (from BRL, Gaithersburg, MD) and Nhe I (from New England Biolabs) using New England BioLabs 10 x buffer #2 (10mM Tris-Hcl, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM OTT, pH 7.9).

The resulting trimmed *Nco* I and *Nhe* I oligonucleotides fragment of the correct size were purified away from the DNA inserts by electrophoresis in a 4 percent polyacrylamide gel. The CC49 scFv inserts were electroeluted and ethanol precipitated (Sambrook). The vector for ligation with these inserts had been prepared from pSCFV33H digested with *Nco* I and *Nhe* I. Competent *E. coli* AG1 cells (Strategene, La Jolla, CA) were transformed with the ligated DNA and plated on LB plates containing 20 μg/mL chloramphenicol.

Four clones were chosen from each transformation to prepare miniprep plasmid DNA (Sambrook) for restriction enzyme analysis. Based on these results single clones were chosen; *E. coli* AG1/pSCFV UHM5.8 *E. coli* and AG1/pSCFV UHM5.2.

The DNA and amino acid sequences of the CC49 species, pSCFV UHM8.1 (A), 5.8 (B) and 5.2 (C), are given in Figure 8.

Preparation of Total Cellular Protein and *Escherichia Coli* Periplasimic Shockates

For analysis of total cell CC49 scFv activity or preparation of samples for sodium dodecyl sulfate polyacrylamide gel electrophonesis (SDS-PAGE), *E. coli* (pSCFV UHM8.1; pSCFV 5.8; or pSCFV 5.2) liquid cultures (2.5 mL LB supplemented with 20 μg/mL chloramphenicol) were grown overnight at 37°C. Cells from 1.5 mL of culture were pelleted in a microcentrifuge, the supernatant discarded, and the cell pellets resuspended in 0.5 mL phosphate buffered saline (PBS) (Sigma Chemical). Sample tubes were placed in an ethanol ice bath before sonication (MSE Soniprep 150, UK) using 3 cycles of disruption at 14 microns amplitude for 15 seconds each and cool down for 45 seconds in between. The sonicated cell debris was pelleted in a microcentrifuge, and the supernatant, representing the total, soluble protein fraction, was filtered through a Millex-GV 0.22 μ filter disc (Millipore, Bedford, MA).

For purification of the CC49 scFv$^{(X)}$ derivatives, where x = 8.1, 5.8 and 5.2, *E. coli* periplamsic fractions were prepared from 1.0 L overnight cultures. A 1.0 L culture was divided into 4x250 mL portions and centrifuged at 5,000 rpm, for 10 minutes in a Sorvall GS-3 rotor. The pelleted cells were washed and resuspended in 100 mL each of 10 mM Tris-HCl pH 7.3 containing 30 mM NaCl. The cells were again pelleted at 5,000 rpm for 10 minutes. Cells were washed with a total of 100 mL of 30 mM Tris-HCl (pH 7.3) and pooled into 1 tube. To this was added 100 mL of 30 mM Tris-HCl pH 7.3 containing 40 percent w/v sucrose and 2.0 mL of 10 mM EDTA, pH 7.5. The mixture was kept at ambient temperature, with occasional shaking for 10 minutes. The hypertonic cells were centrifuged as above. The cells where then quickly resuspended in ice cold 0.5 mM $MgCl_2$ and the suspension kept on ice for 10 minutes with occasional shaking. The cells were centrifuged as above and the shockate, containing the *E. coli* periplasmic fraction was further clarified by passing through a 0.2 μ filter apparatus (Nalge Co., Rochester, NY). This material was concentrated using Centriprep 30 and Centricon 30 ultrafiltration devices (Amicon Beverly, MA) to a final volume of 1-2 mL.

For isolation and analysis of just cytoplasmic proteins, previously shocked *E. coli* cells (0.5-1.0 mg - wet weight) were resuspended in 0.5 mL PBS and sonicated as above. The cytosol was recovered by microcentri-fugation and filtered through a 0.2 μ filter device.

Prior to further purification, the shockate was dialyzed against 50 mM sodium acetate buffer, pH 4.95, using a System 500 microdialyzer unit (Pierce Chemical, Rockford, IL), with 3-4 exchange of buffer. An 8,000 molecular weight (MW) cut-off membrane was used.

Purification of CC49 Single Chain Monomer and Dimer Species

The general purification of the single chain antibody species is given in Scheme 1. The procedure under column 1 was initially used for purification of antibodies from pSCFV UHM8.1 and was later modified as given under column 2. Single chain antibody species derived from pSCFV UHM5.2 were purified as given under column 3. Final purification of the pSCFV UHM5.8 products was not completed.

Scheme I

Periplasmic Fraction From		
1.	2.	3.
E. coli pSCFV UHM8.1	E. coli pSCFV UHM8.1	E. coli pSCFV UHM5.2
↓	↓	↓
Mono S	Mono S	Superdex 75
50 mM NaOAC, pH 4.95 (Figure 9)	50 mM NaOAc, pH 5.20 (Gave smaller pellet upon dialysis) All active fractions pooled	Take monomer fractions (Figure 10)
↓	↓	↓
2 Pools of Activity	Mono Q	Mono Q
Take Pool 2	20 mM Tris-HCL, pH 7.60	20 mM Bis-Tris Propane-HCl, pH 6.80 (Figure 11)
↓	↓	↓
Mono Q	Mono S	
20 mM Tris-HCl, pH 7.60 (Figure 10)	50 mM MES, pH 5.60	
↓	↓	↓
Superdex 75	Superdex 75	Superdex 75
Result: pure dimer partially pure monomer (Figure 11)	Result: (a) Pure dimer - Elutes at 43 min. using PBS buffer and 0.2 ml/min flow rate. (b) pure monomer-elutes at 52 min. using PBS and 0.2 mL/min. flow rate.	Result - pure scFv. Elutes at 49.5 min. using PBS and 0.2 mL/min flow rate. (Figure 12)

A. CC49 scFv 8.1

For single chain antibody species from pSCFV UHM8.1, about half of the *E. coli* AG1/pSCFV UHM8.1 periplasmic shockate prepared from 1 L of culture as described above was dialyzed overnight against 50 mM sodium acetate buffer, pH 4.95. Precipitated proteins were pelleted by microcentrifugation and the supernatant was applied to a cation exchange column (Mono S HR5/5 HPLC column, Pharmacia, Piscataway, N.J.). A linear gradient program utilizing 50 mM sodium acetate pH 4.95 as buffer A and 50 mM sodium acetate pH 4.95 containing 0.3 M NaCl as buffer B was run as follows:.

Time (min)	Flow Rate (mL/min)	Percent B
0	1.5	0
5.0	1.5	0
35.0	1.5	30
40.0	1.5	50
45.0	1.5	100
50.0	1.5	100
55.0	1.5	0

When the periplasmic fraction was chromatographed over Mono S, two areas of activity were observed (Figure 9) and the two pools kept separate for the next step. The lanes 1-9 for SDS-PAGE correspond to a crude periplasmic fraction (lane 1) and fractions 1, 2, 3, 12, 16, 21, 26 and 43, lanes 2-9 respectively, with a Western transfer of the samples from a duplicate gel, indicate product in fractions 26 and 43 (lanes 17 and 18).

Pool 2 from the Mono S purification was concentrated to below 250 µL using a Centricon 30 device and dialyzed against buffer A (20 mM Tris-HCl, pH 7.60) for rechromatography on the Mono Q HR5/5 anion exchange column (Pharmacia, Piscataway, N.J.). Buffer B was the same as buffer A, but in 0.5 M NaCl. The gradient program used was the same as for the Mono S run above, but for this Mono Q run the Mono S pool 2 sample was changed to 100 percent B the gradient was at 16 percent B.

The anion exchange chromatography and SDS-PAGE of the Mono S Pool 2 sample is given in Figure 10. The arrow on the chromatogram indicates the point in the gradient of Buffer B (16 percent) where the percent of B was changed to 100 and held for 5 minutes. Lanes 1-9 correspond to fractions 1-3 of a similar chromatography (not shown) done on Pool 1 (Figure 9); a sample of Pool 1; a sample of Pool 2; and fractions 1-4 from the anion exchange

chromatography of Pool 2. Lanes 10-18 represent the same fractions as in lanes 1-9 in a Western transfer from a duplicate gel.

The final step in the purification utilized a Superdex 75 HR10/30 gel filtration column (Pharmacia). Potassium phosphate buffer (0.175 M, pH 6.85) was used as the mobile phase, at a flow rate of 0.2 mL/min. Fraction 1 from the Mono Q purification was applied to the Superdex column and the resulting chromatogram is shown in Figure 11A-1. Both peaks emerging at 42 and 50.5 minutes were active in the competition assay, with considerably more activity being observed in the first peak (42 minutes). On SDS-PAGE and Western transfer (Figure 11B) a band for the correct molecular weight of monomeric single chain antibody was observed. Lanes B1 and 8 are ChCC49 Fab; lanes 2 and 9, fraction 14; lanes 3 and 10, fraction 15; lanes 4 and 11 fraction 16; lanes 5 and 12, fraction 17,; lanes 6 and 13, fraction 18; and lanes 7 and 14, fraction 19. Application of a ChCC49 Fab fragment to the gel filtration column (Figure 11 A-2), using the same conditions as for the scFv sample, indicated that the scFv peak emerging at 42 minutes contained a protein that was larger than the Fab, which emerged at 45.8 minutes. When the peak emerging at 42 minutes was subjected to the reducing condition of SDS-PAGE, a monomeric unit on the gel and Western was seen, indicating a con-covalently linked dimeric (Fv2) form of the single chain antibody. Further evidence of a unique dimeric entity was obtained when an IEF gel was run.

IEF of fractions 15 (lanes C1 and C3) and 17 (lanes C2 and C4), indicates there is a distinct difference between the pI of the Fv2 (lanes 1 and 3) and the monomeric scFV (lanes 2 and 4). Samples in lanes 1 and 2 were stained with Coomassie Brilliant Blue R-250, while lanes 3 and 4 represent a Western transfer of the corresponding lanes of a duplicate IEF gel. The faint FAID14 positive band in Figure 11C, lane 3, above the main Fv2 band is not known. It could be a trace degradation product or some higher multimer.

A second purification system was then developed to optimize the purification of the monomer, dimer and multimer species of the pI 8.2 single chain antibodies. The procedure is outlined in column two of Scheme 1. The buffer system for purification with the Mono S cation exchange column was also changed to 50 mM sodium acetate pH 5.2 and 50 mM 2-(N-morpholino)ethane sulfonic acid (MES).

The protocol which afforded pure CC49 scFv and Fv2 8.2 was as follows: A periplasmic fraction was dialyzed and applied to mono S using a 50 mM sodium acetate buffer system, as before, but at pH 5.20. At this pH less protein precipitated than at pH 4.95 but the results were much the same in terms of the chromatography. Next, the Mono Q column was utilized as above. After this, a second Mono S run was introduced for the active Mono Q fractions. The buffer system used was: buffer A (50 mM MES, pH 5.6) and buffer B being the same as buffer A in 0.5 M NaCl. The monomer and dimer were finally purified using the Superdex 75 HR10/30 column and PBS as the buffer.

B. CC49 scFv5.2 and Fv2-5.2

The periplasmic shockate from a 1.0 liter culture was prepared as described above. The strategy for purification used here was to separate the monomer from the dimer in a gel filtration step first. A Superdex 75 prep grade HR 26/60 preparative column was used. The concentrated crude sample (0.5 mL) was chromatographed at a flow rate of 1.3 mL/min. using 0.175 M potassium phosphate buffer pH 6.8. See Figure 12 for the chromatogram. The elution times for the molecular weight standards: (1) bovine IgG (Pharmacia), MW = 153 Kd; (2) chicken ovalbumin, MW = 44 Kd; and (3) horse myoglobin, MW 17 Kd from a separate run are as indicated by the arrows.

SDS-PAGE analysis of the fractions was as follows: lanes 1 and 14, ChCC49 Fab; lanes 2-13 correspond to fractions 3, 8, 10, 12, 14, 16, 18, 19, 20, 22, 23 and 24 respectively and lanes 15-24 correspond to fractions 25-33 and 35, respectively.

Both the resulting dimer and monomer pools were concentrated to 300 µL with centricon 30 devices and dialyzed overnight against 20 mM (1,3-bis[tris(hydroxymethyl)-methylamino]propane (Bis-Tris propane)-Cl pH 6.8 buffer and applied to the Mono Q HR5/5 column for the monomer and dimer purification. The same gradient program was used as described earlier with buffer B being 20 mM Bis-Tris propane-Cl pH 6.8 in 0.5 M NaCl. The active Mono Q fractions were pooled and concentrated to 130 µL. The sample was applied to the Superdex 75 HR 10/30 column to verify purity of the monomer and exchange the buffer to PBS.

Antibody Competition

Using purified monomer and dimer, it was possible to quantitate them accurately (Table 1) and compare them directly in a competition assay to a Fab, which is monovalent, and the CC49 whole antibody, which is divalent. The results of this study are given in Figure 13. The results indicate the dimer and whole IgG have similar activity. These results further support the data of the existence of the dimer, indicating the dimer is folding in a way that gives two active, accessible binding sites.

Table 1: Characterization of CC49 Antibody Species

Amino Acid Composition	SCFV UHM 8.1	Fv2 8.1	SCFV UHM 5.8	Fv2 5.8	SCFV UHM 5.2	Fv2 5.2	SCFV UHH	ChCC49 V_L[1]	ChCC49 V_H ($83C_H1$)[1]	CC49 V_L[2]	CC49 V_H[2]
-Ala	20	40	20	40	20	40	21	12	15	10	24
-Cys	4	8	4	8	4	8	4	5	5	5	12
-Asp	18	36	20	40	21	42	19	9	8	11	18
-Glu	9	18	10	20	11	22	9	10	6	9	22
-Phe	8	16	8	16	8	16	8	7	8	7	19
-Gly	17	34	17	34	17	34	18	13	17	13	21
-His	2	4	2	4	2	4	2	2	4	2	11
-Ile	6	12	6	12	6	12	8	4	3	7	15
-Lys	24	48	24	48	24	48	22	16	14	15	32
-Leu	21	42	21	42	21	42	18	20	15	17	23
-Met	2	4	2	4	2	4	3	1	1	2	6
-Asn	7	14	7	14	7	14	8	7	9	10	21
-Pro	9	18	10	20	11	22	9	11	11	11	34
-Gln	15	30	15	30	15	30	14	14	10	11	21
-Arg	4	8	4	8	4	8	5	5	4	6	7
-Ser	35	70	35	70	34	68	32	35	32	35	47
-Thr	16	32	16	32	16	32	17	15	20	18	40
-Val	16	32	16	32	16	32	16	18	20	14	41
-Trp	5	10	5	10	5	10	5	3	4	4	9
-Tyr	15	30	16	32	16	32	15	13	9	13	13
TOTAL	253	506	258	516	260	520	253	220	215	220	436
E_{280}	47,980	95,960	49,320	98,640	49,320	98,640	47,980	68,880		215	750
E_{230} 0.1%	1.73	1.73	1.74	1.74	1.72	1.72	1.73	1.46		1.49	
MW[3]	27,749	55,498	28,369	56,738	28,623	57,246	27,736	24,125	22,956	24,294	48,037
pI	8.12	8.29-8.46	5.84	6.10-6.38	5.23	5.35	6.39	8.67	9.22	8.21	7.22

[1]The MW of the ChCC49 Fab from these components is 47,081 daltons.
[2]The MW of the CC49 IgG1 from these components is 144,662 daltons. Carbohydrate weight was not included.
[3]The values given are adjusted for disulfide bond formation between Cys residues.

The activity of the purified CC49 scFv5.2 was compared with the ChCC49 Fab in a competition assay (Figure 14) and the scFv was found to be equivalent to the Fab. This indicates one can add certain features to the carboxyl terminus of a scFv construction, as in this case the addition of Pro-Glu-Asp-Pro-Glu-Asp-Tyr-Asp, that do not affect the binding site. Therefore, other modifying sequences can be contemplated which alter the overall characteristics of the molecule.

Amino-Terminal Amino Acid Analysis

The amino terminal sequencing data gave the following result: NH2-?-I-V-M-S-Q-S-P-S?-T?-L. The first residue couldn't be unequivocally assigned, but its positioning indicates that signal peptidase had cleaved at the end of the *pel* B signal sequence, and released a correctly processed mature product. The residues indicated at positions 2-11 correspond to the sequence predicted by the DNA sequence (See Figure 8). The yields for Ser and Thr at positions 9 and 10 were low, but in general, Ser and Thr yields are known to be lower than for the other amino acids. Based on these data, one can conclude that the *pel* B signal sequence is effective in directing the secretion of the single chain products to the *E. coli* periplasm. It is apparent that one need not be limited to using the *pel* B leader, and that the same results, as far as processing and secretion of the product is concerned, can be obtained by using other signal sequences such as *omp* A and *pen* PC, and others. One can include these sequences in the PCR oligo when making the construction upstream from the mature protein.

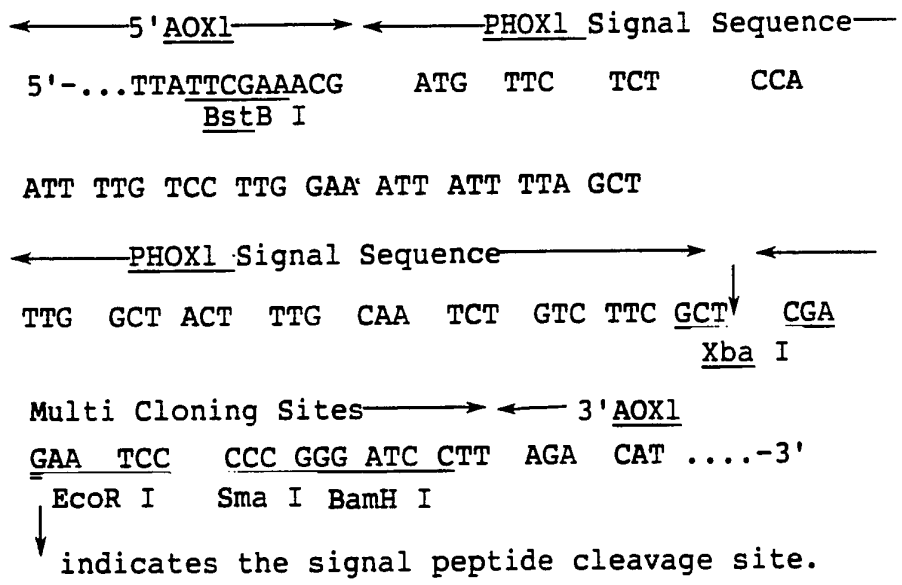
Temperature Stability Study of CC49 Fv2 8.1

Approximately 3 μg aliquots of CC49 Fv2 8.1 kept at 37°C were analyzed at intervals over 24 hours by gel filtration chromatography. A Superdex 75 HR10/30 column was utilized, using PBS as the mobile phase, at a flow rate of 0.5 mL/min.

The results of samples taken at various time points over 24 hours at 37°C indicated that the Fv2 remained intact (eluted at the same position as the starting material), and that no degradation products were observed.

Example 2 Cloning of CC49 Single Chain Antibody Species into the Yeast *Pichia pastoris*

For expression of single chain antibody species in yeast, the strain *Pichia pastoris* GTS115 *HIS4* (NRRL Y-15851) carrying a defective histidine dehydrogenase gene was used as a host cell. The plasmid pHIL-S1, available from Phillips Petroleum Company, was used as the expression plasmid modified as described below. The original sequence and features in the 5' region of pHIL-S1 are as follows:



DNA Sequencing

The dideoxy chain termination method of Sanger (Sambrook et al. *supra*) was used for DNA sequencing of the CC49 scFv genes present in pPY21 and pPY22. A Sequenase™ sequencing kit (United States Biochemical, Cleveland, OH) was used for the sequencing reactions, according to the manufacturer's directions. The radiolabel used was α-P32 dATP (3,000 Ci/mmol) obtained from Amersham (Arlington Heights, IL), lot no. AC9048. Double loadings were done using a BRL Model S2 sequencing apparatus and a premixed 6% Gel-Mix sequencing gel (BRL). After electrophoresis, gels were dried for 30 min using a Model 483 Slab Gel Dryer (Biorad, Richmond, CA) and exposed to Kodak X-OMat film (Sigma Chemical, St. Louis, MO) for 1-24 h. The sequencing oligonucleotides used for priming (0.5 pmol each) were:

1) AOX1P (23-mer), 5'-TTTAACGACAACTTGAGAAGATC-3';
2) TAGVLFR2 (20-mer), 5'-TGGTACCAGCAGAAACCAGG-3';
3) TAGVLCDR3 (Mixed 22-mer), 5'-GTCAGCA(AG)TATTATAG(CT)TATCC-3' and;
4) TAGVHCDR2 (21-mer), 5'-ATGGATTGGATATTTTTCTCC-3'

Construction of pPY1

A clone of *E. coli* AG1 transformed with pHIL-S1 was used to isolate purified pHIL-S1 plasmid using Qiagen's midi plasmid prep procedure (Chatsworth, CA). Three

micrograms of purified pHIL-S1 were treated with *Sst* I (Biological Research Laboratories, BRL) (10 units) in a volume of 40 µL for 45 minutes and then 2 µL of 1.5 M NaCl and 20 units (2 µL) of *Sal* I (BRL) were added (see Figure 15A). After the reaction proceeded for about 45 minutes, the sample was loaded onto a (16 X 18 cm) 4 percent polyacrylamide gel (PAG).

In a separate reaction, pBluescript II KS + (5 µg) purchased from Stratagene, was treated with *Sst* I (12.5 units) in a volume of 40 µL for 45 min. (see Figure 15B). The buffer was then modified to favor *Sal* I activity, by adding 2λ of 1.5 M NaCl, and then 25 units of *Sal* I were added. After another 45 min at 37°C, this sample was also loaded on the 4 percent PAG. DNA fragments were separated by electrophoresis at 90 volts over 2.5 h.

pHIL-S1 gave 2 fragments, a larger one at 5.9 Kbp containing the ampicillin resistance gene and a smaller fragment at 2.5 Kbp containing most of the 5'*AOX*1 region and multiple cloning site. Both fragments were cut out of the gel and isolated by electroelution and ethanol precipitation.

For the pBluescript II digest, 2 bands were visualized, one corresponding to the vector piece at about 2.8 Kbp and a small fragment from the multiple cloning site. The 2.8 Kbp fragment was recovered as above. To make pPY1 (Figure 15C), the pBluescript II *Sst I*/*Sal* I 2.8 Kbp fragment (~100 mg) was used in a ligation reaction with the 2.5 Kbp pHIL-S1 fragment (~100 mg). The ligation kit components were from Stratagene, while the T4 DNA ligase was a New England Biolabs product. Ligation commenced at 18°C, with a cooling gradient down to 4°C overnight. *E. coli* SURE (Stratagen, LaJolla, CA) was transformed with this ligation mix (1 µL and 5 µL samples). After overnight growth on Luria Broth (Sambrook et al., *Supra*) plates containing 100 µg/mL ampicillin, uncolored colonies (plasmids containing inserts) out--numbered blue colonies (without inserts) by roughly 10:1. Five of the uncolored colonies were picked for plasmid screening with *Sst* I and *Sal* I. All 5 gave the restriction pattern expected based on the plasmid construction (2 bands at 2.8 Kbp and 2.5 Kbp). The plasmid from a clone designated #4 was tested further and found to have the correct sites for *Bst*B I, *Xho* I (2 sites), *Eco*R V and *Bam*H I, and was thus picked as the representative clone for pPY1.

Construction of pPY2

Using the plasmid mini prep DNA of pPY1 from above, 2.5 µg were treated with 20 units of *Eco*R V (BRL) in a reaction volume of 45 µL at 37°C for 30 min. A single linearized DNA fragment at 5.3 Kbp was purified from a 3.75 percent PAG as described above. A linker DNA fragment with single *Eco*R I and *Not* I sites was then ligated into the blunt ended *Eco*R V site as follows.

The oligos ECONOT, a 20-mer: 5'-GAATTCTTAGCGGCCGCTTG-3' for the top strand and TONOCE, a 20-mer: 5'-CAAGCGGCCGCTAAGAATTC-3' for the bottom strand were synthesized and purified for annealing. A 60 fold molar excess of annealed linker over the *Eco*R V treated pPY1 fragment (5.7 X 10^{-14} moles -20 mg) were ligated using T4 DNA ligase and temperature conditions as above. Competent *E. coli* AG1 cells were transformed with aliquots

(1 and 5 µL) of this ligation mix and plated (75 or 150 µL) onto LB agar plates containing 100 µg/mL ampicillin. Twelve clones were picked for plasmid screening with *Eco*R I. Out of these, 10 clones had the correct two *Eco*R I fragments of roughly 600 bp and 4.7 Kbp. Four of these clones were selected for DNA sequencing of their plasmids for verification and orientation of the linker sequence. The oligo used for the sequencing (0.5 pmol) was ECOVNHESEQ, a 19-mer: 5'-TGCGCATAGAAATTGCATC-3'. Two of the plasmids had the correct sequence and desired orientation, one was chosen as the representative pPY2 clone (see Figure 15D).

Construction of pPY21 and pPY22

A common vector derived from pPY2 was used to generate both pPY21 and pPY22. Two micrograms of pPY2 mini-prep DNA were treated with 24 units of *Bst*B I (New England Biolabs) in a 20 µL reaction volume at 65°C for 15 min. At this point, 1.5 M NaCl was added (1.2 µL) with 12 units (1.2 µL) of *Bam*H I (New England Biolabs) and the reaction allowed to proceed for 15 min at 37°C. This sample was loaded on a 4 percent PAG, and the pPY2 fragment with *Bst*B I and *Bam*H I ends of roughly 5.2 Kbp was recovered by electroelution and ethanol precipitation.

The PCRs to generate 2 CC49 scFv inserts for the pPY2 vector were performed as follows. Oligos (100 pmol each) targeting pSCFV UHM5.2, prepared as described in Example 1 (5 ng) were:

1) PY49VLI, a 92-mer,
5'-ACATTTCGAAACGATGCTTTTGCAAGCTTTCCTTTTCCTTTTGGCTG GTTTTGCAGCTAAGATATCTGCTGACATTGTGATGTCACAGTCTC-3'; and

2) pPY523, a 36-mer,
5'-AAATGGATCCTATTAGTCATAGTCTTCAGGGTCTTC-3'.

Taq polymerase was purchased from Perkin Elmer Cetus (Norwalk, CT) and used in conjunction with the GeneAmp™ kit, according to the manufacturer's directions. The conditions for the PCR were: 30 cycles of 94°C for 30 sec, 52°C for 30 sec and 72°C for 1 min. On the final cycle, the last elongation step (72°C) was 2 min. An MJ Research (Watertown, MA) thermal cycler was used.

For the PCR of the second CC49 scFv insert, oligos (100 pmol each) targeting pSCFV UHM8.1 (see example 1) (5 ng) were:

1) PY49VL1, as above; and

2) PSC49VH, a 40-mer,
5'-ATGTGGATCCCTATTAGTAGGAGACGGTGACTGAGGTTCC-3'.

The reaction was set up and carried out as described for pSCFV UHM5.2, above. The PCR products were purified free of reactants by electrophoresis in a 4 percent PAG. The desired bands from the pSCFV UHM5.2 and pSCFV UHM8.1 targets were visualized at the expected

875 bp and 854 bp sizes respectively. The DNA was recovered by electroelution and ethanol precipitation.

The PCR inserts were prepared for ligation by trimming the ends off of each with *Bst*B I at the 5' ends and *Bam*H I at the 3' ends, using the same reaction conditions as described above for the pPY2 vector. After this treatment, the DNA inserts were again purified using a 4 percent PAG, subsequent electroelution and ethanol precipitation. The DNA inserts generated from pSCFV UHM5.2 and pSCFV UHM8.1 were used to make pPY21 and pPY22, respectively (Figure 15E). The ligation reactions regenerating pPY21 and pPY22 were set up (20 µL total volume) using 100 mg of pPY2 vector for each, and a 1:1 stoichiometric equivalent of the prepared PCR inserts from pSCFV UHM5.2 and pSCFV UHM8.1 respectively. The ligation kit components and T4 DNA ligase were from Stratagene and used according to their directions. The ligation reactions were incubated at 15°C for 2 hours, and 2 µL from each used to transform *E. coli* AG1 competent cells. Samples were plated onto LB agar containing 100 µg/mL ampicillin. Seven clones each for pPY21 and pPY22 were picked for screening of their plasmid DNAs, using the *Bst*B I-*Bam*H I double digest described above. A single clone (#6) was obtained with the correct DNA fragments for pPY21, while four clones (#11-14) were obtained for pPY22. The clone for pPY21 and pPY22 clone #12 were selected for DNA sequencing of the insert DNAs containing the CC49 scFv constructions. Both clones contained the correct sequence for CC49 scFv, 5.2 (pPY21) and 8.1 (pPY22), respectively, and were used for the final expression plasmid constructions.

The DNA and amino acid sequences of CC49 scFv in pPY21 and pPY22 are given in Figure 16. In these scFv constructions, the V_L gene came first followed by the V_H and was joined by a 25 amino acid linker which is in bold and underlined. The oligonucleotides used as DNA sequencing primers are shown above the DNA sequence lines. Restriction enzyme sites are as indicated, and underlined. The SUC2 signal peptide is underlined; the putative first amino acid of these mature products is indicated by (+ 1). Complementarity determining CDRs regions segments for the CC49V_L and CC49V_H are italicized in bold. Sequences for pPY21, giving negatively charged proteins (CC49, scFv5.2, Fv(2n)-5.2) are indicated by sequence A; and sequences for pPY22, giving close to neutrally charged scFv products (CC49, scFv8.1P, Fv-(2n)-8.1P) are represented by sequence B.

Construction of pPY31 and pPY32

The *Sst* I-*Sal* I DNA fragments of pPY21 and pPY22 containing the CC49 scFv constructions were isolated and purified, to be used as inserts with the pHIL-S1 vector fragment (5.9 Kbp) already described above. Three micrograms of pPY21 and pPY22 plasmid mini-prep DNAs from above (same clones as were sequenced) were treated with 9 units each of *Sst* I(BRL) in a 30 µL reaction at 37°C for 20 min. The samples were electrophoresed on a 4 percent PAG. The resulting bands at 3.2 Kbp (containing the DNA of interest) and at 2.8 Kbp were isolated together, since the resolution was not adequate for good resolution on the PAG. These pairs of

DNA inserts were then ligated with the pHIL-S1 *Sst* I-*Sal* I vector fragment (100 mg) in a 1:1 stoichiometric ratio, in a 20 µL reaction volume for 2h at 15°C to form pPY31 and pPY32 (Figure 15F). Competent *E. coli* AG1 cells were transformed with 2.5 µL aliquots of the ligation mixes and plated onto LB agar plates containing 100 µg/mL ampicillin. Six potential clones each of pPY31 and pPY32 were selected for plasmid screening. The resulting mini-prep DNAs were digested with an *SstI-SalI* double digest, as described above for the insert preparation. Three clones had the correct 2 bands (3.2 Kbp and 5.9 Kbp) for pPY31, one was chosen as the pPY31 representative. Two clones had the correct bands for pPY32 and one of them was chosen as the representative for transformation into *Pichia*.

Transformation of *Pichia pastoris* GTS115 with pPY31 and pPY32

The procedure of Becker and Guarente for [*Methods in Enzymology*, 194, 182 (1990)]. electroporation of yeast cells was followed with some modifications, as described below, to introduce the CC49 scFv genes into *Pichia*. Yeast extract peptone glucose (YEiD) medium (125 mL) was inoculated for overnight culture at 30°C from a stock of *P. pastoris* GTS115 stored at 4°C. The cells were harvested the next day when the $O.D._{600nm}$ was between 0.8-0.9. After washing with sterile water and 1M sorbitol, the cells were resuspended in ice--cold 1 M sorbitol (150 µL) and held on ice. Aliquots of pPY31 or pPY32 (2 µg/µL) linearized with *Bgl* II, totaling 5 µg and 10 µg were added to 40 µL each of the *Pichia* electro competent cells and transferred to pre-chilled (on ice) 0.2 cm sterile electroporation cuvettes (Biorad). Each sample was pulsed at 1.5 kV, 25 µF and 200 ohms, using a Biorad Gene Pulser with Pulse Controller. The time interval of the pulsers was 4.7-4.8 msec. Immediately after each pulse, 1.0 mL of cold 1M sorbitol was added to the cuvette, and three RDB plates for each of the 4 transformations (see U.S. Patents 4,855,231, 4,879,231 and 4,808,537) were plated (200 µL each). Plates were incubated at 30°C. Four days later, 36 colonies (numbered 1-36) transformed with pPY31 and 35 colonies (numbered 37-71) transformed with pPY32 were randomly picked and inoculated into 2.0 mL of BMGY media and streaked on MD and MM plates. All were grown at 30°C. BMGY is buffered minimal glycerol-complex medium containing 1 m potassium phosphate (pH 6.0); 13.4 g/L yeast nitrogen base with ammonium sulfate; 400 µg/L biotin; 10 g/L yeast extract; and 20 g/L peptone. MD is a medium containing 13.4 g/L yeast nitrogen base; 400 µg/L biotin; and 20 g/L dextrose. MM is the same as MD with 5 mL/L of methanol substituted for the dextrose.

Clones able to grow on MM pates are not *AOX*$^-$, implying that the Bgl II fragment did not incorporate at the *AOX*1 locus, but at some other chromosomal location. The MM plates have only methanol as a carbon source. After 2 days of growth in the BMGY medium, 9 potential *AOX*$^-$ mutant lines (observed by slow growth on the MM plate, 3 for PPY31, designated Nos. 4, 31, 32 and 6 for pPY32, designated Nos. 39, 44, 60, 61, 65 and 71 and an *AOX*$^+$ control (Clone No. 11, from the pPY31 set) were transferred to BMMY medium (2.0 mL each). BMMY medium is the same as BMGY with 5 ml/L methanol substituted for glycerol.

Aliquots of methanol (20 µL) were added to each tube, roughly every 10-14 h. Samples (100 µL) from each culture were taken 32 h after methanol induction and the biological activity determined by a competition assay described in Example 1. SDS-PAGE 10-20 percent gradient gels were run on 10 µL aliquots taken 32 and 100 h post induction. Western transfer and detection by biotinylated FAID14 was also done for the 100 hour samples.

The relative anti TAG-72 activities were evaluated for the methanol slow (*AOX*-) clones, along with some normal growing clones on MM medium. Biotinylated CC49 was used as the competitor in a competition assay. The results are presented in Figure 17, where the percent competition for a given sample was calculated based on OD_{405nm}-OD_{450nm} readings of the average of triplicate samples assayed using the following formula:

$$\frac{\text{zero competition - Sample}}{\text{zero competition - 100\% competition}} \times 100 = \% \text{ competition}$$

The zero competition value was obtained from a 0.2 micron filtered BMMY medium sample, while the 100 percent competition value was obtained using a 25 µL sample of CC49 IgG at 5 µg/mL. The *Pichia* samples for assay were prepared by microcentrifugation of the culture sample and filtration through a 0.22 µ filter. For pPY31 the results indicated that the methanol slow clones 4 and 32 competed very well (Fig. 17) as did clone 31, which by observation of growth on the MM plate could not be categorized as methanol slow. Clones 11 and 17 were producing little or no scFv. For pPY32, the methanol slow clones 39, 60, 61, 65 and 71 all gave excellent competition. Clones 38, 44 and 50 gave little or no competition.

The SDS-PAGE-Western data corroborated the competition ELISA results. The main product of the crude *Pichia* culture supernatant observed on SDS-PAGE-Western was the scFv product. The *Pichia* products correlated well with the previously obtained *E. coli* product equivalents in terms of size on the SDS-PAGE and activity in the competition assay. In the case of the pI 8.1 *Pichia* products, a very slightly slower mobility was observed relative to the Western positive band representing the *E. coli* CC49 scFv I8.1 product. This is due to the increase in molecular weight of the *Pichia* product due to the carboxyl terminal tyrosine residue weighing approximately 78 daltons more than the corresponding serine residue at the same position in the *E. coli* product.

Purification of Single Chain Antibody Species from *P. pastoris* GTS115 pPY31 Culture

Pichia pastoris carrying the plasmid pPY31 produces a CC49 single chain antibody having a pI of about 5.2. Starter cultures (20 mL BMGY medium) was inoculated (Clone #4 above) and grown for 26.5 hours at 30°C. This was used then to inoculate 800 mL BMGY medium in a 2.8 L Fernbach flask. The culture was grown at 30°C for 24 hours with shaking at 150 rpm. The *Pichia* cells were pelleted at 10°C using 2 tubes and a G53 rotor (Sorvall) run at 5,000 rpm for 10 minutes. The cells from each tube were aseptically resuspended in BMMY

medium (40 mL) to which 75 µL of methanol were added and continued to shake at 30°C. Methanol (200 µL) was added approximately every 12 hours over a 36 hour growth period.

Culture supernatants (containing the single chain antibody products) were harvested by spinning the cultures at 5,500 rpm in a SS34 rotor (Sorvall) for 5 minutes. The supernatant (about 66 mL) was further clarified by filtering through a 0.22 µ pore size filter device (Nalge) and then concentrated to 10 mL using a Centriprep 10 device (Amicon). Half of this material was applied to a Superdex 75 HR26/60 gel filtration column (Pharmacia), at a flow rate of 1.3 mL/min using PBS as the buffer. The gel filtration profile (See Figure 18) gave three pools of single chain species indicating the formation of monomeric single chain antibody (scFv, fraction 19-23), dimers (scFv2, fractions 13-15) and multimeric forms (scFv(2n) where $n>1$, fractions 8-10). On the SDS-PAGE insert, lane "S" indicates the identical purified protein standard CC49 scFv5.2 derived from *E. coli* pSCFV UHM5.2 (Example 1). In the processing of running SDS-PAGE, the non-covalent interactions in a dimer or multimer are caused to break apart, showing that the dimer and multimer forms correspond to the same size as the basic monomeric unit.

TABLE I

GEL FILTRATION ANALYSIS OF CC49 SCFV PI = 5.2 SPECIES[1]

Single Chain Antibody Species	Elution Time (min.)	Elution Volume (mL)	K_{av}[2]	Predicted MW (Kd)	Observed MW (Kd)	Notes
A. Monomer: scFv	21.1	12.66	.320	28.6	30.5	Derived from fractions 26 and 27, Figure 2A, pool 1
B. Dimer: Fv2	18.8	11.28	.237	57.2	68	Derived from fractions 41 and 42, Figure 2B, pool 2
C. Tetramer: Fv4	17.5	10.5	.191	114.5	120	Derived from fractions 40 and 41, Figure 2Ca, pool 3
D. Hexamer: Fv6	17.0	10.2	.173	171.7	180	Derived from fractions 42 and 43, Figure 2Cb, pool 4
E. Octomer: Fv8 and above?	-[3]	-[3]	-[3]	229.0	-[3]	Derived from fraction 44, Figure 2Cc

[1]Buffer used was PBS, at a flow rate of 0.6 mL/min. In each case, all peaks were indicative of a pure species with <10 percent of any contamination from other forms or products.

[2]Based on a void volume of 7.32 mL (thyroglobulin, MW=670 Kd) and a column volume of 24 mL. K_{av} values for the MW standards borine IgG at 158 Kd daltons, ovalbumin at 44 Kd daltons, myoglobulin at 17 Kd daltons and cyanocobalamin at 1,350 daltons were 0.178, 0.284, 0.381 and 0.644, respectively.

[3]All of the above samples were run twice, except this one which was only run once. Using a different Superdex 75 column at that time the elution times for the species ScFv, Fv2, Fv4, Fv6 and this putative Fv8 were 16.74, 14.10, 12.78, 12.46 and 12.2 minutes, respectively. No gel filtration standards were run at the same time so that a MW for the putative Fv8 could not be determined.

Each of the single chain antibody pools was further purified by use of a Mono Q HR5/5 anion exchange column as described in Example 1. Each of the various species was then re-chromatographed on an analytical gel filtration column (Superdex 75 HR10/30, Pharmacia-LKB). Table II gives the elution time, elution volume, Kav values and molecular weights determined for the monomeric and various multimeric forms. The predicted molecular weights were predicted by DNASTAR™ protein titrate computer program using the amino acid composition of the polypeptide.

The Anion Exchange Chromatography and SDS-PAGE are given in Figure 19. Relevant parts A, B, and C correspond to the monomer, dimer and higher multimer forms of the scFv species, respectively. The buffer system used and the gradient of Buffer B are also shown. The SDS-PAGE insert (D) has the following samples: molecular weight markers (lane 1); monomer and dimer (lanes 2 and 3); and *E. coli* derived CC49 scFv 5.2 standard (lane 4); tetramer, hexamer and putative octamer (lanes 5, 6, and 7, respectively) and an approximately 34 Kd impurity (lane 8) derived from fraction 45.

The IEF gel and Western, shown in Figure 20 indicate that the monomer and various multimer forms have distinct pI values. Both the *E. coli* and *Pichia* derived monomer forms, have a pI of 4.92 (derived from a graph of distance from cathode vs. pI of the standards), with a minor band at pI 4.97, which is the same as the pI for all multimers. That 2 bands seen in the monomer (lanes 3 and 9) support the hypothesis that there are two charge forms for the monomer in equilibrium, where the pI 4.97 form has a tendency to dimerize. In Figure 20, lanes 1-7 represent bands stained with Coomassie Brilliant Blue R-250 and lanes 8-13 represent a Western transfer of samples corresponding to lanes 2-7. The pI markers (lane 1) were from Sigma: soybean trypsin inhibitor, pI 4.55; bovine β-lactoglobulin A, pI 5.13; and human erythrocyte carbonic anhydrase B, pI 6.57. Lanes 2 and 8 show purified CC49 scFv5.2 derived from *E. coli* pSCFV UHM5.2; lanes 3 and 9 show the same product derived from *Pichia*. Lanes 4 and 10 show a sample of crude culture supernatant from *Pichia* containing all the CC49 single chain antibody species present. Lanes 5 and 11, 6 and 12, and 7 and 13 indicate the *Pichia* dimer, tetramer and hexamer species, respectively.

Purification of Single Chain Antibody Species of *Pichia pastoris* GTS115/pPY32

P. pastoris having the plasmid pPY32 produces CC49 single chain antibodies having a pI of about 8.1. The growth, harvesting and induction of the *AOX*1 promoter were the same as for the pPY31 clone above.

The purity of the 8.1 monomer and dimer (multimeric forms were not analyzed) was verified by SDS-PAGE. The IEF pattern of these molecules indicated that except for carboxyl terminal Ser residue present in the *E. coli* derived CC49 scFv8.1 and Fv28.1, the corresponding *Pichia* products have the same pI values. In *Pichia* the carboxyl terminal residue is Tyr.

The N-terminal amino acid sequencing results indicated that the invertase signal sequence used in constructing these single chain antibodies was accurately being removed by signal peptidase. For the CC49 scFv8.1P and Fv2-8.1P proteins, the following NH_2 terminal residues were determined: Asp-Ile-Val-Met-Ser-Gln-Ser-Pro-Ser-Ser, which matches the predicted amino acid sequence of the mature form, from the DNA sequence (see Figure 16). For the CC49 Fv2-5.2 product, the same results were obtained as above, except that the first and tenth amino acids could not be unequivocally assigned. Based on the above results with the 8.1 pI system, it is reasonable to assume for the 5.2 pI products that the unassigned residues are the same.

From the competition ELISA results (Figure 21A and 21B), it can be observed that the avidity of the single chain species, increases with higher multimer forms. It is possible that the effects seen for the tetramer and hexamer are underestimated and that different concentrations or densities of TAG-72 on the ELISA plate may influence the results. The availability of antigen to some of the binding faces of the hexamer and octamer, for example, may not be sterically available.

Example 3 Biodistribution Characteristics of ^{125}I Labeled CC49 Single Chain Fv Species

The biodistribution of four CC49 single chain antibody species, two monomeric species: CC49 scFv with a pI of 8.1 obtained from *Pichia* (scFv 8.1P) and CC49 with a pI of 5.2 also obtained from *Pichia* (scFv 5.2); and two dimeric species: CC49 Fv2 with a pI of 8.1 obtained from *Pichia* (Fv2-8.1P) and CC49 Fv2 with a pI of 5.2 obtained from *E. coli* (Fv2-5.2), was determined. This was compared with ChCC49 Fab and IgG. The single chain species were labeled with ^{125}I using the Iodo-Beads™ (Pierce, Rockford, IL) method using N-chloro-benzenesulfonamide derivatived polystyrene beads (Iodo-Beads™, Pierce, Rockford, IL). To label the antibodies, initially 3 Iodo-Beads™ were incubated at room temperature for 5 minutes in PBS and 150 µCi carrier-free I-125 (Nordion, 100.9 µCi/µL). At this point, the antibody (0.3 mg in PBS) was added and allowed to react for 8 minutes. Unincorporated isotope and reaction by-products were removed by gel filtration chromatography (Pharmacia Superdex 75 HR10/30 column), using PBS as the buffer at a flow rate of 0.6 mL/minute. An aliquot of the purified protein peak was measured in each case at A_{280nm} and quantitated according to the $E^{1\%}_{280nm}$ values as described in Example 1 for the respective proteins. Levels of radioactivity were determined by measurement in a Capintec Counter, set for reading I-125.

SDS-PAGE and IEF analyses were performed as previously described. Gels were dried and radioactive bands detected by exposure to Kodak XAR X-OMAT film. The observed pI values for the 8.1P species, as determined by IEF are 7.1 and 7.25 for monomer and dimer, respectively, and for the 5.2 species, 4.9 and 5.0 for the monomer and dimer, respectively. Differences between the calculated values (after which the species are officially called) and the observed values indicate that the tertiary structure of these molecules play a role in charge-charge interactions and/or hydrogen bonding which will determine the observed pI values.

Five test groups, each containing 5 female nu/nu (CD-1) mice (obtained from Charles River Breeding Laboratories) bearing LS174T human tumor xenografts, were used to measure the biodistribution of ^{125}I labeled antibody species. The biodistribution of the compounds was measured at various time intervals over 48 hours, after intravenous (tail vein) injection of the compounds. Animals were housed and identified by group as outlined in Section 4.0 of Standard Operation Manual for the Care of Laboratory Animals (SOP-PTG). At the appropriate time, the mice were euthanatized and the radioactivity remaining in samples of blood, liver, spleen, kidney, tumor, lung, GI tract, tail, and remaining carcass were quantified as outlined in Section 4.0 and Section 5.0 of SOP-PTG. The LS174T tumors were produced in the mice according to the procedure set forth in Section 2.0 of SOP-PTG.

For metabolite studies, groups of Balb/c mice were injected intravenously with 1 μCi of one of the antibody species. Mice from each group were anesthetized after 30 minutes, 2h and 5h, urine removed from the bladder, urine counted for radioactivity and then kept frozen at -70°C until HPLC analysis. A Pharmacia Superdex 75 gel filtration HR10/30 column was used for chromatography, using PBS as the eluent at 1.5 mL/minute. The volumes of the injected urine varied from 20-80 μL. The column eluent passed through a dual detector for absorbence at 280nm and for radioactivity.

Gamma Counter #2 (Searle/ND-66) was used to measure radioactivity in standards and tissue samples. Biodistribution of radioactivity in the collected tissues was analyzed using template TEMPL as outlined in Section 5.0 of SOP-PTG. Experimental outliers were treated as outlined in Section 6.0 of SOP-PTG.

Table IV gives the amount of protein and activity of each species injected into the mice for biodistribution studies.

Tables IV, V, VI, VII, VIII, and IX give the biodistribution of ^{125}I CC49 species of scFv5.2, Fv2-5.2, and scFv8.1P, Fv2-8.1P, ChFab and IgG, respectively, based on percent injected dose per organ. Percent injected dose per organ is calculated by determining the ^{125}I in counts per minute (CPM) in each tissue, divided by the injected dose (CPM) multiplied by 100 to give percent.

TABLE III

Summary of Protein and Radioactivity Amounts Used to Inject Nude Mice Bearing LS174T Tumor Xenografts

Study No.	scFV5.2 N9103	FV2-5.2 N9103	scFV8.1P N9105	FV2-8.1P N9105	Ch Fab N9102	IgG. N9101
reg. protein Per mouse	6.85	7.75	5.15	3.85	27.25	21.95
uCi I-125 per mouse	1.12	1.60	1.00	0.75	12.5	4.32
Percent Activity (as determined by BSM binding)	64	895	91	68	64	96
Specific Activity (µCi/µg)	0.163	0.21	0.19	0.19	0.46	0.20

TABLE IV

Biodistribution of ^{125}I Injected as ^{125}I- CC49 scFv Monomer, pI 5.2 (scFV5.2)

Values as Percent Injected Dose/Gram (n = 5)

Tissue	20 Minutes		2 hours		5 Hours		24 Hours		48 Hours	
	Mean	Standard	Mean	Standard	Mean	Standard	Mean	Standard	Mean	Standard
Blood	5.87	0.58	2.43	0.39	1.53	0.22	0.02	0.01	0.01	0.00 n=4,a
Liver	4.08	0.42	1.77	0.16	1.16	0.08	0.28	0.05	0.17	0.02
Spleen	3.35	0.80	1.77	0.09	1.17	0.13	0.19	0.03	0.14	0.03
Kidney	155.25	17.46	51.58	6.37	34.25	0.53	15.76	1.33	9.62	1.69
Tumor	2.80	0.34	2.24	0.13 n=4,a	1.72	0.25	0.58	0.09	0.40	0.08
Lung	5.72	0.88	2.91	0.72	1.04	0.27	0.13	0.11	0.01	0.00
Tumor Weight	0.20	0.04	0.19	0.05	0.27	0.07	0.13	0.07	0.17	0.04

TABLE V

Biodistribution of ^{125}I Injected as ^{125}I- CC49 scFv Dimer, pI 5.2 (Fv2-5.2)
Values as Percent Injected Dose/Gram (n = 5)

Tissue	20 Minutes		2 hours		5 Hours		24 Hours		48 Hours	
	Mean	Standard	Mean	Standard	Mean	Standard	Mean	Standard	Mean	Standard
Blood	29.47	3.26	12.47	1.81	3.95	1.37	0.04	0.02	0.01	0.01
Liver	10.24	0.35 n=4,a	5.51	0.79	2.87	0.54	0.55	0.04	0.30	0.09
Spleen	9.63	2.56	5.45	0.73	2.31	0.30	0.34	0.08	0.22	0.12
Kidney	30.56	0.89 n=4,a	15.85	1.07	8.63	1.22	3.12	0.26	2.07	0.51
Tumor	3.57	0.16 n=4,a	8.91	1.58	8.52	2.35	7.66	1.74	4.96	2.08
Lung	8.65	0.78	4.84	0.80	2.09	0.52	0.07	0.02	0.01	0.01
Tumor Weight	0.17	0.02	0.25	0.11	0.27	0.04	0.18	0.10	0.14	0.06

TABLE VI

Biodistribution of ^{125}I Injected as ^{125}I- CC49 scFv Monomer, pI 8.1 (scFv8.1P)

Values as Percent Injected Dose/Gram

Tissue	20 Minutes n=3		2 hours n=5		5 Hours n-5		24 Hours n=5		48 Hours n=5	
	Mean	Standard	Mean	Standard	Mean	Standard	Mean	Standard	Mean	Standard
Blood	5.31	0.77	2.43	0.81	2.36	0.75	0.01	0.00	0.01	0.00
Liver	5.80	0.96	1.30	0.33	1.05	0.41	0.07	0.01	0.04	0.01
Spleen	3.79	0.03 n=2,a	1.81	0.51	1.42	0.12 n=4,a	0.05	0.02	0.02	0.01
Kidney	150.74	34.76	5.91	0.71	3.05	0.70	0.37	0.03	0.23	0.02
Tumor	3.06	0.38	2.19	0.28	2.09	0.35	0.64	0.06	0.40	0.03 n=4,a
Lung	6.75	1.01	7.25	0.86 n=4,a	1.34	0.32	0.02	0.01 n=4,a	0.00	0.00
Tumor Weight	0.24	0.03	0.16	0.04	0.25	0.10	0.18	0.06	0.16	0.03

TABLE VII

Biodistribution of ^{125}I Injected as ^{125}I-CC49 scFv Dimer, pI 8.1 (Fv2-8.1P)

Values as Percent Injected Dose/Gram

Tissue	20 Minutes n=4		2 hours n=5		5 Hours n=5		24 Hours n-5		48 Hours n=5	
	Mean	Standard	Mean	Standard	Mean	Standard	Mean	Standard	Mean	Standard
Blood	20.55	3.26	3.30	0.60	1.91	0.51	0.01	0.00	0.00	0.00
Liver	15.74	2.00	3.92	0.46	1.81	0.28	0.23	0.03	0.11	0.02
Spleen	10.52	1.94	3.71	0.82	1.73	0.17	0.19	0.07	0.09	0.04
Kidney	31.40	2.16	4.90	0.63	1.71	0.30	0.12	0.02	0.05	0.01
Tumor	1.70	0.45	3.18	0.89	2.23	0.40 n=4,a	1.56	0.35	0.93	0.18
Lung	5.77	1.26	3.05	0.86 n=4,b	1.15	0.33	0.03	0.04	0.00	0.00
Tumor Weight	0.25	0.13	0.21	0.08	0.35	0.08	0.14	0.02	0.15	0.05

TABLE VIII

Biodistribution of ^{125}I Injected as 125I ChCC49 Fab (Ch Fab), Values as Percent Injected Dose/Gram (n = 5)

Tissue	15 Minutes n=4		30 Minutes n=3		2 Hours n=5		5 Hours n=5		24 Hours n=5	
	Mean	Standard	Mean	Standard	Mean	Standard	Mean	Standard	Mean	Standard
Blood	17.11	3.85	6.06	0.63	4.68	0.42	1.96	0.39	0.16	0.02
Liver	6.82	0.45	4.32	0.38	2.78	0.58	1.19	0.26	0.43	0.02 n=4,a
Spleen	5.92	1.54	3.28	0.80	2.84	0.77	1.13	0.31	0.24	0.00 n=4,a
Kidney	154.17	22.39	167.64	9.52	23.81	7.39	5.46	1.23	1.29	0.06 n=4,a
Tumor	2.54	0.56	2.36	0.19	3.03	0.13	1.75	0.20	1.03	0.10
Lung	6.08	0.47	4.94	1.02	2.56	0.17	1.27	0.31	0.13	0.02
Tumor Weight	0.26	0.14	0.36	0.10	0.33	0.12	0.20	0.09	0.11	0.02

a One tissue value rejected due to Outlier Statistic >p-95 value.

TABLE IX
Biodistribution of 125I Injected as 125I-IgG
Values as Percent Injected Dose/Gram

Tissue	5 Hours n=5		24 Hours n=5		48 Hours n=4		120 Hours n=5	
	Mean	Standard	Mean	Standard	Mean	Standard	Mean	Standard
Blood	22.13	1.05	13.73	1.25	10.24	0.91	8.14	0.35 n=4,a
Liver	7.87	0.58	3.96	0.22 n=4,a	2.77	0.12	2.13	0.09 n=4,a
Spleen	5.06	0.45	3.05	0.62	2.14	0.22	1.56	0.26
Kidney	3.52	0.65	1.58	0.41	1.77	0.02 n=3,a	1.53	0.12 n=4,a
Tumor	10.85	2.51	30.12	10.09	55.49	1.50 n=3.,a	58.21	12.56
Lung	6.04	0.56	3.80	0.62	3.11	0.39	2.18	0.34 n=4,a
Tumor Weight	0.19	0.03	0.17	0.03	0.17	0.02	0.18	0.07

a One tissue value rejected due to Outlier Statistic >p-95 value.
b One tumor value rejected-outside acceptable weight range.

The differences in pharmacokinetics for the various sizes and charges of the CC49 antibody species are demonstrated by the data in tables IV - IX. In general, the smaller the molecule, the faster it clears the blood. The 25-50 K dalton size molecules (all except the IgG) clear the blood within 24 hours. The negatively charged Fv2-5.2 dimer has the greatest amount in the blood at 2 hour (Table V). Both dimers accumulate in the tumor to the largest extent over a 24 hour time period (Tables V and VII), excluding the IgG.

A major difference between the antibody species is observed in retention by the kidneys. The negatively charged monomer species, CC49 scFv5.2, clears the most slowly (Table IV). This is surprising in that the negative charge should have prevented electrostatic interaction with the negatively charged glomerular cells of the kidney. The nominally neutral charged species, scFv8.1P cleared more quickly (Table VI). This trend was also true for the two dimers, but clearance was much better for these relative to the monomers. It is unexpected that the negatively charged dimer would have the best tumor localization values (Table V). The data also shows that a relatively small molecule (58 Kd) has staying power on the tumor. Both dimers out performed the monomers and chimeric CC49 Fab molecule in this regard.

Results from metabolic studies indicated that after injection with ^{125}I labeled scFv 5.2 and Fv2-5.2, only one radioactive metabolite was observed in the urine, free ^{125}I, as determined by gel filtration chromatography. No larger peptides are observed at any of the time points. These data indicate that dehalogenation is occurring within the body and that free I-125 is able to be readily excreted into the urine.

The tumor to tissue ratios 8 to 24 hours post injection indicated the dimers would be effective in radioimmunoguided surgery procedures. Surgery may be performed in a more timely fashion, rather than 2 to 3 weeks post injection, as is presently necessitated by the biodistribution kinetics of whole antibody.

Example 4 Construction, Purification and Characterization of Anti TAG-72 Single Chain Antibody Species with the Human Subgroup IV V_L

To provide for a rapid and convenient procedure to determine the effectiveness of the single chain dimers and multimers of the present invention, a unique octapeptide sequence -NH_2-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-COOH, called the FLAG™ peptide (a trademark of International Biotechnologies Inc., CT) was used. This sequence is recognized by International Biotechnology Incorporated's (IBI) mouse M2 monoclonal antibody. Placing the FLAG™ sequence on two annealing oligonucleotides in pSCFV UHH leads to a single chain antibody having the human subgroup 4 V_L and CC49 V_H with the FLAG peptide attached at the carboxyl terminus.

The final plasmid derived from pSCFV UHH containing FLAG™ is designated pSC49FLAG. The nomenclature for the single chain proteins produced from pSC49FLAG is as follows: for the monomer-H4L49HF scFv, for the dimer - H4L49HF Fv2 and for the tetramer-

H4L49HF Fv4. The "H4L" portion refers to the human Subgroup IV light chain V_L region, "49H" refers to the CC49 V_H and "F" indicates that the FLAG™, peptide is attached.

Construction of pSC49FLAG

A general outline for obtaining the plasmid pSC49FLAG is shown in Figure 22 and the procedure described below.

a) Isolation of pSCFVUHH *Xho II/Nhe I* Vector Fragment

Approximately 5 µg of plasmid pSCFV UHH (Example 1) in 15 µL of water was used from the Magic Mini-prep system (Promega). To this was added 5.4 µL of 10X Buffer #2 (New England Biolabs), 45 units of *Xho I* (New England Biolabs), 15 units of *Nhe I* and 24 µL of water. The reaction proceeded for 1 hour at 37°C. The sample was then loaded with running dyes on a 4 percent polyacrylamide gel, electrophoresed and purified by electroelution (Sambrook et al, *supra*). The obtained DNA pellet was dissolved in 20 µL of water.

b) Construction of pATDFLAG

Plasmid pSCFV UHH treated with *Xho I* and *Nhe I* from above was used in a ligation reaction with annealed FLAG and FLAGNC oligos.

FLAGC:

5'-TCGAGACAATGTCGCTAGCGACTACAAGGACGATGATGACAAATAAAAAC-3'

FLAGNC:

5'-CTAGGTTTTTATTTGTCATCATCGTCCTTGTAGTCGCTAGCGACATTGTC-3'

Equimolar amounts (3 µM of each of the oligos) FLAGC and FLAGNC in a total volume of 62.5 µL) were mixed together using a ligation buffer diluted to 1X (Stratagene). The sample was heated to 95°C for 2 min and was allowed to cool to below 35°C over 10 min before use in the ligation reaction below.

COMPONENT	AMOUNT
pSCFVUHH *XhoI/NheI* vector (~100 ng)	1.0 µL
ANNEALED FLAGC/FLAGNC	0.85 µL
10X Ligation buffer	2 µL
T4 DNA Ligase	1 µL
10 MM ATP	2 µL
ddH_2O	13.2 µL

The reaction was carried out using the components and amounts indicated above. Starting at 18°C, and then being cooled gradually to 4°C overnight. Competent *E. coli* Ag1 cells (Stratagene) were transformed with 3 µl of the above ligation reaction and colonies selected using LB/CAM 20 plates. A clone having the appropriate *Nhe I/Xho I* and *Nhe I/Xho I* restriction patterns was selected for DNA sequencing.

The oligonucleotide used to verify the sequence of the FLAG™ linker in pATDFLAG was called PENPTSEQ: 5'-CTTTATGTAAGATGATGTTTTG-3. This oligonucleotide is derived from the non-coding strand of the *pen*P terminator region (See Figure 23). Figure 23 gives the amino acid and DNA sequence in pATDFLAG. The amino acid sequence corresponding to the FLAG™ peptide is given in bold italics. The FLAG™ peptide is out of frame relative to the $H4V_L$-linker sequence which ends at the *Xho* I site. The DNA sequencing primer, PENPTSEQ, is shown in bold. Nucleotides 824-478 inclusive were verified using the PENPTSEQ primer. At position 624, a 'T' nucleotide present in the original $H4V_L$ sequence has been changed to a 'C' nucleotide resulting in a Val to Ala amino acid residue 89 substitution. These differences are indicated in shadow. The CDR amino acid sequences of H_4VL are shown in bold, while the UNIHOPE linker sequence is in bold and underlined. DNA sequencing was performed using the Sequenase™ sequencing kit (U.S. Biochemical, Cleveland, OH) following the manufacturer's directions.

c) Generation of pSC49FLAG

The plasmid pATDFLAG (approximately 5 µg, purified from a 2.5 mL culture by the Magic Miniprep system (Promega) was treated at 37°C for 1 h as indicated in the protocol given below:

DNA - 17 µL
10X buffer (New England Biolabs #2) - 6 µL
Xho I (New England Biolabs) - 2.5 µL (25 units)
Nhe I (New England Biolabs) - 3.5 µL (17.5 units)
H_2O - 31 µL

The resulting vector fragment was purified from a 4 percent PAG, electroeluted and ethanol precipitated. The obtained DNA was dissolved in 20 µL water.

The CC49 V_H insert DNA was obtained from a PCR amplification protocol (Perkin Elmer Cetus) as instructed by the manufacturer. The target DNA (uncut) containing the CC49 V_H was pSCFV UHM 8.1 (Example 1) (0.5 mg). The 5' oligo used for the PCR (100 pmol) was UNI-3 (Example 1). The 3' oligo used (100 pmol) was called SC49FLAG, a 33-mer as follows:

Nhe I
5'-TATTGCTAGCTGA GGA GAC GGT GAC TGA GGT TC-3'.

The resulting amplified DNA was purified from a 4 percent PAG, dissolved in 20 µL water, and then treated at 37°C for 1 h as indicated in the protocol given below:

DNA - 10 µL (~2 µg)
10X buffer (New England Biolabs #2) - 6 µL
Xho I (New England Biolabs) - 2.5 µL (25 units)
Nhe I (New England Biolabs) - 3.5 µL (17.5 units)
water - 38 µL

The resulting insert fragment was purified and dissolved in 20 µL water. The vector and insert fragments from above were set up in a ligation reaction at 18°C going down to 4°C overnight as follows:

DNA vector (pATDFLAG-*Xho I/Nhe I*) - 1.5 µL
DNA insert (PCR amplified CC49 V_H-*Xho I/Nhe I*)-0.8 µL
10X ligation buffer (Stratagene) - 2 µL
10 mM ATP - 2 µL
T4 DNA ligase - 1 µL
water - 12.7 µL

Competent *E. coli* AG1 cells (Stratagene) were transformed using the ligation reaction from above, as directed by the manufacturer. Clone number 1, was selected for further work, and for verification of the constructed DNA sequence. The oligos used as primers for the DNA sequencing were as follows:

1) PENPR1, a 21-mer: 5'-AAC ACT GTA GGG ATA GTG GAA-3';
2) PENPR2, a 20-mer: 5'-GTC TCC CTC CGT TTG AAT AT-3';
3) TAGVLFR2, a 20-mer: 5'-TGG TAC CAG CAG AAA CCA GG-3;
4) UHVHSEQ, a 22-mer: 5'-GAT GCT GCG AAG AAG GAT GAC G-3';
5) TAGVHCDR2, a 21-mer: 5'-ATGG ATT GGA TAT TTT TCT CC-3' and;
6) TAG VH FR4, a 21-mer: 5'-ACT GGG GTC AAG GAA CCT CAG-3'.

The DNA and amino acid sequence of pSC49FLAG is given in Figure 24. Asp = 1 indicates the first amino acid from H4V_L of the mature single chain antibody species. The UNIHOPE linker is in bold and underlined. The complementarity determining region sequences are shown in bold, while the FLAG peptide is in bold italics. Nucleotides corresponding to the *Nhe* I site in pSCFV UHH are in bold and underlined (position 1154-1159). The 'C' at position 1155 and the 'A' at position 1157 are italicized within this sequence and indicate differences from the published *penP* terminator sequence. The DNA sequencing was carried out using the Sequenase™ kit mentioned above.

The predicted pI and molecular weight (MW) of each of the protein species produced was obtained from the DNASTAR™ Protein-Titrate computer program (Madison, WI).

Purification of H4L49HF Single Chain Products

A 10 mL M2 antibody affinity column was prepared (gel purchased from IBI) and incorporated on the HPLC system described under general experimental procedures. The H4L49HF crude protein sample was prepared as follows. One liter (2 X 500 mL) of *E. coli* AG1/pSC49FLAG was grown overnight at 37°C in LB broth containing 20 µg/mL chloramphenicol. The cells were recovered by centrifugation of the culture at 5,000 rpm for 10 min at 5°C in a Sorvall GS-3 rotor (duPont). The cell pellet was resuspended in 100 mL of PBS, and subjected to sonication (MSE Soniprep 150) for 5 bursts at 14 microns amplitude lasting for 15 seconds each, and cooling in an ethanol-ice bath for 1 minute in between. The sonicate was

ultracentrifuged at 26,000 rpm for 2 hours in a Type Ti30 rotor (Beckman Instruments). The resulting supernatant was filtered through a 0.22 membrane device (Nalge Co., Rochester, NY) and finally concentrated to 22 mL using Centriprep 30 (Amicon) devices. The sonicate was pumped onto the affinity column at 2 mL/min with stops lasting for 1 min twice during the application, to allow interaction with the affinity ligand.

PBS was used to wash the column of any unbound material, and when the absorbance at 276 nm returned substantially to the baseline, the buffer was changed to 0.1M glycine-HCl, at pH 3.1. Elution of a peak began 4 minutes later, the fractions of which were immediately neutralized using 1 M Tris pH 8.8 and PANPEHA indicator (range 0-14) pH paper strips (Schleicher Schull). The eluted protein was concentrated to 200 µL using Centriprep 30 and Centricon 30 (Amicon) devices.

The resulting single chain antibody species were separated by gel filtration using a Pharmacia Superdex 75 Prep Grade HR 26/60 column and PBS as the eluent. The flow rate was initially 0.4 mL/min, and then increased to 1.2 mL/min during the chromatography. Three pools of fractions from this run were made, and then tested analytically on a Superdex 75 HR10/30 column for purity and characteristic elution time. Biorad gel filtration standards were also chromatographed on this column using the same buffer (PBS) and a 0.6 mL/min flow rate, to generate a standard curve for comparison with the elution times of the single chain antibody species. The three species obtained were concentrated, quantitated at OD 280 nm and stored frozen at -20°C.

The results of the purification and characterization of the H4L49HF single chain products is given in Figure 25. Panel A shows the M2 affinity chromatography profile for *E. coli* pSC49FLAG sonicate preparation. Stars (*) indicate points in the application of sample when the flow was stopped for 30 seconds. The arrow indicates the point at which the buffer was changed and the flow chart increased to 2.0 mL/min. Fractions were 2 minutes long.

Panel B represents an analytical gel filtration chromatogram obtained from application of 0.5 percent of the total pooled sample from fractions 19-21 shown in A.

Panel C shows a 10-20 percent gradient SDS-PAGE of samples stained with Coomassie Brilliant Blue R-250 (Lanes 1-6) or Western transferred (lanes 7-11) and probed with biotinylated FAID14. Lane 1: prestained molecular weight standards (Diversified Biotech); lanes 2-4 and 7-9 are purified H4L49HF scFv, Fv2 and Fv4, respectively; lanes 5 and 10; *E. coli* pSC49FLAG sonicate sample, the same as that as applied to M2 affinity column; and lanes 6 and 11, *Pichia* CC49scFv 5.2.

Amino Terminal Sequencing

Samples of the three protein species of H4L49HF (10 µg each) were desalted by 3 successive water washings using Centricon 30 devices and freeze-dried. Amino terminal sequencing was determined by standard Edman degradation techniques.

Determination of Anti TAG-72 Activity

a) anti-FLAG ELISA

A. Taq Elisa

Binding activities of the purified monomer and multimer forms of H4L49HF as well as sonicates of *E. coli* expressing these species were determined by ELISA (Enzyme Linked Immunosorbant Assay) on 96 well PVC plates coated with a 1/300 dilution of the TAG-72 prep (040191). The plates were prepared and blocked for two hours with 1 percent bovine serum albumin (BSA) in PBS with 0.025 percent sodium azide at 37°C. After blocking, the plates were washed three times with 0.025 percent Tween 20 solution and stored at 4°C.

Aliquots of 50 µL of sample at each concentration were added in duplicate to the 96 well plate. The starting concentrations for the purified monomer, dimer, and tetramer were 10 µg/mL for each, and 1:2 dilutions were made to a final concentration of 0.005 µg/mL. The samples added to the plate included the purified monomer, dimer, tetramer, and various crude sonicates.

After adding the samples, the plate went through a series of incubations at 37°C. The first incubation was for 1 hour with sample. The plate was washed five times with 0.025 percent Tween 20. Binding of FLAG™ containing single chain species was detected by subsequent incubations with murine anti-FLAG MAb, M2 (International Biotechnologies cat: IB13010) at 1/250 dilution. The plate was incubated for 1 hour removed, and washed. Alkaline phosphatase conjugated goat anti-mouse polyclonal antibody (GAMIG-ALPH, Southern Biotechnology Associates) was added at 50 µL per well. The plate was incubated for 1 hour, removed, and washed. The diluent for both antibodies was in 1 percent BSA in PBS with 0.025 percent sodium azide. The phosphatase substrate (Kirkegaard & Perry Laboratories, Inc.) was prepared by mixing 1 ml of 5X buffer (Kirkegaard & Perry Kit), 4 mL water, and one substrate tablet (Kirkegaard & Perry Kit). To each well, 50 µL were added. Color development was terminated after 15 minutes with 50 µL/well of 1 M sodium hydroxide. The ELISA plate was read with the Molecular Devices Kinetic Microplate Reader using the Delta Soft© Computer program.

B. BSM ELISA

Bovine submaxillary mucin (BSM) was applied to a PVC plate at concentrations ranging from 5.0 mg/ml to 0.00008 mg/ml decreasing at 1:2 dilutions in water. A blank of 50 µL of PBS with 0.025 percent azide was added as a control. The plate was allowed to dry at room temperature overnight in a biological safety cabinet. The plate was blocked using 200 µL of 1 percent BSA in PBS. The plate was incubated at 37°C for 50 minutes. The plate was removed from incubation and washed three times with 0.025 percent Tween 20. Dilutions (1/3, 1/9, 1/27) of a crude sonicate of pSC49FLAG were applied to the BSM control wells.

After obtaining results from the above ELISA, a BSM concentration of 60 µg/ml was selected for further plating. The diluent was water. The BSM at 60 µg/ml was loaded at

50 µL per well using a multi-channel pipet and dried and blocked as above. The plates were washed three times with 0.025 percent Tween 20 and stored at 4°C. Incubation and detection of FLAG-containing species was performed as above, using the M2 anti-FLAG antibody.

The relative binding of H4L49HF single chain species by TAG of BSM ELISA are given in Figure 26. Figure 26A shows results from TAG ELISA for purified monomer, dimer and tetramer. Figure 26B shows results from TAG ELISA for *E. coli* pSC49FLAG sonicate containing monomer and multimer species. Figure 26C shows results from various concentrations of the single chain species reacting with BSM. Figure 26D is ELISA on BSM with pSC49FLAG sonicate.

In Figure 26A, starting with concentrations of 10 ug/ml, 1:3 dilutions were performed and the relative binding to TAG-coated plates was determined. It can be seen that the tetramer species gave the greatest binding, followed by dimer, and eventually by monomer. Using DeltaSoft software (BioMetallics, Princeton, NJ), and assuming the dimer as the standard curve, the tetramer activity was 1.3 to 5 times greater than the dimer activity. The monomer activity was only 0.5 to 0.8 percent of the dimer activity.

b) Competition ELISA

This assay was performed as described in Example 1. The formula used to calculate percent competition is as follows:

$$\frac{\text{No Competition - Sample reading}}{\text{No Competition - Background}} \times 100$$

Absorbance values for the samples were measured at 405 nm - 450 nm. The average of duplicate readings was used. Initially samples (25 µL) were applied to the TAG-72 coated microliter plates at 2.0×10^{-10} /mL. Biotin CC49 (4 µg/µL diluted 1:20,000 - used 25 µL) diluted the samples by a factor of 2. Serial dilutions (1:2) were performed, resulting in a total of 10 concentrations for each sample. The "no competition" sample consisted of 25 µL of antibody buffer (1 percent BSA in Tris buffered saline (Pierce Chemical Co.) + 0.025 percent Tween and 25 µL of the biotinylated CC49 competitor. Background samples were formulated with either antibody buffer with no added CC49 biotin, or a 5 µg/mL solution of CC49 IgG in antibody buffer. Both give essentially the background reading.

The results of the competition ELISA are given in Figures 27 and 28, Figure 27 based on moles of antibody binding site and Figure 28 based on moles of antibody. These results show that the extra FLAG™ binding sites on the dimer and tetramer did not skew the results in favor of the multimers.

Immunohistochemistry (IHC)

Specimens. A total of 7 specimens of colorectal adenocarcinoma, including four primary adenocarcinomas and three intra-abdominal metastases (two mesenteric metastases and one hepatic metastasis), were used in these studies. Areas of reactive or normal tissue were also present for assessment of immunoreactivity of non-neoplastic tissues. Sequential 5 µm

thick sections were prepared. One section was stained with hematoxylin and eosin (H and E) with other sections used for the various IHC procedures.

The purified H4L49HF Fv2 product was used at 0.33, 1.7 and 8.3 μg/mL levels as the primary probing agent for the specimens. Sonication (as per Example 1) of a 2.5 mL overnight culture of *E. coli* pSC49FLAG provided a crude test probe material and was used for the specimens. These sonicates contained a mixture of H4L49HF scFv, Fv2, Fv4 etc. whose concentration and composition was not known.

Immunohistochemistry procedures (IHC). IHC was performed using a modification of the avidin-biotin-peroxidase complex (ABC) method (Hsu et al., *J. Histochem. Cytochem.*, 29, 577-580, 1981). The method utilized a commercial anti-mouse IgG kit (Vectastain Peroxidase Mouse IgG, Vector Laboratories, Burlingame, CA). Briefly, the technique was as follows:

1) Sequential 5-6 μ sections of the test tumors were deparaffinized, rehydrated, and endogenous peroxidase activity was quenched.

The sections were then sequentially flooded with the following reagents:

2) Normal equine blocking serum (Vectastain kit) for 20 minutes.

3) Add primary antibody for 45 minutes, either 200 μL of H4L49HF Fv2 or sonicate of *E. coli* pSC49FLAG (from 2.5 mL of overnight cell growth), and add to sequential sections of the tumor. Diluent (PBS containing 0.1 percent BSA, Sigma Chemicals Co. St. Louis, MO) was used on one section from every tumor at this step to serve as a negative control.

4) M-2 (International Biotechnologies Inc., San Pedro, CA), murine anti-FLAG MAb, was then used at a dilution of 1:100 for 45 minutes.

5) Secondary antibody, biotinylated anti-mouse IgG (Vectastain kit), for 45 minutes.

6) ABC reagent for 45 minutes.

7) The chromagen, 0.05 percent diaminobenzidine, in 0.02 percent H_2O_2 for 3.5 minutes.

8) The slides were counterstained with Meyer's hematoxylin (Sigma Diagnostics, St. Louis, MO), dehydrated, and coverslipped by routine methods.

All reactions were conducted at room temperature. The slides were rinsed between each step, except following step 2, in three washes of PBS for at least five minutes each; the final rinse contained 0.1 percent BSA. IHC reactivity was evaluated for both the acellular and cellular parts of the tumor as well as for adjacent non-neoplastic structures

The H4L49HF purified dimer stained both cellular and acellular areas of the test colon carcinomas. Mucinous acellular material from the neoplastic cells was extensively reactive. Viable neoplastic cells had more variable reactivity with some tumors having high numbers of reactive cells and other tumors having few reactive cells. The specific cellular location of the staining was also variable within and between the different specimens. The

atypical cytoplasm and/or (atypical) surface adjacent stained acellular material was most frequently stained. Lesser members of cells had diffuse cytoplasmic staining. There was little reactivity of H4L49H Fv2 with limited numbers of non-neoplasmic tissues examined except reactive colon mucosa immediately adjacent CRC. In this mucosa, the mucin vacuoles of goblet cells and the free mucin on the surface was reactive. The staining diminished with the distance from the tumor/reactive mucosa junction. Crude *E. coli* pSC49FLAG sonicate, containing all the H4L49HF scFv species (monomer, dimer, multimer, etc.) also gave similar results, indicating that these single chain antibodies are specific for tumor tissues. A control sonicate from *E. coli* pATDFLAG gave no staining of the TAG-72 positive areas.

Claims:

1. A protein comprising a dimer or multimer of single chain polypeptides wherein the polypeptides are non-covalently linked and each polypeptide comprises

(a) a first polypeptide comprising an antigen binding portion of a variable domain of an antibody;

(b) a second polypeptide comprising an antigen binding portion of a variable domain of an antibody; and

(c) a peptide linker linking the first and second polypeptides (a) and (b) into a single chain polypeptide having affinity for an antigen.

2. The protein of Claim 1 wherein the first polypeptide is an antigen binding portion of a heavy chain variable region of an antibody and the second polypeptide is an antigen binding portion of a light chain variable region of an antibody.

3. The protein of Claim 1 wherein the first polypeptide is an antigen binding portion of a light chain variable region of an antibody and the second polypeptide is an antigen binding portion of a heavy chain variable region of an antibody.

4. The protein of Claim 3 wherein the polypeptide is a dimer.

5. The protein of any one of Claims 1 to 4 conjugated to an imaging marker.

6. The protein of Claim 5 wherein the imaging marker is ^{125}I, ^{131}I, ^{111}In, ^{105}Rh, ^{153}Sm, ^{67}Cu, ^{67}Ga, ^{166}Ho, ^{177}Lu, ^{186}Re, ^{188}Re or ^{99m}Tc.

7. A composition comprising a protein of any one of Claims 1 to 6 in a pharmaceutically acceptable carrier.

8. The protein of any one of Claims 1 to 4 conjugated to a therapeutic agent.

9. The protein of Claim 8 wherein the therapeutic agent is a radionuclide, drug or biological response modifier or toxin.

10. The composition of Claim 9 wherein the radionuclide is ^{131}I, ^{90}Y, ^{105}Rh, ^{47}Sc, ^{67}Cu, ^{212}Bi, ^{211}At, ^{67}Ga, ^{125}I, ^{186}Re, ^{188}Re, ^{177}Lu, ^{99m}Tc, ^{153}Sm, ^{123}I or ^{111}In.

11. A composition of any one of Claims 8 to 10 in a pharmaceutically acceptable carrier.

12. A method of imaging the internal structure of an animal comprising administering to the animal an effective amount of a composition of Claim 6 and measuring detectable radiation associated with the animal.

13. A method for producing a dimer or multimer of single chain polypeptides wherein the polypeptides are non-covalently linked and each polypeptide comprises (a) a first polypeptide comprising an antigen binding portion of a variable domain of an antibody; (b) a second polypeptide comprising an antigen binding portion of a variable domain of an antibody; and (c) a peptide linker linking the first and second polypeptides (a) and (b) into a single chain polypeptide having affinity of an antigen, the method comprising:

(i) providing a genetic sequence coding for a single chain polypeptide;

(ii) transforming a host cell with the genetic sequence;

(iii) expressing the genetic sequence in the host cell; and

(iv) recovery of single chain polypeptides which have non-covalently linked to form dimers and multimers.

14. The method of Claim 13 wherein genetic sequence coding for a single chain polypeptide contains a sequence encoding for a signal peptide which will direct secretion of the single chain polypeptide out of the cytoplasm of the host cell.

15. The protein of any one of Claims 1-4 wherein the linker is encoded by a nucleotide sequence of

5'-CTTAGTGCGGACGATGCGAAAAAGGATGCTGCGAAGAAGGATGACGCTAAG AAAGACGATGCTAAAAAGGACCTC-3'.

16. The protein of any of Claims 1-4 wherein the linker has the amino acid sequence Leu Ser Ala Asp Asp Ala Lys Lys Asp Ala Ala Lys Lys Asp Asp Ala Lys Lys Asp Asp Ala Lys Lys Asp Leu.

17. A method of detecting an antigen in or suspected of being in a sample which comprises:

(a) contacting the sample with the protein of any one of Claims 1 to 4; and

(b) detecting whether the protein has bound to the antigen.

FIGURE 1

CONSTRUCTION OF PLASMID pSCFV31

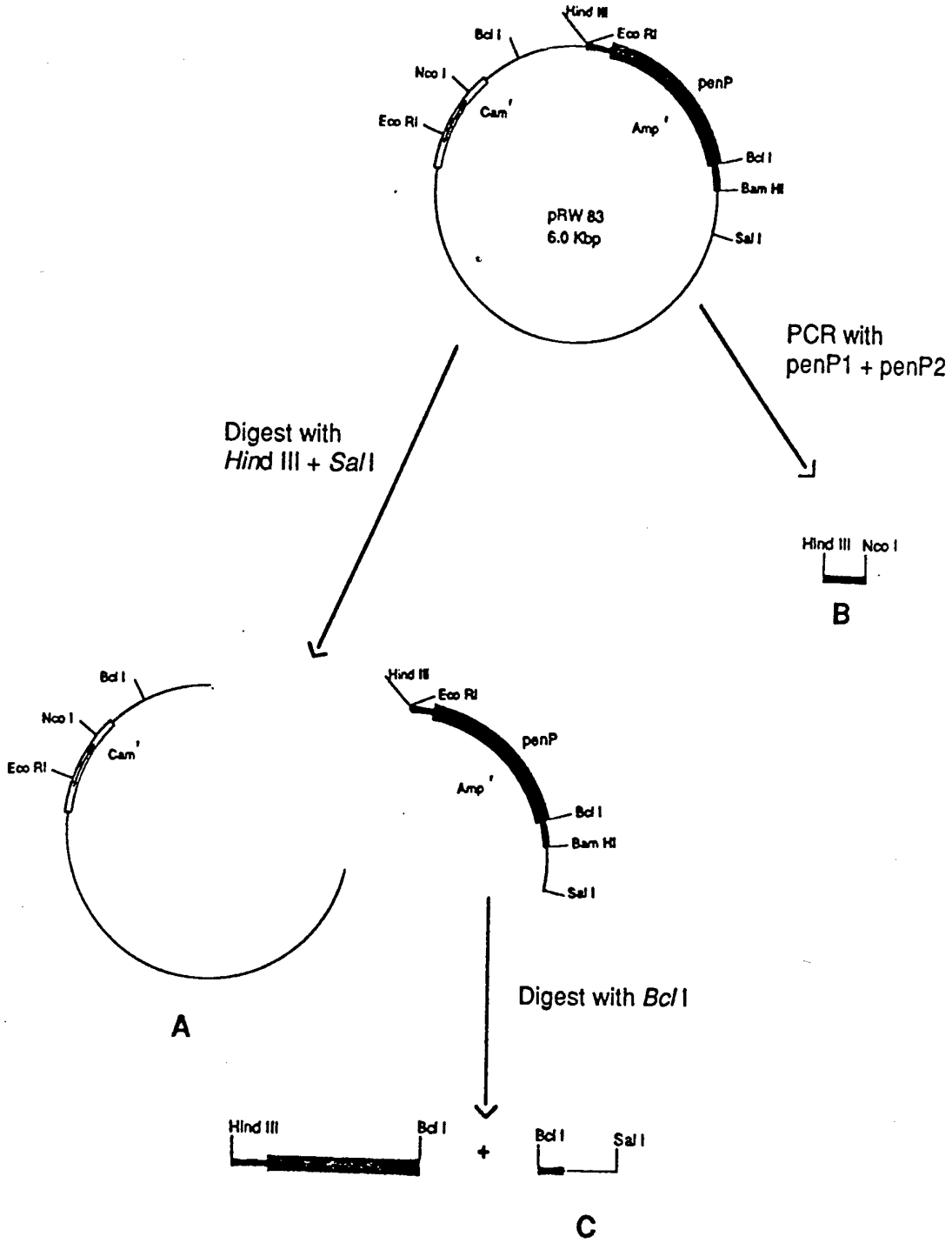


FIGURE 2

CONSTRUCTION OF PLASMID pSCFV31

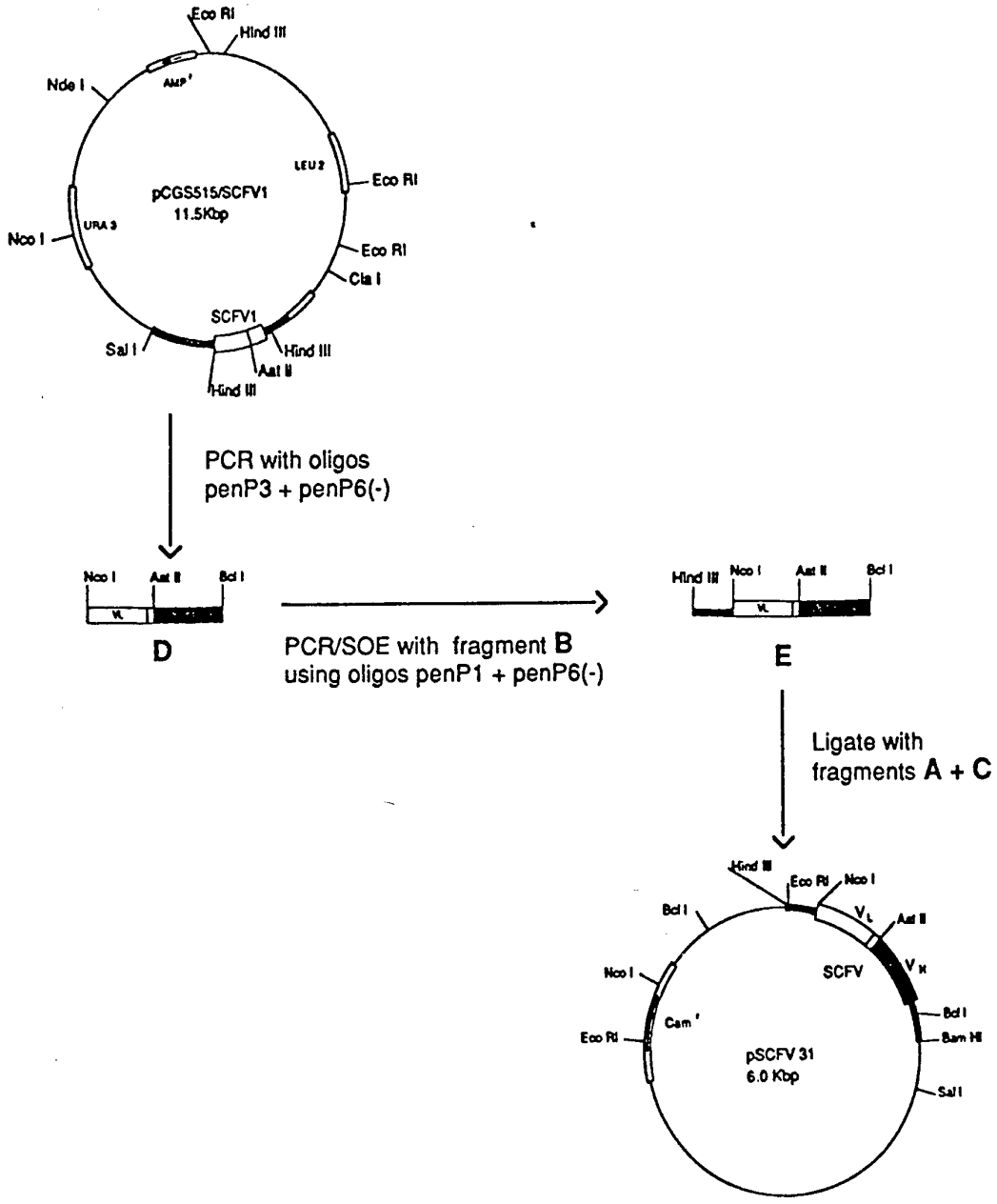


FIGURE 3

```
                          Met Leu
AAAAACTAT AAGCTCCATG ATG CTT

Leu Gln Ala Phe Leu Phe Leu Leu Ala
TTG CAA GCT TTC CTT TTC CTT TTG GCT

Gly Phe Ala Ala Lys Ile Ser Ala Asp
GGT TTT GCA GCC AAA ATA TCT GCA GAC

Ile Val Met Thr Gln Ser Pro Asp Ser
ATC GTG ATG ACC CAG TCT CCA GAC TCC

Leu Ala Val Ser Leu Gly Glu Arg Ala
CTG GCT GTG TCT CTG GGC GAG AGG GCC

                  :------------CDR1L--
Thr Ile Asn Cys Lys Ser Ser Gln Ser
ACC ATC AAC TGC AAG TCC AGC TGC AAG

-----------------------------------
Val Leu Tyr Ser Ser Asn Asn Lys Asn
GTT TTA TAC AGC TCC AAC AAT AAG AAC

-----------:
Tyr Leu Ala Trp Tyr Gln Gln Lys Pro
TAC TTA GCT TGG TAC CAG CAG AAA CCA

Gly Gln Pro Pro Lys Leu Leu Ile Tyr
GGA CAG CCT CCT AAG CTG CTC ATT TAC

:----------CDR2L-----------:
Trp Ala Ser Thr Arg Glu Ser Gly Val
TGG GCA TCT ACC CGG GAA TCC GGG GTC

Pro Asp Arg Phe Ser Gly Ser Gly Ser
CCT GAC CGA TTC AGT GGC AGC GGG TCT

Gly Thr Asp Phe Thr Leu Thr Ile Ser
GGG ACA GAT TTC ACT CTC ACC ATC AGC

Ser Leu Gln Ala Glu Asp Val Ala Val
AGC CTG CAG GCT GAA GAT GTG GCA GT-
```

FIGURE 3 Continued

```
           :----------CDR3L--------
Tyr Tyr Cys Gln Gln Tyr Tyr Ser Tyr
TAT TAC TGT CAG CAA TAT TAT AGT TAT

-----------:
Pro Leu Thr Phe Gly Gly Gly Thr Lys
CCT CTC ACT TTC GGC GGA GGG ACC AAG

   :-------------------------------
Val Lys Glu Ser Gly Ser Val Ser Ser
GTG AAG GAG TCA GGT TCG GTC TCC TCA

-------LINKER----------------------
Glu Gln Leu Ala Gln Phe Arg Ser Leu
GAA CAA TTG GCC CAA TTT CGT TCC TTA

---:
Asp Val Gln Leu Gln Gln Ser Asp Ala
GAC GTC CAG TTG CAG CAG TCT GAC GCT

Glu Leu Val Lys Pro Gly Ala Ser Val
GAG TTG GTG AAA CCT GGG GCT TCA GTG

Lys Ile Ser Cys Lys Ala Ser Gly Tyr
AAG ATT TCC TGC AAG GCT TCT GGC TAC

           :----------CDR1H----:
Thr Phe Thr Asp His Ala Ile His Trp
ACC TTC ACT GAC CAT GCA ATT CAC TGG

Val Lys Gln Asn Pro Glu Gln Gly Leu
GTG AAA CAG AAC CCT GAA CAG GGC CTG

                 :-------------------
Glu Trp Ile Gly Tyr Phe Ser Pro Gly
GAA TGG ATT GGA TAT TTT TCT CCC GGA

-------------------CDR2H-----------
Asn Asp Asp Phe Lys Tyr Asn Glu Arg
AAT GAT GAT TTT AAA TAC AAT GAG AGG

-----------:
Phe Lys Gly Lys Ala Thr Leu Thr Ala
TTC AAG GGC AAG GCC ACA CTG ACT GCA
```

FIGURE 3 Continued

```
Asp Lys Ser Ser Ser Thr Ala Tyr Val
GAC AAA TCC TCC AGC ACT GCC TAC GTG

Gln Leu Asn Ser Leu Thr Ser Glu Asp
CAG CTC AAC AGC CTG ACA TCT GAG GAT

                                   :---
Ser Ala Val Tyr Phe Cys Thr Arg Ser
TCT GCA GTG TAT TTC TGT ACA AGA TCC

---CDR3H-----------:
Leu Asn Met Ala Tyr Trp Gly Gln Gly
CTG AAT ATG GCC TAC TGG GGT CAA GGA

Thr Ser Val Thr Val Ser
ACC TCA GTC ACC GTC TCC TAG TGA


AGCTTGGAAC ACCACACAAA CCATATCCAA A
```

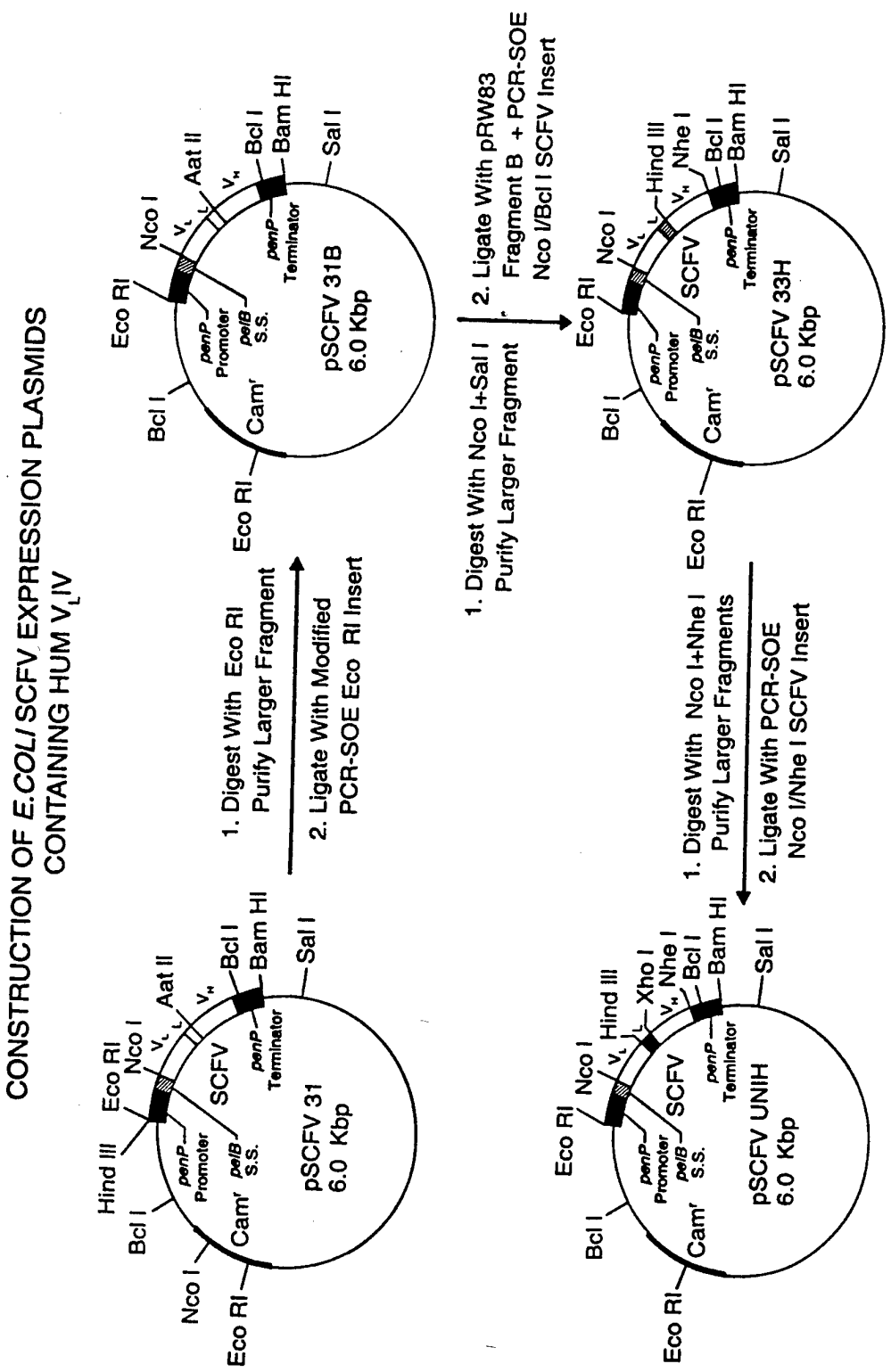

CONSTRUCTION OF E.COLI SCFV EXPRESSION PLASMIDS
CONTAINING HUM VLIV
Eco RI
Nco I
VL
L
Aat II
VH
Bcl I
Bam HI
Sal I
penP Promoter
pelB S.S.
Cam'
penP Terminator
pSCFV 31B
6.0 Kbp
Eco RI
Bcl I
1. Digest With Eco RI
Purify Larger Fragment
2. Ligate With Modified
PCR-SOE Eco RI Insert
Hind III
Eco RI
Nco I
VL
L
Aat II
VH
Bcl I
Bam HI
Sal I
penP Promoter
pelB S.S.
SCFV
Cam'
penP Terminator
pSCFV 31
6.0 Kbp
Bcl I
Nco I
Eco RI
1. Digest With Nco I+Sal I
Purify Larger Fragment
2. Ligate With pRW83
Fragment B + PCR-SOE
Nco I/Bcl I SCFV Insert
Eco RI
Nco I
VL
L
Hind III
Nhe I
VH
Bcl I
Bam HI
Sal I
penP Promoter
pelB S.S.
SCFV
Cam'
penP Terminator
pSCFV 33H
6.0 Kbp
Bcl I
Eco RI
1. Digest With Nco I+Nhe I
Purify Larger Fragments
2. Ligate With PCR-SOE
Nco I/Nhe I SCFV Insert
Eco RI
Nco I
VL
L
Hind III
Xho I
Nhe I
VH
Bcl I
Bam HI
Sal I
penP Promoter
pelB S.S.
SCFV
Cam'
penP Terminator
pSCFV UNIH
6.0 Kbp
Bcl I
Eco RI

FIGURE 4

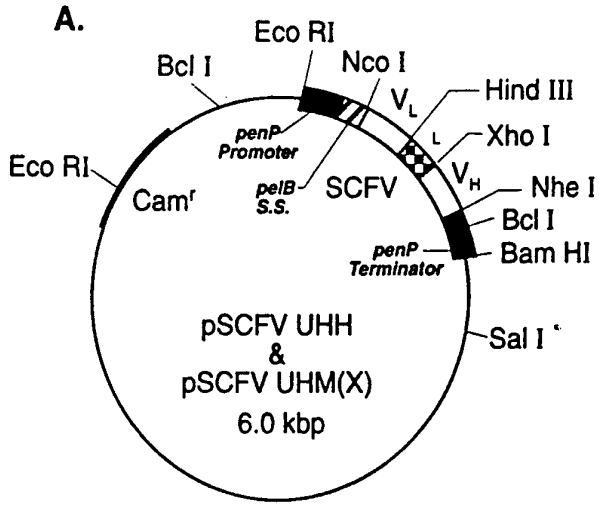
FIGURE 5

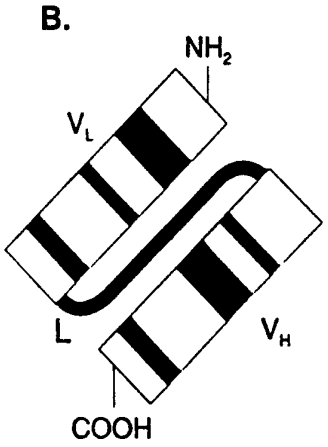
```
     Cla I
3' ATCGATAAAA TTTATTGAGA ATTTGTTTAT TATGATTAAC AGAGGTAAAA GCCAGTATAT   3438
3' TAGCTATTTT AAATAACTCT TAAACAAATA ATACTAATTG TCTCCATTTT CGGTCATATA

TACTGATTAA TATAGGTAAA AGGCAGTTAA GAAATTGGGA ATGCTTTCTC TTCTGCTTTC     3498
ATGACTAATT ATATCCATTT TCCGTCAATT CTTTAACCCT TACGAAAGAG AAGACGAAAG

TTCTACGATG CACAAGGCGT TTCACATTTA TGCCCCTATG AAAATTACTA GGCTGTCCTA     3558
AAGATGCTAC GTGTTCCGCA AAGTGTAAAT ACGGGGATAC TTTTAATGAT CCGACAGGAT

GTCATTAGAT CTTTCAGCAG TTTGTAGTTT TAGAGCTTCT AAGTTGACTT CTGTCTTTTC     3618
CAGTAATCTA GAAAGTCGTC AAACATCAAA ATCTCGAAGA TTCAACTGAA GACAGAAAAG

TATTCATACA ATTACACATT CTGTGATGAT ATTTTTGGCT CTTGATTTAC ATTGGGTACT     3678
ATAAGTATGT TAATGTGTAA GACACTACTA TAAAAACCGA GAACTAAATG TAACCCATGA
       HUMLIN1(-)

TTCACAACCC ACTGCTCATG AAATTTGCTT TTGTACTACT GGTTGTTTTT GCATAGGCCC     3738
AAGTGTTGGG TGACGAGTAC TTTAAACGAA AACATGATGA CCAACAAAAA CGTATCCGGG

CTCCAGGCCA CGACCAGGTG TTTGGATTTT ATAAACGGGC CGTTTGCATT GTGAACTGAG     3798
GAGGTCCGGT GCTGGTCCAC AAACCTAAAA TATTTGCCCG GCAAACGTAA CACTTGACTC

                                Met Val Leu Gln Thr Gln Val Leu Ile
CTACAACAGG CAGGCAGGGG CAGCAAG ATG GTG TTG CAG ACC CAG GTC TTC ATT     3852
GATGTTGTCC GTCCGTCCCC GTCGTTC TAC CAC AAC GTC TGG GTC CAG AAG TAA

Ser Leu Leu Leu Trp Ile Ser G:Intron
TCT CTG TTG CTC TGG ATC TCT G GTGA GGAATTAAAA AGTGCCACAG TCTTTTCAGA   3908
AGA GAC AAC GAG ACC TAG AGA C CACT CCTTAATTTT TCACGGTGTC AGAAAAGTCT
                                                      HUMLIN2(-)

GTAATATCTG TGTAGAAATA AAAAAAATTA AGATATAGTT GGAAATAATG ACTATTTCCA     3968
CATTATAGAC ACATCTTTAT TTTTTTTAAT TCTATATCAA CCTTTATTAC TGATAAAGGT

    Bam HI
ATATGGATCC AATTATCTGC TGACTTATAA TACTACTAGA AAGCAAATTT AAATGACATA     4028
TATACCTAGG TTAATAGACG ACTGAATATT ATGATGATCT TTCGTTTAAA TTTACTGTAT
```

FIGURE 5 Continued

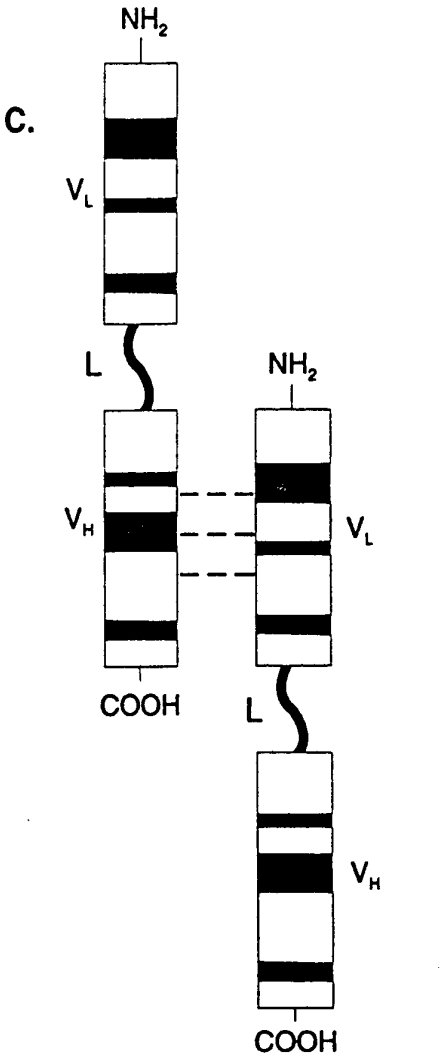
```
TTTCAATTAT ATCTGAGACA GCGTGTATAA GTTTATGTAT AATCATTGTC CATTACTGAC      4088
AAAGTTAATA TAGACTCTGT CGCACATATT CAAATACATA TTAGTAACAG GTAATGACTG

                        +1
      ly Ala Tyr Gly Asp Ile Val Met Thr Gln Ser Pro Asp Ser Leu
TACAG GT GCC TAC GGG GAC ATC GTG ATG ACC CAG TCT CCA GAC TCC CTG      4137
ATGTC CA CGG ATG CCC CTG TAG CAC TAC TGG GTC AGA GGT CTG AGG GAC

                                                  :---------------
Ala Val Ser Leu Gly Glu Arg Ala Thr Ile Asn Cys Lys Ser Ser Gln
GCT GTG TCT CTG GGC GAG AGG GCC ACC ATC AAC TGC AAG TCC AGC CAG      4185
CGA CAC AGA GAC CCG CTC TCC CGG TGG TAG TTG ACG TTC AGG TCG GTC

Ser Val Leu Tyr Ser Ser Asn Asn Lys Asn Tyr Leu Ala Trp Tyr Gln
AGT GTT TTA TAC AGC TCC AAC AAT AAG AAC TAC TTA GCT TGG TAC CAG      4233
TCA CAA AAT ATG TCG AGG TTG TTA TTC TTG ATG AAT CGA ACC ATG GTC
                              HUMLCDR1(-)

                                                  :------CDR 2----
Gln Lys Pro Gly Gln Pro Pro Lys Leu Leu Ile Tyr Trp Ala Ser Thr
CAG AAA CCA GGA CAG CCT CCT AAG CTG CTC ATT TAC TGG GCA TCT ACC      4281
GTC TTT GGT CCT GTC GGA GGA TTC GAC GAG TAA ATG ACC CGT AGA TGG

-----------:
Arg Glu Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr
CGG GAA TCC GGG GTC CCT GAC CGA TTC AGT GGC AGC GGG TCT GGG ACA      4329
GCC CTT AGG CCC CAG GGA CTG GCT AAG TCA CCG TCG CCC AGA CCC TGT

Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Ala Glu Asp Val Ala Val
GAT TTC ACT CTC ACC ATC AGC AGC CTG CAG GCT GAA GAT GTG GCA GTT      4377
CTA AAG TGA GAG TGG TAG TCG TCG GAC GTC CGA CTT CTA CAC CGT CAA

          :----------------CDR 3-------------:
Tyr Tyr Cys Gln Gln Tyr Tyr Ser Tyr Pro Leu Thr Phe Gly Gly Gly
TAT TAC TGT CAG CAA TAT TAT AGT TAT CCT CTC ACT TTC GGC GGA GGG      4425
ATA ATG ACA GTC GTT ATA ATA TCA ATA GGA GAG TGA AAG CCG CCT CCC

Thr Lys Val Val Ile Lys A                    Hind III
ACC AAG GTG GTG ATC AAA C GTAAGTACAC TTTTCTAAG CTT 3' human Cκ
4466
TGG TTC CAC CAC TAG TTT G CATTCATGTG AAAAGATTC GAA 5' insert
```

ANTI TAG-72 SINGLE CHAIN ANTIBODY
EXPRESSION PLASMIDS AND PRODUCTS





SINGLE CHAIN Fv MONOMER
(scFv)



SINGLE CHAIN Fv Dimer
(Fv2)

FIGURE 6

FIGURE 7

DNA AND AMINO ACID SEQUENCES OF H4V$_L$-CC49 V$_H$ SCFV

```
                                  Cla I   EcoR I
5'-C TCA TGT TTG ACA GCT TAT CAT CGA TGA ATT CCA TCA CTT CCC TCC      46

GTT CAT TTG TCC CCG GTG GAA ACG AGG TCA TCA TTT CCT TCC GAA AAA      94

ACG GTT GCA TTT AAA TCT TAC ATA TAT AAT ACT TTC AAA GAC TAC ATT     142

TGT AAG ATT TGA TGT TTG AGT CGG CTG AAA GAT CGT ACG TAC CAA TTA     190

TTG TTT CGT GAT TGT TCA AGC CAT AAC ACT GTA GGG ATA GTG GAA AGA     238

GTG CTT CAT CTG GTT ACG ATC AAT CAA ATA TTC AAA CGG AGG GAG ACG     286

        -22
        Met Lys Tyr Leu Leu Pro Thr Ala Ala Ala Gly Leu Leu Leu
ATT TTG ATG AAA TAC CTA TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA     334

                      Nco I       +1
Leu Ala Ala Gln Pro Ala Met Ala Asp Ile Val Met Thr Gln Ser Pro
CTC GCT GCC CAA CCA GCC ATG GCC GAC ATC GTG ATG ACC CAG TCT CCA     382

     10                                        20
Asp Ser Leu Ala Val Ser Leu Gly Glu Arg Ala Thr Ile Asn Cys Lys
GAC TCC CTG GCT GTG TCT CTG GGC GAG AGG GCC ACC ATC AAC TGC AAG     430

                      30                                        40
Ser Ser Gln Ser Val Leu Tyr Ser Ser Asn Asn Lys Asn Tyr Leu Ala
TCC AGC CAG AGT GTT TTA TAC AGC TCC AAC AAT AAG AAC TAC TTA GCT     478

                                  50
Trp Tyr Gln Gln Lys Pro Gly Gln Pro Pro Lys Leu Leu Ile Tyr Trp
TGG TAC CAG CAG AAA CCA GGA CAG CCT CCT AAG CTG CTC ATT TAC TGG     526

               60                                        70
Ala Ser Thr Arg Glu Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly
GCA TCT ACC CGG GAA TCC GGG GTC CCT GAC CGA TTC AGT GGC AGC GGG     574

                              80
Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Ala Glu Asp
TCT GGG ACA GAT TTC ACT CTC ACC ATC AGC AGC CTG CAG GCT GAA GAT     622

     90                                        100
Val Ala Val Tyr Tyr Cys Gln Gln Tyr Tyr Ser Tyr Pro Leu Thr Phe
GTG GCA GTT TAT TAC TGT CAG CAA TAT TAT AGT TAT CCT CTC ACT TTC     670

                      110          Hind III                     120
Gly Gly Gly Thr Lys Val Val Ile Lys Leu Ser Ala Asp Asp Ala Lys
GGC GGA GGG ACC AAG GTG GTG ATC AAG CTT AGT GCG GAC GAT GCG AAA     718
```

FIGURE 7 Continued

```
                                                130
Lys Asp Ala Ala Lys Lys Asp Asp Ala Lys Lys Asp Asp Ala Lys Lys
AAG GAT GCT GCG AAG AAG GAT GAC GCT AAG AAA GAC GAT GCT AAA AAG     766

    Xho I   140                                             150
Asp Leu Glu Val Gln Leu Gln Gln Ser Asp Ala Glu Leu Val Lys Pro
GAC CTC GAG GTT CAG TTG CAG CAG TCT GAC GCT GAG TTG GTG AAA CCT     814

                            160
Gly Ala Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr
GGG GCT TCA GTG AAG ATT TCC TGC AAG GCT TCT GGC TAC ACC TTC ACT     862

    170                                             180
Asp His Ala Ile His Trp Val Lys Gln Asn Pro Glu Gln Gly Leu Glu
GAC CAT GCA ATT CAC TGG GTG AAA CAG AAC CCT GAA CAG GGC CTG GAA     910

                    190                                     200
Trp Ile Gly Tyr Phe Ser Pro Gly Asn Asp Asp Phe Lys Tyr Asn Glu
TGG ATT GGA TAT TTT TCT CCC GGA AAT GAT GAT TTT AAA TAC AAT GAG     958

                                    210
Arg Phe Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr
AGG TTC AAG GGC AAG GCC ACA CTG ACT GCA GAC AAA TCC TCC AGC ACT    1006

            220                                     230
Ala Tyr Val Gln Leu Asn Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr
GCC TAC GTG CAG CTC AAC AGC CTG ACA TCT GAG GAT TCT GCA GTG TAT    1054

                            240
Phe Cys Thr Arg Ser Leu Asn Met Ala Tyr Trp Gly Gln Gly Thr Ser
TTC TGT ACA AGA TCC CTG AAT ATG GCC TAC TGG GGT CAA GGA ACC TCA    1102

    250
Val Thr Val Ser Ser ***     Nhe I
GTC ACC GTC TCC TCA TAA AAA GCT AGC GAT GAA TCC GTC AAA ACA TCA    1150

TCT TAC ATA AAG TCA CTT GGT GAT CAA GCT CAT ATC ATT GTC CGG CAA    1198

TGG TGT GGG CTT TTT TTG TTT TCT ATC TTT AAA GAT CAT GTG AAG AAA    1246

AAC GGG AAA ATC GGT CTG CGG GAA AGG ACC GGG TTT TTG TCG AAA TCA    1254

                                        BamH I
TAG GCG AAT GGG TTG GAT TGT GAC AAA ATT CGG ATC C-3'              1291
```

FIGURE 8

DNA AND AMINO ACID SEQUENCES OF CC49 SCFV SPECIES

```
                                  Cla I   EcoR I
5'-C TCA TGT TTG ACA GCT TAT CAT CGA TGA ATT CCA TCA CTT CCC TCC      46

GTT CAT TTG TCC CCG GTG GAA ACG AGG TCA TCA TTT CCT TCC GAA AAA      94

ACG GTT GCA TTT AAA TCT TAC ATA TAT AAT ACT TTC AAA GAC TAC ATT     142

TGT AAG ATT TGA TGT TTG AGT CGG CTG AAA GAT CGT ACG TAC CAA TTA     190

TTG TTT CGT GAT TGT TCA AGC CAT AAC ACT GTA GGG ATA GTG GAA AGA     238

GTG CTT CAT CTG GTT ACG ATC AAT CAA ATA TTC AAA CGG AGG GAG ACG     286

        -22
        Met Lys Tyr Leu Leu Pro Thr Ala Ala Ala Gly Leu Leu Leu
ATT TTG ATG AAA TAC CTA TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA     334

                      Nco I      +1
Leu Ala Ala Gln Pro Ala Met Ala Asp Ile Val Met Ser Gln Ser Pro
CTC GCT GCC CAA CCA GCC ATG GCC GAC ATT GTG ATG TCA CAG TCT CCA     382

     10                                        20
Ser Ser Leu Pro Val Ser Val Gly Glu Lys Val Thr Leu Ser Cys Lys
TCC TCC CTA CCT GTG TCA GTT GGC GAG AAG GTT ACT TTG AGC TGC AAG     430

                      30                                        40
Ser Ser Gln Ser Leu Leu Tyr Ser Gly Asn Gln Lys Asn Tyr Leu Ala
TCC AGT CAG AGC CTT TTA TAT AGT GGT AAT CAA AAG AAC TAC TTG GCC     478

                                  50
Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu Ile Tyr Trp
TGG TAC CAG CAG AAA CCA GGG CAG TCT CCT AAA CTG CTG ATT TAC TGG     526

               60                                        70
Ala Ser Ala Arg Glu Ser Gly Val Pro Asp Arg Phe Thr Gly Ser Gly
GCA TCC GCT AGG GAA TCT GGG GTC CCT GAT CGC TTC ACA GGC AGT GGA     574

                              80
Ser Gly Thr Asp Phe Thr Leu Ser Ile Ser Ser Val Lys Thr Glu Asp
TCT GGG ACA GAT TTC ACT CTC TCC ATC AGC AGT GTG AAG ACT GAA GAC     622

     90                                        100
Leu Ala Val Tyr Tyr Cys Gln Gln Tyr Tyr Ser Tyr Pro Leu Thr Phe
CTG GCA GTT TAT TAC TGT CAG CAG TAT TAT AGC TAT CCC CTC ACG TTC     670

                      110          Hind III                     120
Gly Ala Gly Thr Lys Leu Val Leu Lys Leu Ser Ala Asp Asp Ala Lys
GGT GCT GGG ACC AAG CTG GTG CTG AAG CTT AGT GCG GAC GAT GCG AAA     718
```

FIGURE 8 Continued

```
                                                130
Lys Asp Ala Ala Lys Lys Asp Asp Ala Lys Lys Asp Asp Ala Lys Lys
AAG GAT GCT GCG AAG AAG GAT GAC GCT AAG AAA GAC GAT GCT AAA AAG      766

    Xho I   140                                             150
Asp Leu Glu Val Gln Leu Gln Gln Ser Asp Ala Glu Leu Val Lys Pro
GAC CTC GAG GTT CAG TTG CAG CAG TCT GAC GCT GAG TTG GTG AAA CCT      814

                            160
Gly Ala Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr
GGG GCT TCA GTG AAG ATT TCC TGC AAG GCT TCT GGC TAC ACC TTC ACT      862

    170                                     180
Asp His Ala Ile His Trp Val Lys Gln Asn Pro Glu Gln Gly Leu Glu
GAC CAT GCA ATT CAC TGG GTG AAA CAG AAC CCT GAA CAG GGC CTG GAA      910

                        190                                 200
Trp Ile Gly Tyr Phe Ser Pro Gly Asn Asp Asp Phe Lys Tyr Asn Glu
TGG ATT GGA TAT TTT TCT CCC GGA AAT GAT GAT TTT AAA TAC AAT GAG      958

                                    210
Arg Phe Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr
AGG TTC AAG GGC AAG GCC ACA CTG ACT GCA GAC AAA TCC TCC AGC ACT     1006

            220                                     230
Ala Tyr Val Gln Leu Asn Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr
GCC TAC GTG CAG CTC AAC AGC CTG ACA TCT GAG GAT TCT GCA GTG TAT     1054

                            240
Phe Cys Thr Arg Ser Leu Asn Met Ala Tyr Trp Gly Gln Gly Thr Ser
TTC TGT ACA AGA TCC CTG AAT ATG GCC TAC TGG GGT CAA GGA ACC TCA     1102

       250
A. Val Thr Val Ser | Ser ***
   GTC ACC GTC TCC | TCA TAA                                      1120
       250
B. Val Thr Val Ser | Pro Glu Asp Tyr Asp Ser ***
   GTC ACC GTC TCC | CCT GAG GAC TAT GAC TCC TAA                   1135
       250                                  260
C. Val Thr Val Ser | Pro Glu Asp Pro Glu Asp Tyr Asp ***
   GTC ACC GTC TCC | CCT GAA GAC CCT GAA GAC TAT GAC TAA           1141

   Nhe I
AAA GCT AGC GAT GAA TCC GTC AAA ACA TCA TCT TAC ATA AAG TCA CTT     1189

GGT GAT CAA GCT CAT ATC ATT GTC CGG CAA TGG TGT GGG CTT TTT TTG     1237

TTT TCT ATC TTT AAA GAT CAT GTG AAG AAA AAC GGG AAA ATC GGT CTG     1285

CGG GAA AGG ACC GGG TTT TTG TCG AAA TCA TAG GCG AAT GGG TTG GAT     1333

                BamH I
TGT GAC AAA ATT CGG ATC C-3'                    1354(A); 1369(B); 1375(C)
```

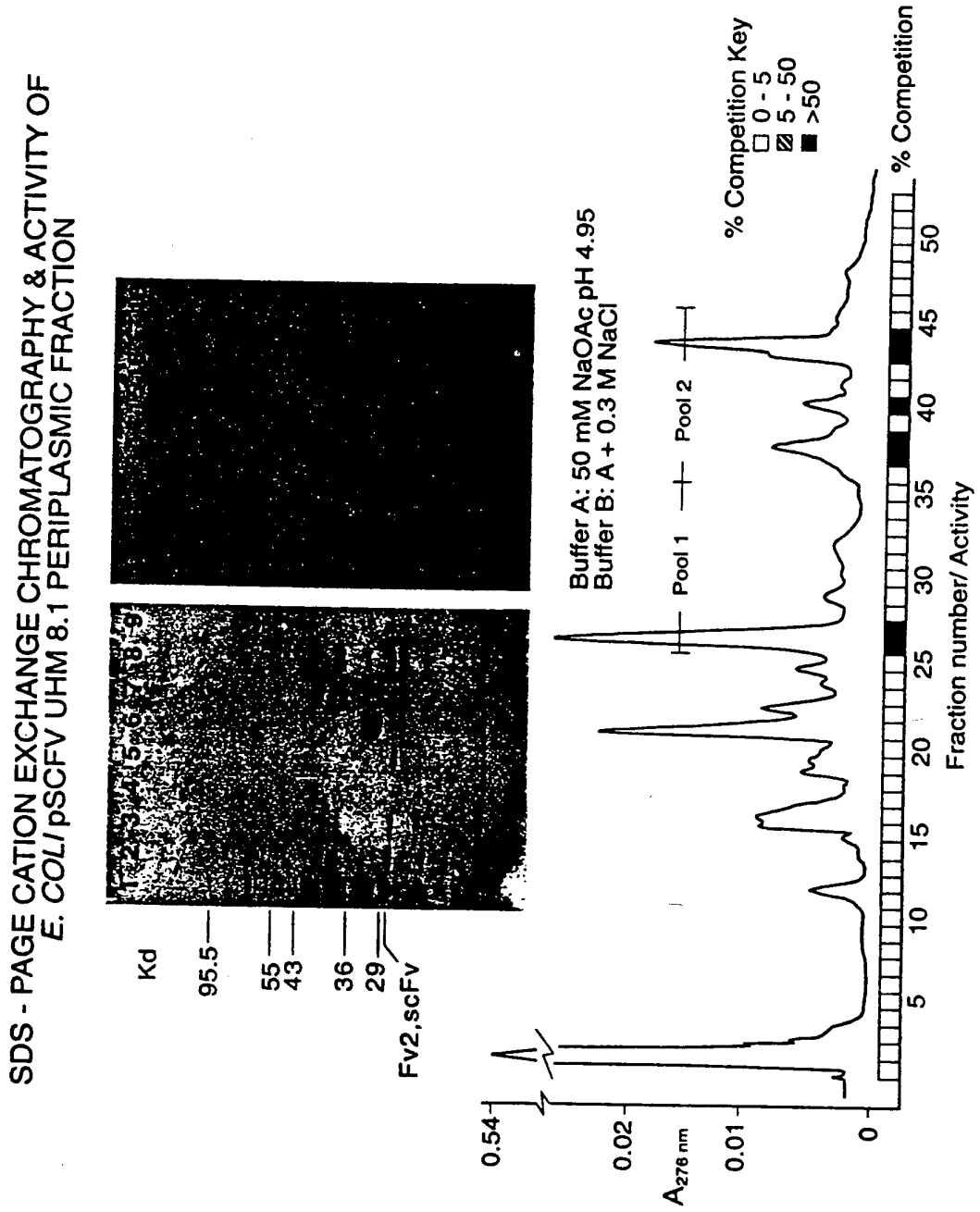
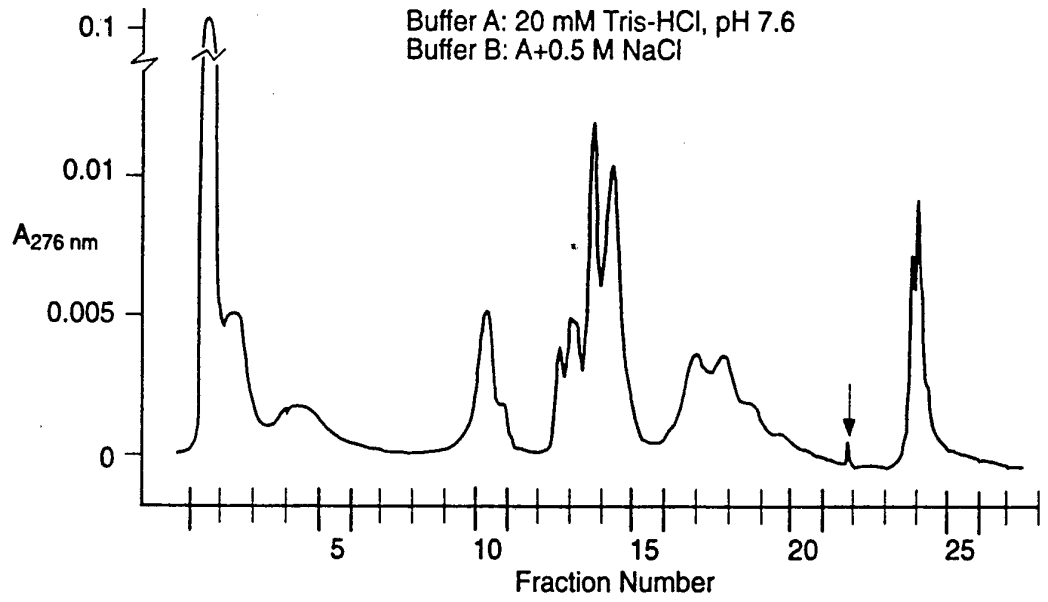

SDS - PAGE CATION EXCHANGE CHROMATOGRAPHY & ACTIVITY OF
E. COLI pSCFV UHM 8.1 PERIPLASMIC FRACTION
Kd
95.5
55
43
36
29
Fv2,scFv
1 2 3 4 5 6 7 8 9
Buffer A: 50 mM NaOAc pH 4.95
Buffer B: A + 0.3 M NaCl
Pool 1
Pool 2
% Competition Key
0 - 5
5 - 50
>50
% Competition
0.54
0.02
0.01
0
$A_{278\ nm}$
5 10 15 20 25 30 35 40 45 50
Fraction number/ Activity

FIGURE 9

ANION EXCHANGE CHROMATOGRAPHY & SDS-PAGE OF MONO S
POOL 2 *E.COLI* pSCFV UHM 8.1 PERIPLASMIC FRACTION



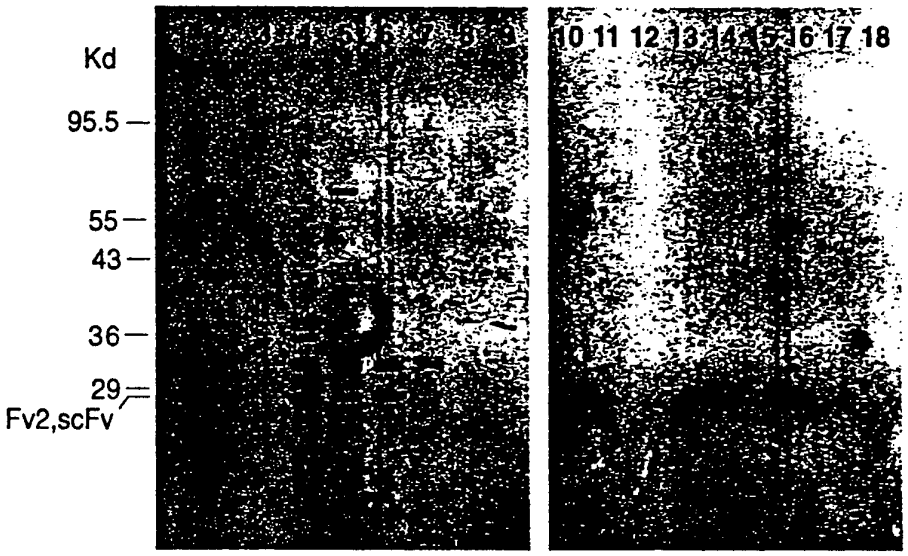


FIGURE 10

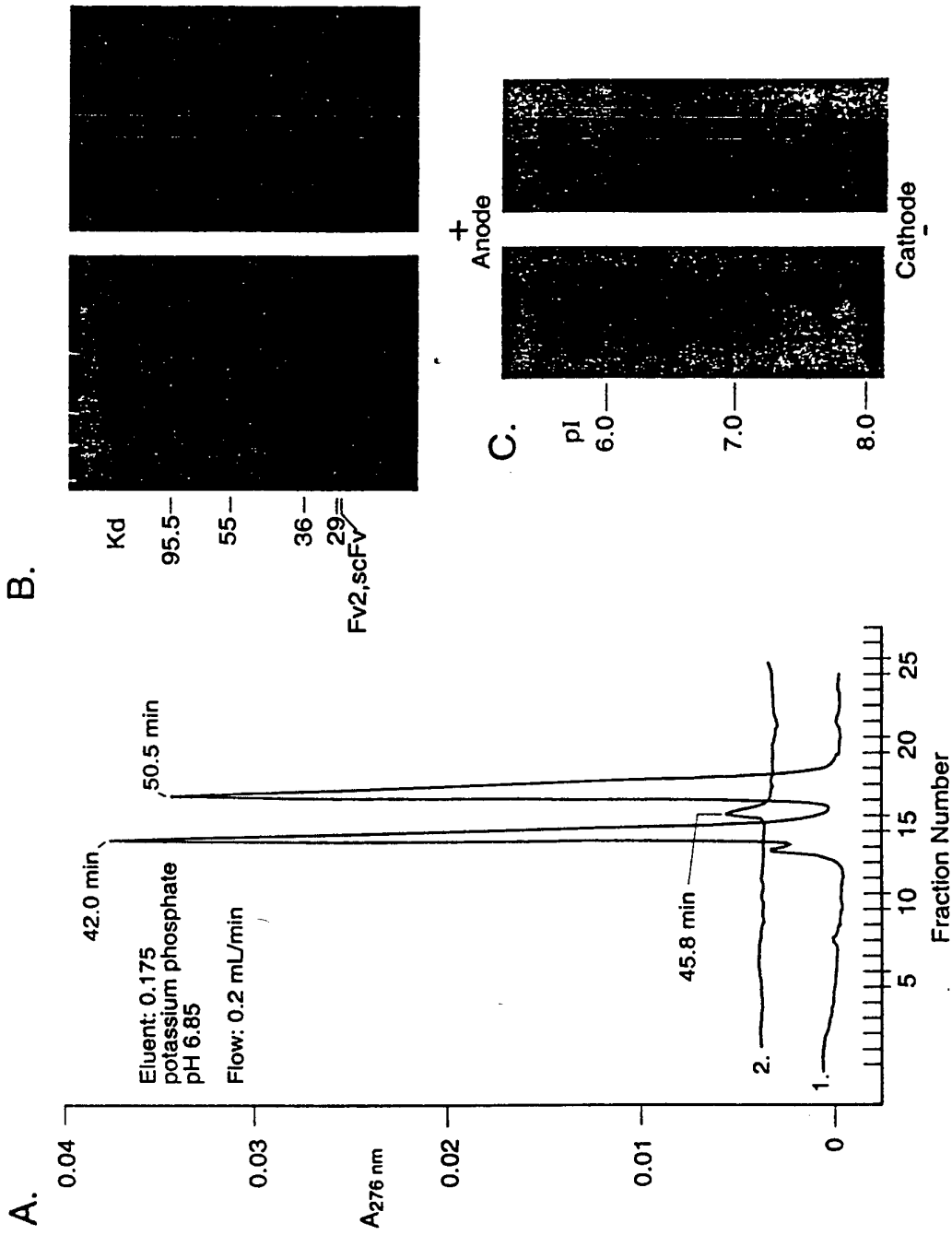

GEL FILTRATION, SDS - PAGE & IEF CORRELATION IN THE DISCOVERY OF FV2
A.
B.
C.
Kd
95.5
55
36
29
Fv2,scFv
Anode
Cathode
pI
6.0
7.0
8.0
0.04
0.03
0.02
0.01
0
$A_{276\ nm}$
42.0 min
50.5 min
45.8 min
Eluent: 0.175 potassium phosphate pH 6.85
Flow: 0.2 mL/min
1.
2.
5
10
15
20
25
Fraction Number

FIGURE 11

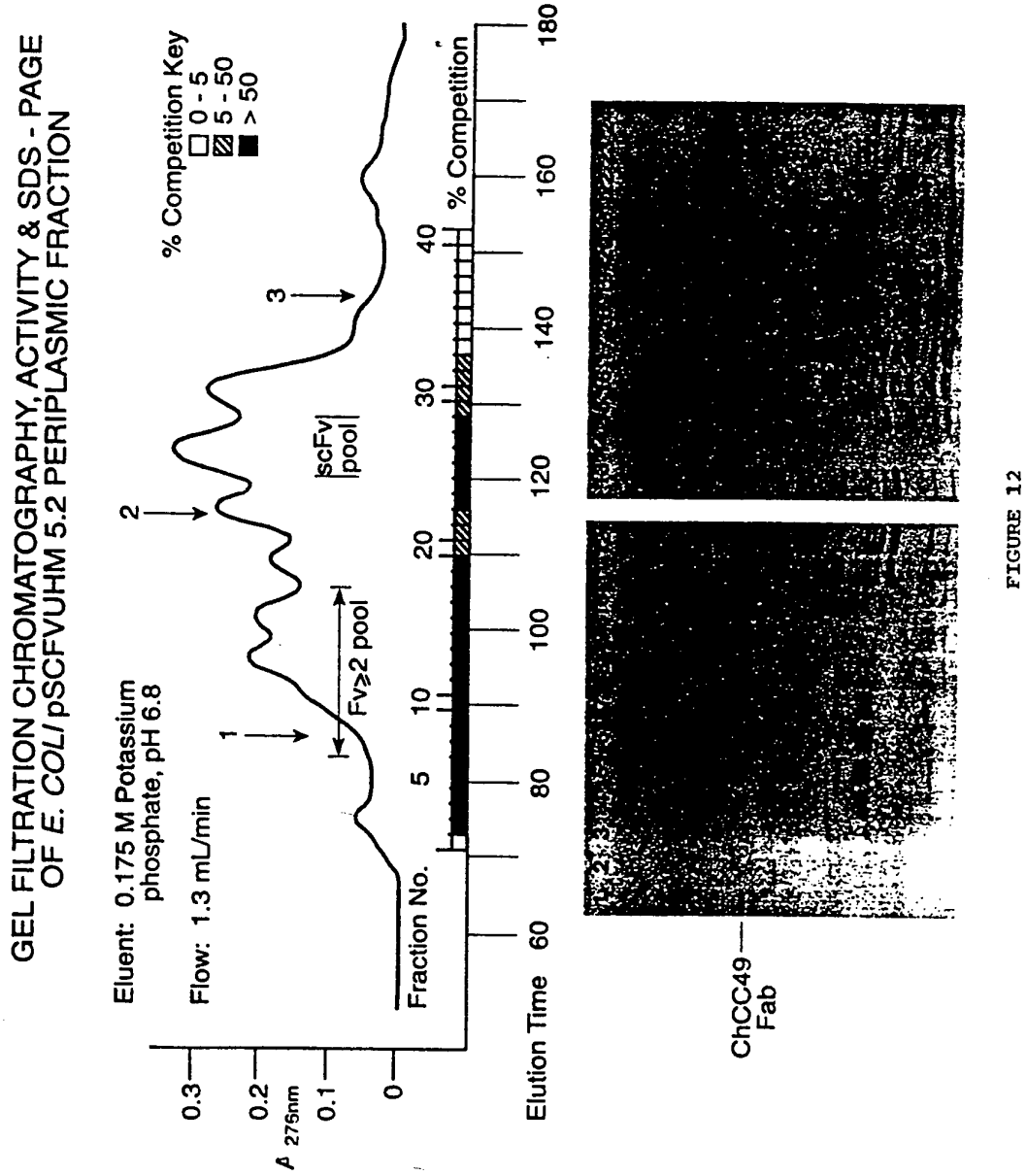

GEL FILTRATION CHROMATOGRAPHY, ACTIVITY & SDS - PAGE
OF E. COLI pSCFVUHM 5.2 PERIPLASMIC FRACTION
Eluent: 0.175 M Potassium phosphate, pH 6.8
Flow: 1.3 mL/min
% Competition Key
0 - 5
5 - 50
> 50
1
2
3
Fv≥2 pool
scFv pool
A 275nm
0.3
0.2
0.1
0
Fraction No.
5
10
20
30
40
% Competition
Elution Time
60
80
100
120
140
160
180
ChCC49 Fab

FIGURE 12

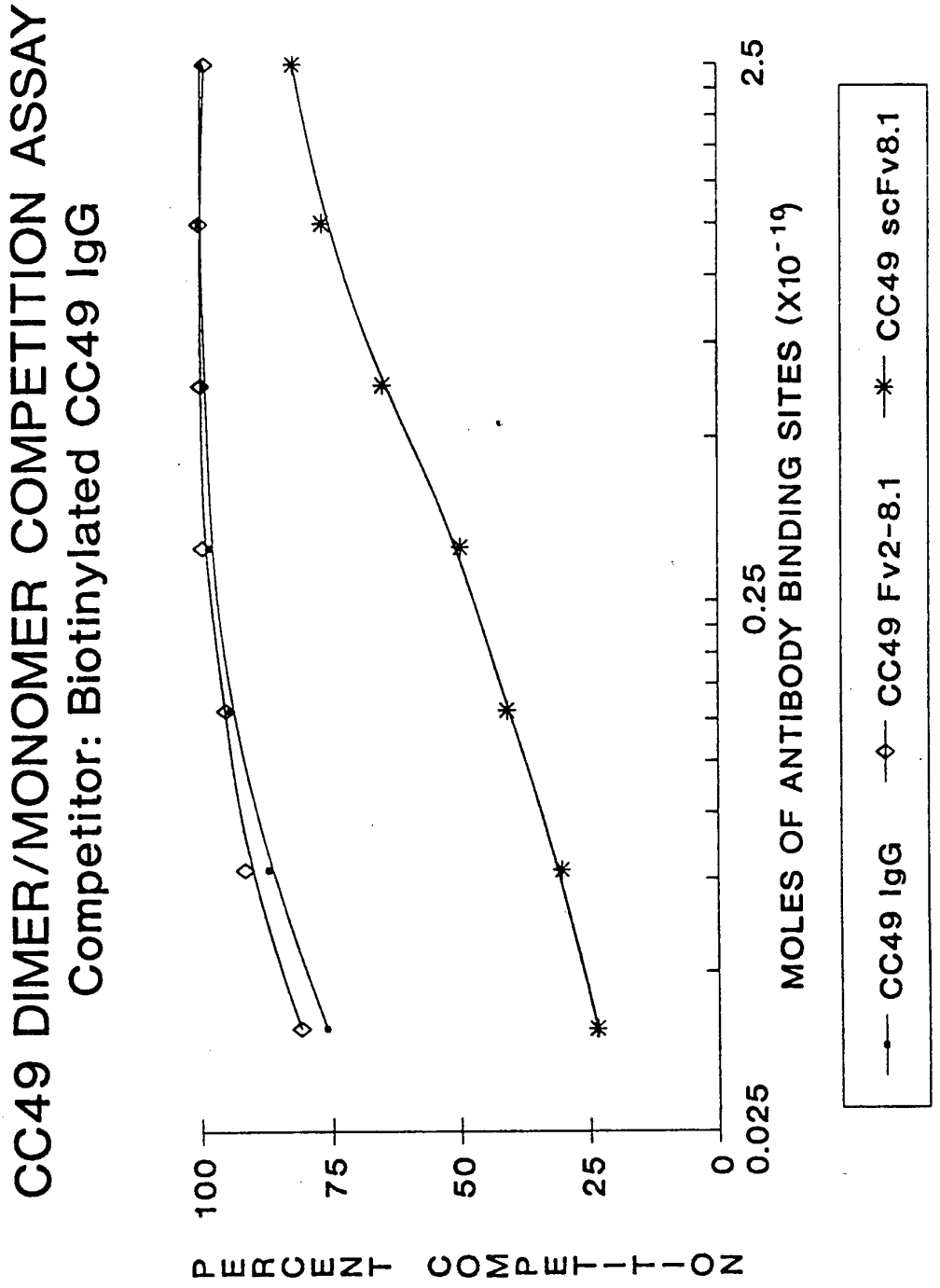


FIGURE 13 (Panel 5)

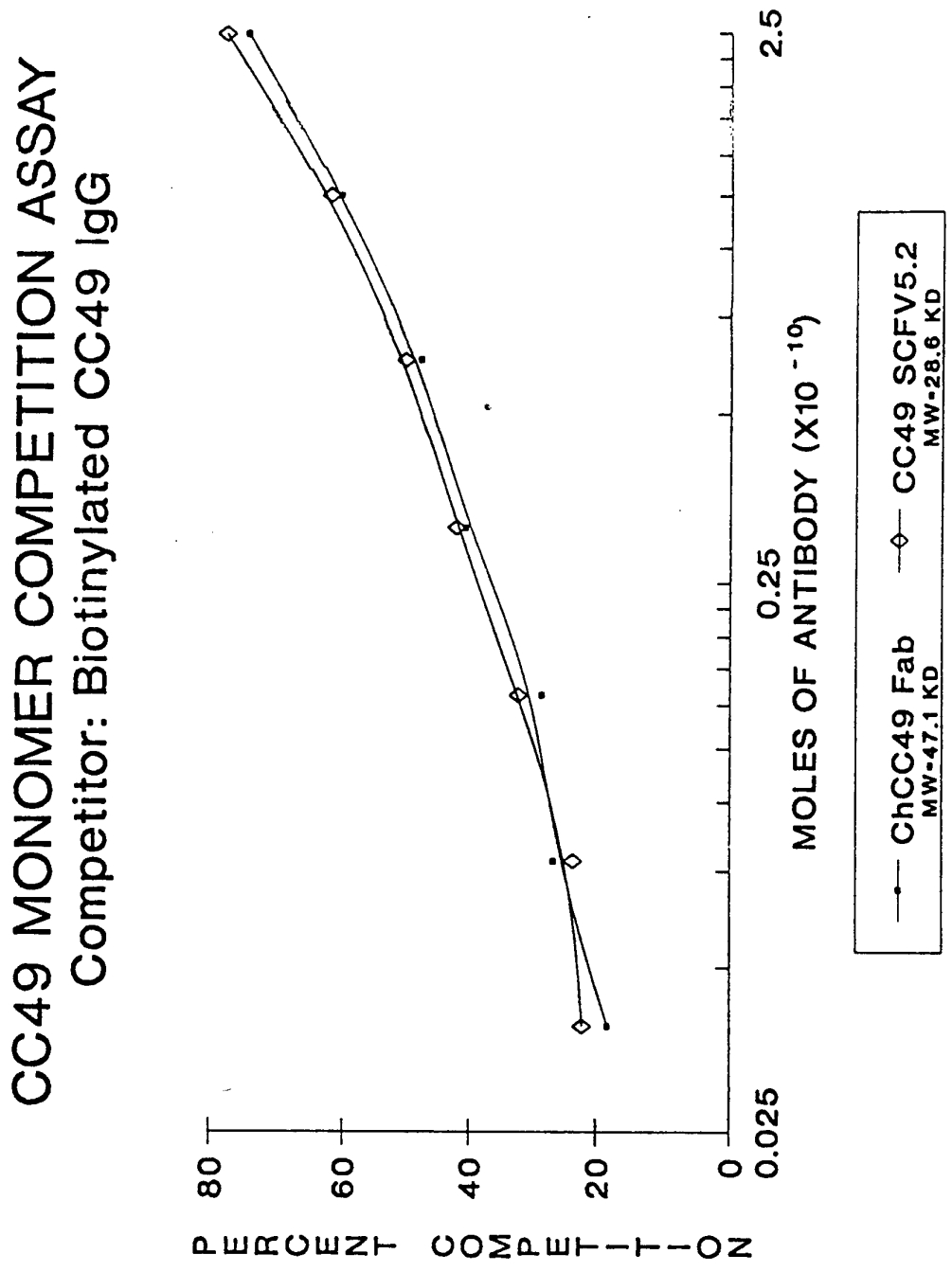

CC49 MONOMER COMPETITION ASSAY
Competitor: Biotinylated CC49 IgG
PERCENT COMPETITION
80
60
40
20
0
0.025
0.25
2.5
MOLES OF ANTIBODY (X10 -10)
ChCC49 Fab
MW-47.1 KD
CC49 SCFV5.2
MW-28.6 KD

FIGURE 14

PLASMID CONSTRUCTIONS LEADING TO pPY31& pPY32

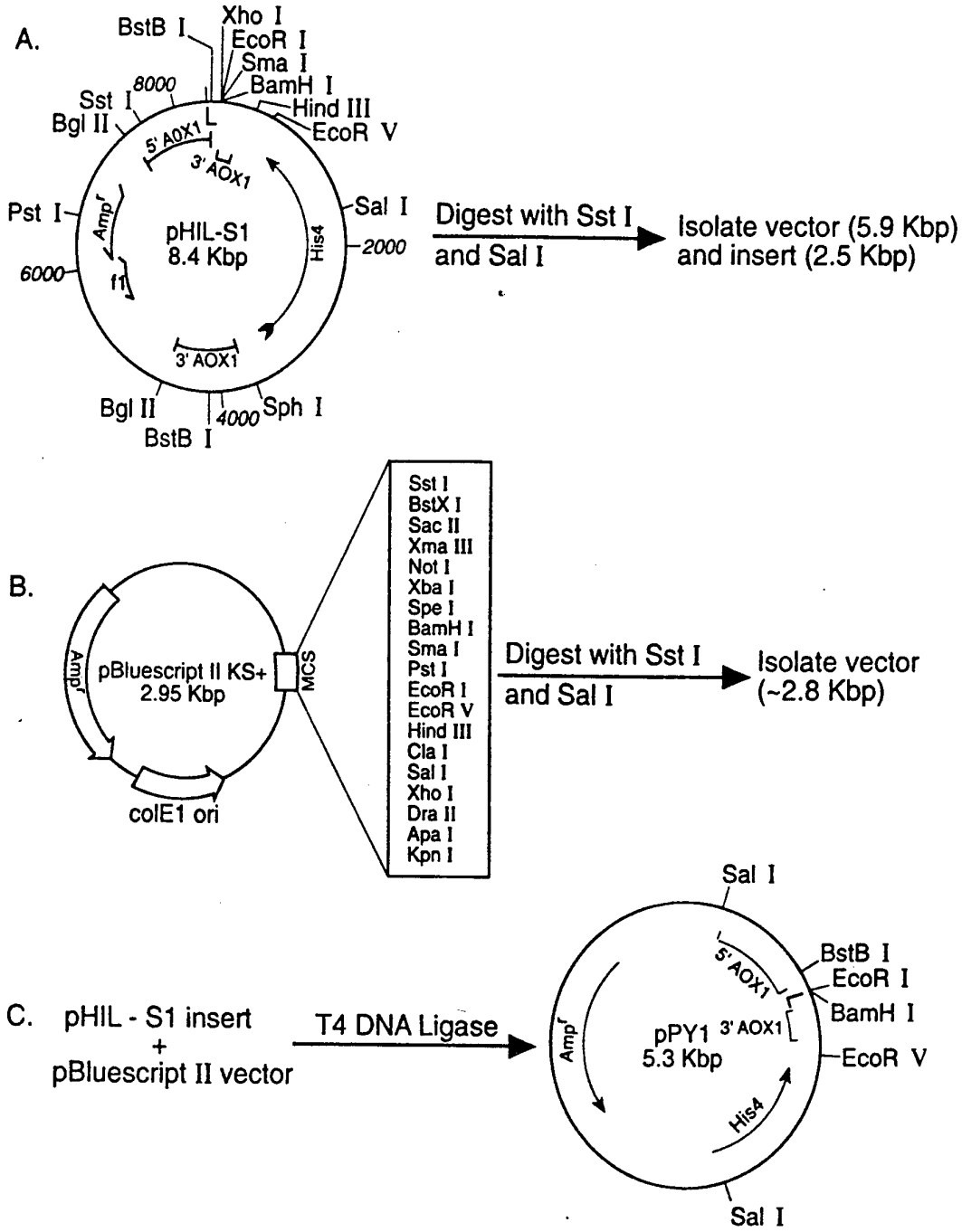
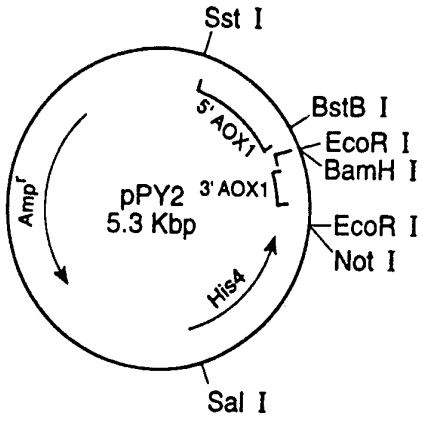


FIGURE 15

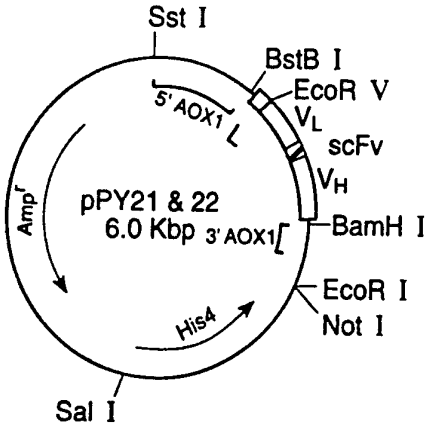
D.

pPY1 → Digest with EcoR V, Ligate with annealed linker



E.

pPY2 → Digest with BstB I + BamH I, Ligate with CC49 scFv PCR products



F.

pPY21& pPY22 → Digest with Sst I + Sal I, Ligate with pHIL - S1 vector fragment from A

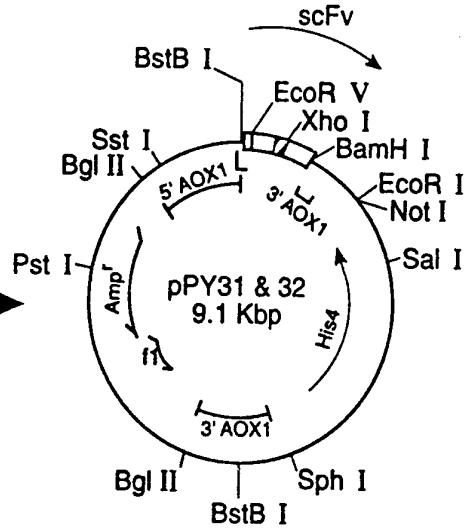


FIGURE 15
Continued

FIGURE 16

DNA AND AMINO ACID SEQUENCE OF CC49 SCFV IN pPY21 & pPY22

```
                                  AOX1P
                          TTTAACGACAACTTGAGAAGATC                    BstB I
5'-AOX1----------------------------------(AAAAAACAAC)TAATTATTC                    9

          -22
          Met Lys Tyr Met Leu Leu Gln Ala Phe Leu Phe Leu Leu Ala
GAA ACG ATG AAA TAC ATG CTT TTG CAA GCT TTC CCT TTC CTT TTG GCT                   57

                          EcoR V          +1
Gly Phe Ala Ala Lys Ile Ser Ala Asp Ile Val Met Ser Gln Ser Pro
GGT TTT GCA GCT AAG ATA TCT GCT GAC ATT GTG ATG TCA CAG TCT CCA                  105

     10                                                  20
Ser Ser Leu Pro Val Ser Val Gly Glu Lys Val Thr Leu Ser Cys Lys
TCC TCC CTA CCT GTG TCA GTT GGC GAG AAG GTT ACT TTG AGC TGC AAG                  153

                          30                                              40
Ser Ser Gln Ser Leu Leu Tyr Ser Gly Asn Gln Lys Asn Tyr Leu Ala
TCC AGT CAG AGC CTT TTA TAT AGT GGT AAT CAA AAG AAC TAC TTG GCC                  201

          TAGVLFR2
TGG TAC CAG CAG AAA CCA GG              50
Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu Ile Tyr Trp
TGG TAC CAG CAG AAA CCA GGG CAG TCT CCT AAA CTG CTG ATT TAC TGG                  249

               60                                                 70
Ala Ser Ala Arg Glu Ser Gly Val Pro Asp Arg Phe Thr Gly Ser Gly
GCA TCC GCT AGG GAA TCT GGG GTC CCT GAT CGC TTC ACA GGC AGT GGA                  297

                                   80
Ser Gly Thr Asp Phe Thr Leu Ser Ile Ser Ser Val Lys Thr Glu Asp
TCT GGG ACA GAT TTC ACT CTC TCC ATC AGC AGT GTG AAG ACT GAA GAC                  345

                                  TAGVLCDR3
                                  A       T
     90                    GT CAG CAG TAT TAT AGC TAT CC
Leu Ala Val Tyr Tyr Cys Gln Gln Tyr Tyr Ser Tyr Pro Leu Thr Phe
CTG GCA GTT TAT TAC TGT CAG CAG TAT TAT AGC TAT CCC CTC ACG TTC                  393

                          110             Hind III                        120
Gly Ala Gly Thr Lys Leu Val Leu Lys Leu Ser Ala Asp Asp Ala Lys
GGT GCT GGG ACC AAG CTG GTG CTG AAG CTT AGT GCG GAC GAT GCG AAA                  441

                                       130
Lys Asp Ala Ala Lys Lys Asp Asp Ala Lys Lys Asp Asp Ala Lys Lys
AAG GAT GCT GCG AAG AAG GAT GAC GCT AAG AAA GAC GAT GCT AAA AAG                  489

     Xho I   140                                                150
Asp Leu Glu Val Gln Leu Gln Gln Ser Asp Ala Glu Leu Val Lys Pro
GAC CTC GAG GTT CAG TTG CAG CAG TCT GAC GCT GAG TTG GTG AAA CCT                  537
```

FIGURE 16 Continued

```
                                  160
Gly Ala Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr
GGG GCT TCA GTG AAG ATT TCC TGC AAG GCT TCT GGC TAC ACC TTC ACT     585

    170                                           180              A
Asp His Ala Ile His Trp Val Lys Gln Asn Pro Glu Gln Gly Leu Glu
GAC CAT GCA ATT CAC TGG GTG AAA CAG AAC CCT GAA CAG GGC CTG GAA     633

        TAGVHCDR2   190
TGG ATT GGA TAT TTT TCT CC                                   200
Trp Ile Gly Tyr Phe Ser Pro Gly Asn Asp Asp Phe Lys Tyr Asn Glu
TGG ATT GGA TAT TTT TCT CCC GGA AAT GAT GAT TTT AAA TAC AAT GAG     681

                                  210
Arg Phe Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr
AGG TTC AAG GGG AAG GCC ACA CTG ACT GCA GAC AAA TCC TCC AGC ACT     729

            220                                       230
Ala Tyr Val Gln Leu Asn Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr
GCC TAC GTG CAG CTC AAC AGC CTG ACA TCC GAG GAT TCT GCA GTG TAT     777

                                  240
Phe Cys Thr Arg Ser Leu Asn Met Ala Tyr Trp Gly Gln Gly Thr Ser
TTC TGT ACA AGA TCC CTG AAT ATG GCC TAC TGG GGT CAA GGA ACC TCA     825

        250                                       260
A.  Val Thr Val Ser | Pro Glu Asp Pro Glu Asp Tyr Asp *** ***
    GTC ACC GTC TCC | CCT GAA GAC CCT GAA GAC TAT GAC TAA TAG        867

        250
B.  Val Thr Val Ser | Tyr *** ***
    GTC ACC GTC TCC | TAC TAA TAG                                   846

BamH I
GGATCCTTAG----------AOX1 TERMINATOR-3'  877/856
```

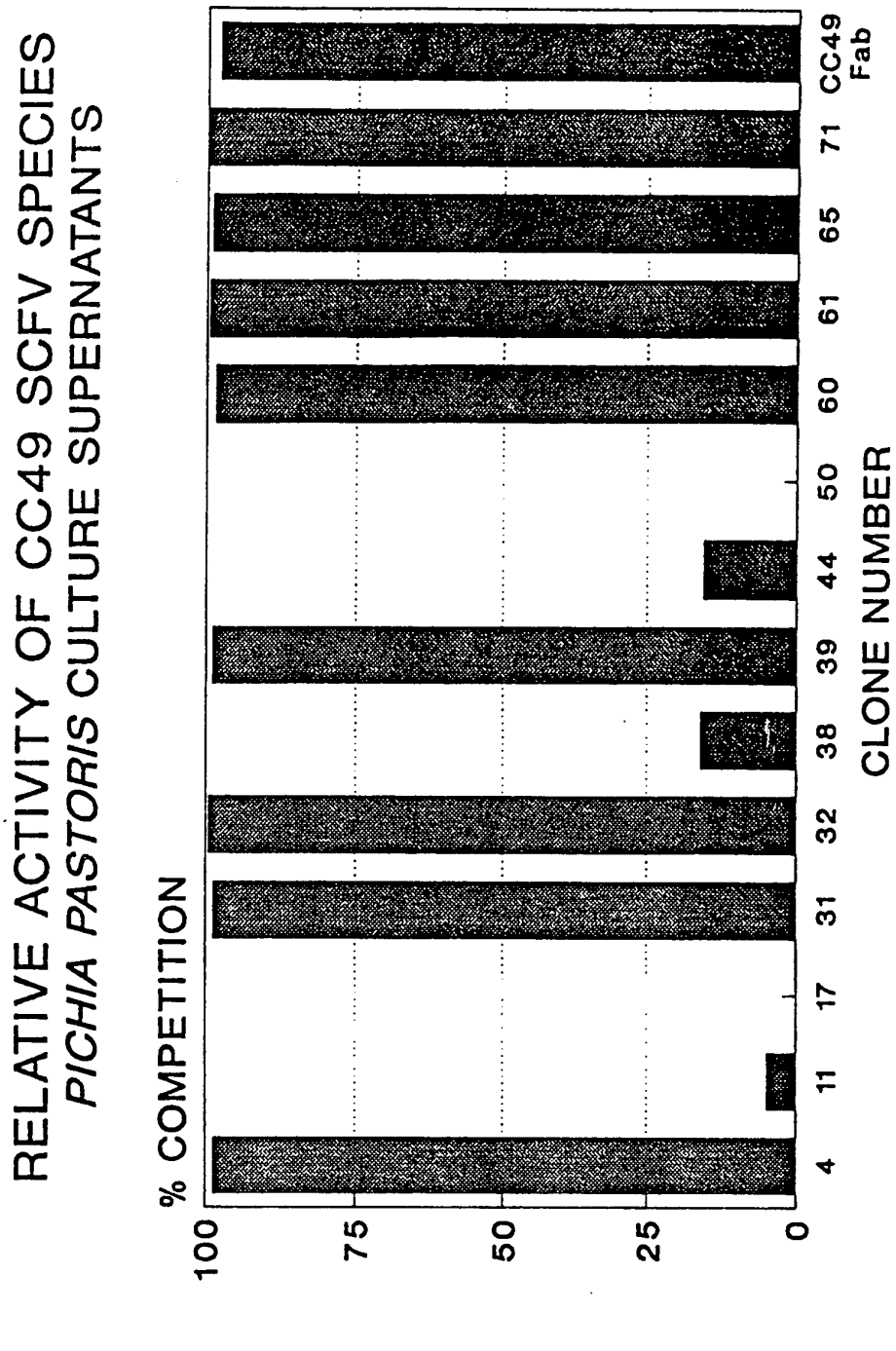

RELATIVE ACTIVITY OF CC49 SCFV SPECIES
PICHIA PASTORIS CULTURE SUPERNATANTS
% COMPETITION
100
75
50
25
0
4
11
17
31
32
38
39
44
50
60
61
65
71
CC49 Fab
CLONE NUMBER
CLONES 1-36: pPY31
CLONES 37-71: pPY32

FIGURE 17

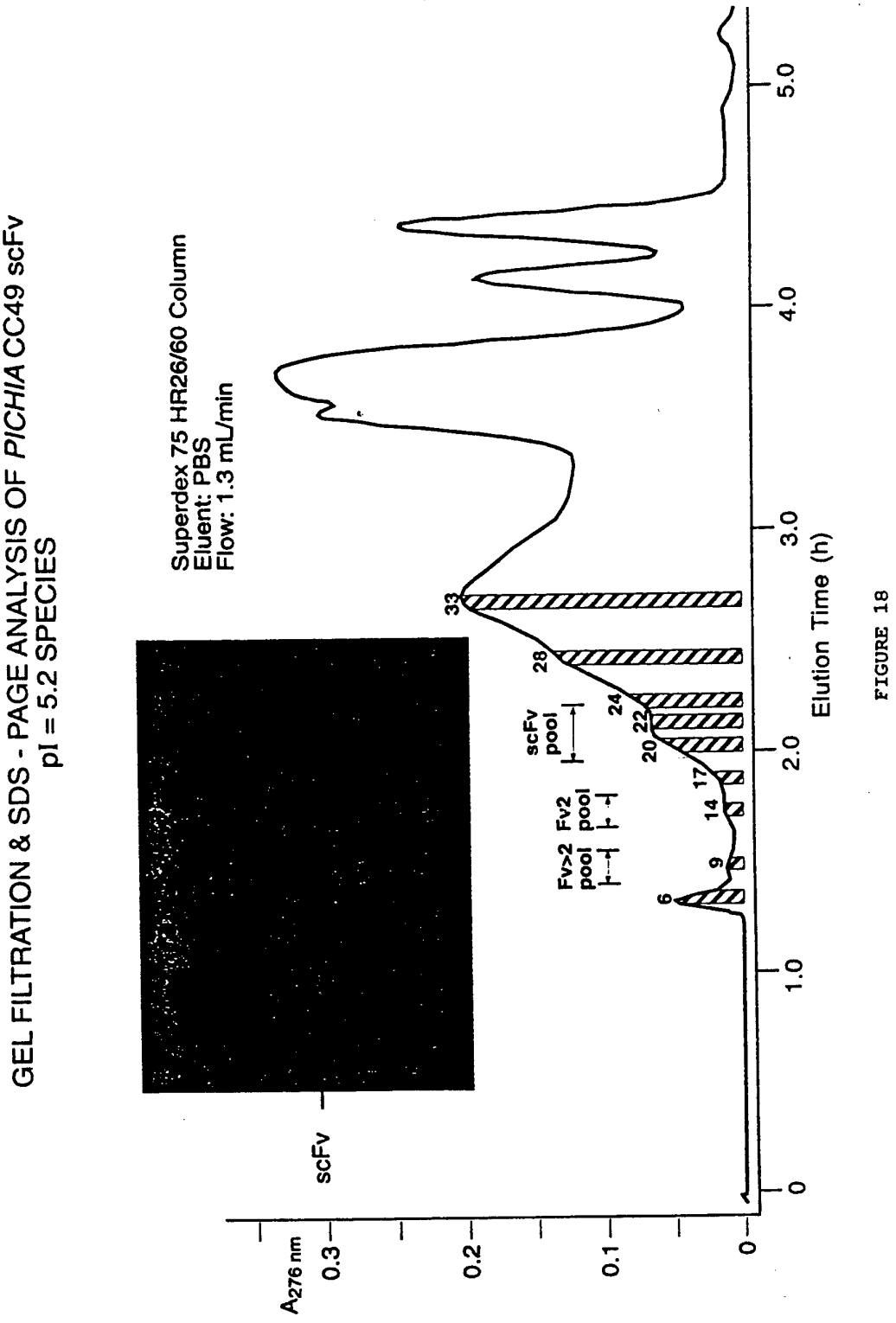

GEL FILTRATION & SDS - PAGE ANALYSIS OF PICHIA CC49 scFv
pI = 5.2 SPECIES
Superdex 75 HR26/60 Column
Eluent: PBS
Flow: 1.3 mL/min
scFv
A276 nm
0.3
0.2
0.1
0
0
1.0
2.0
3.0
4.0
5.0
Elution Time (h)
33
28
24
22
20
17
14
9
6
scFv pool
Fv2 pool
Fv>2 pool

FIGURE 18

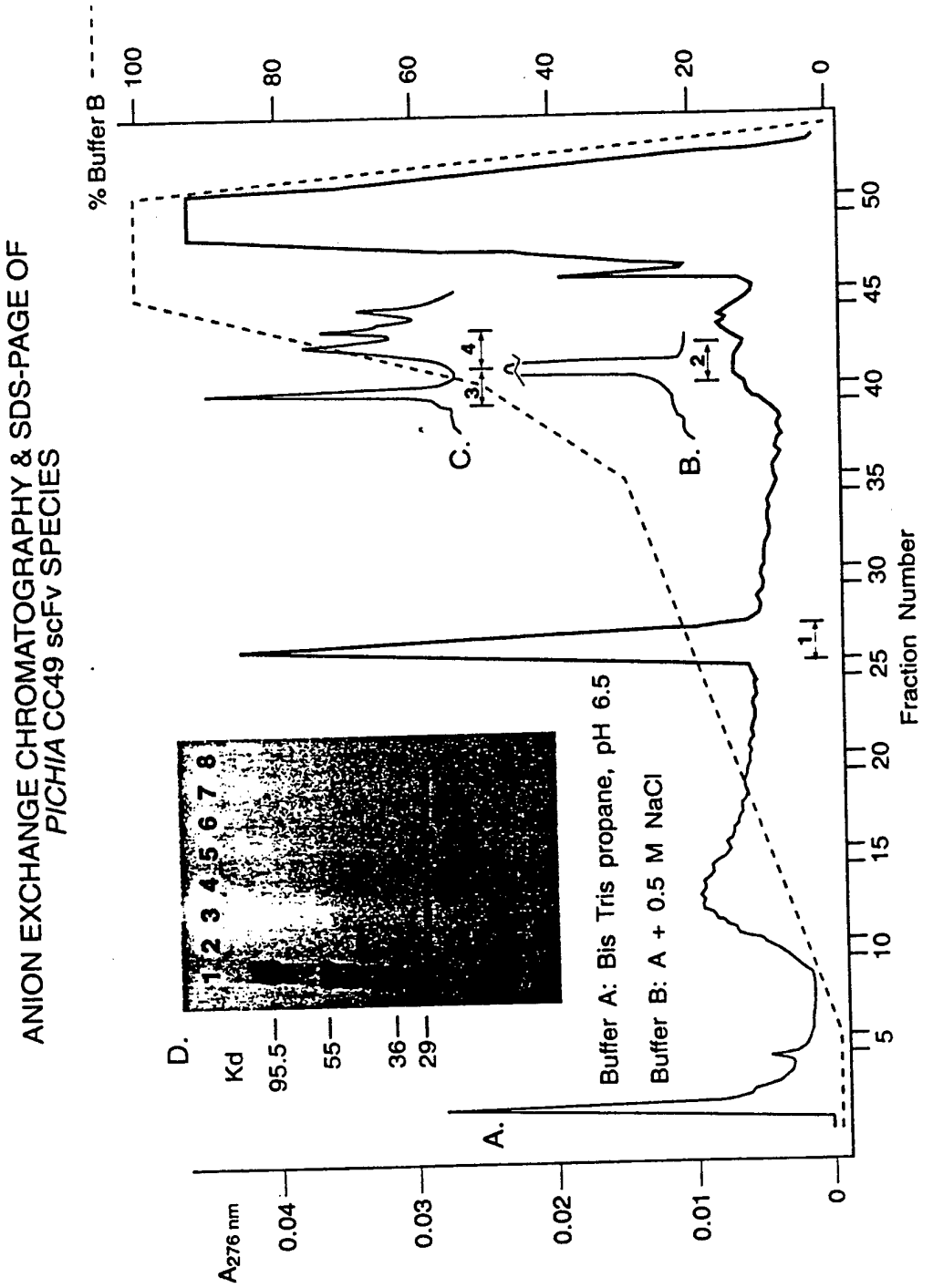

ANION EXCHANGE CHROMATOGRAPHY & SDS-PAGE OF
PICHIA CC49 scFv SPECIES
% Buffer B
100
80
60
40
20
0
A.
B.
C.
D.
Kd
95.5
55
36
29
1 2 3 4 5 6 7 8
Buffer A: Bis Tris propane, pH 6.5
Buffer B: A + 0.5 M NaCl
$A_{276\ nm}$
0.04
0.03
0.02
0.01
0
5 10 15 20 25 30 35 40 45 50
Fraction Number

FIGURE 19

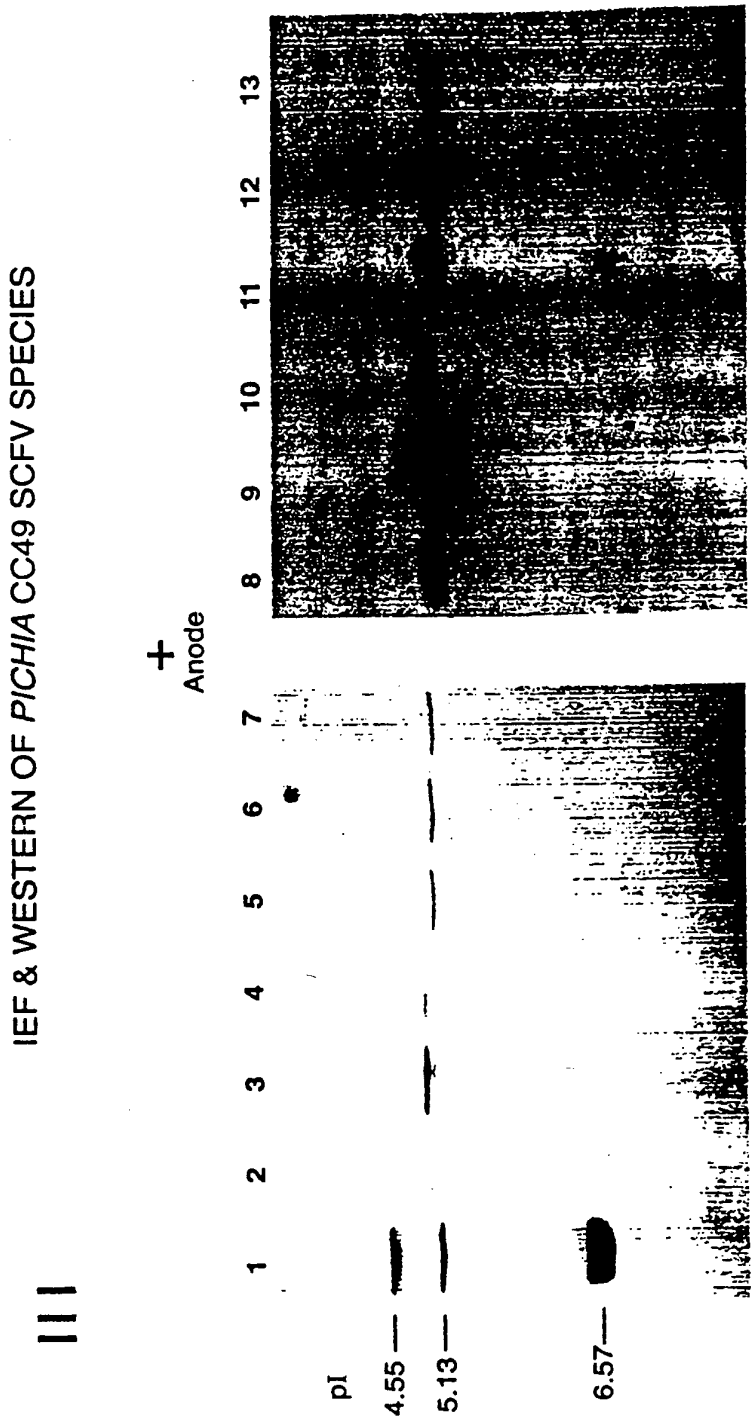

IEF & WESTERN OF PICHIA CC49 SCFV SPECIES
+
Anode
Cathode
-
1 2 3 4 5 6 7 8 9 10 11 12 13
pI
4.55
5.13
6.57

FIGURE 20

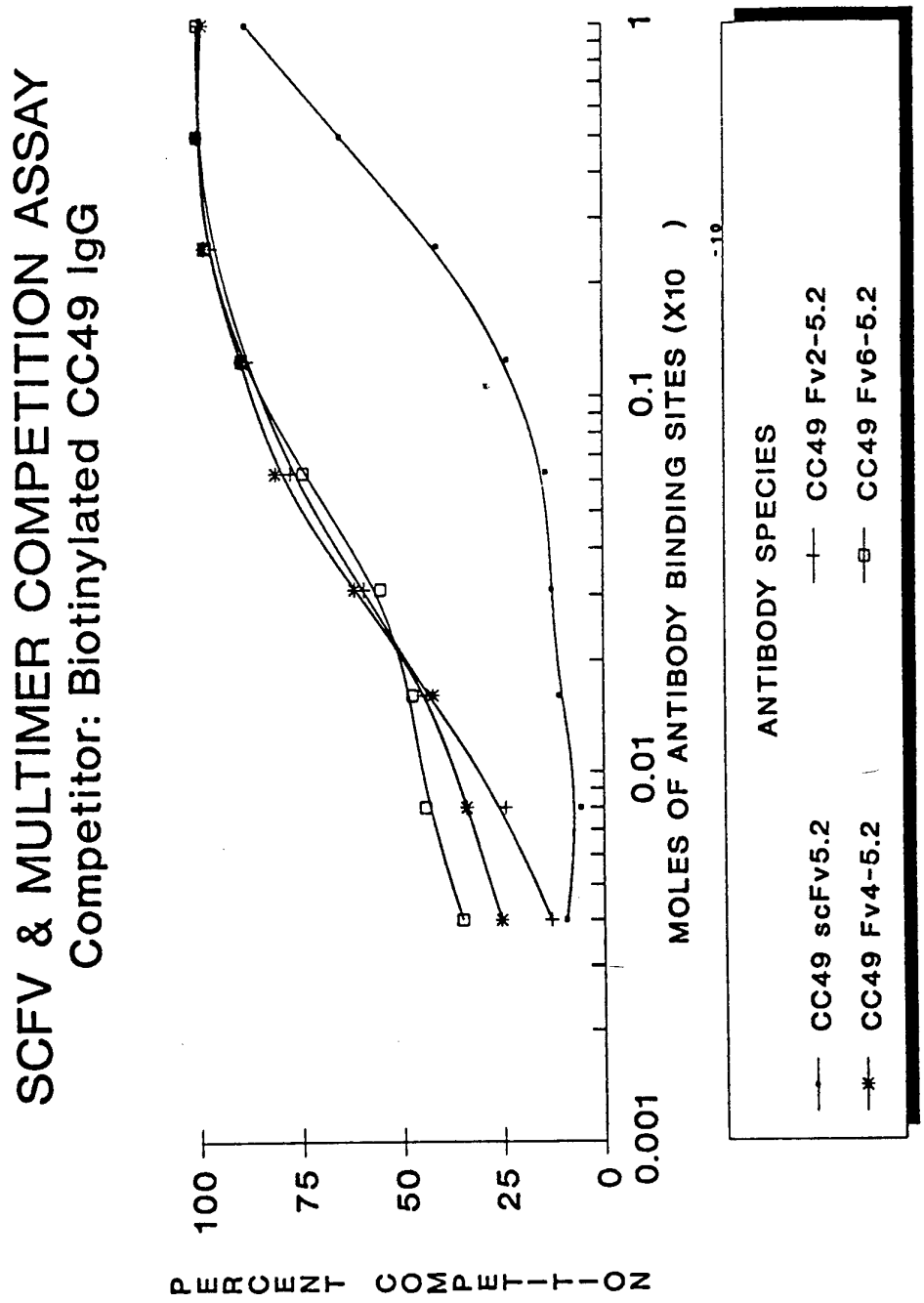

SCFV & MULTIMER COMPETITION ASSAY
Competitor: Biotinylated CC49 IgG
PERCENT COMPETITION
100
75
50
25
0
0.001
0.01
0.1
1
MOLES OF ANTIBODY BINDING SITES (X10)
-10
ANTIBODY SPECIES
CC49 scFv5.2
CC49 Fv2-5.2
CC49 Fv4-5.2
CC49 Fv6-5.2

FIGURE 21

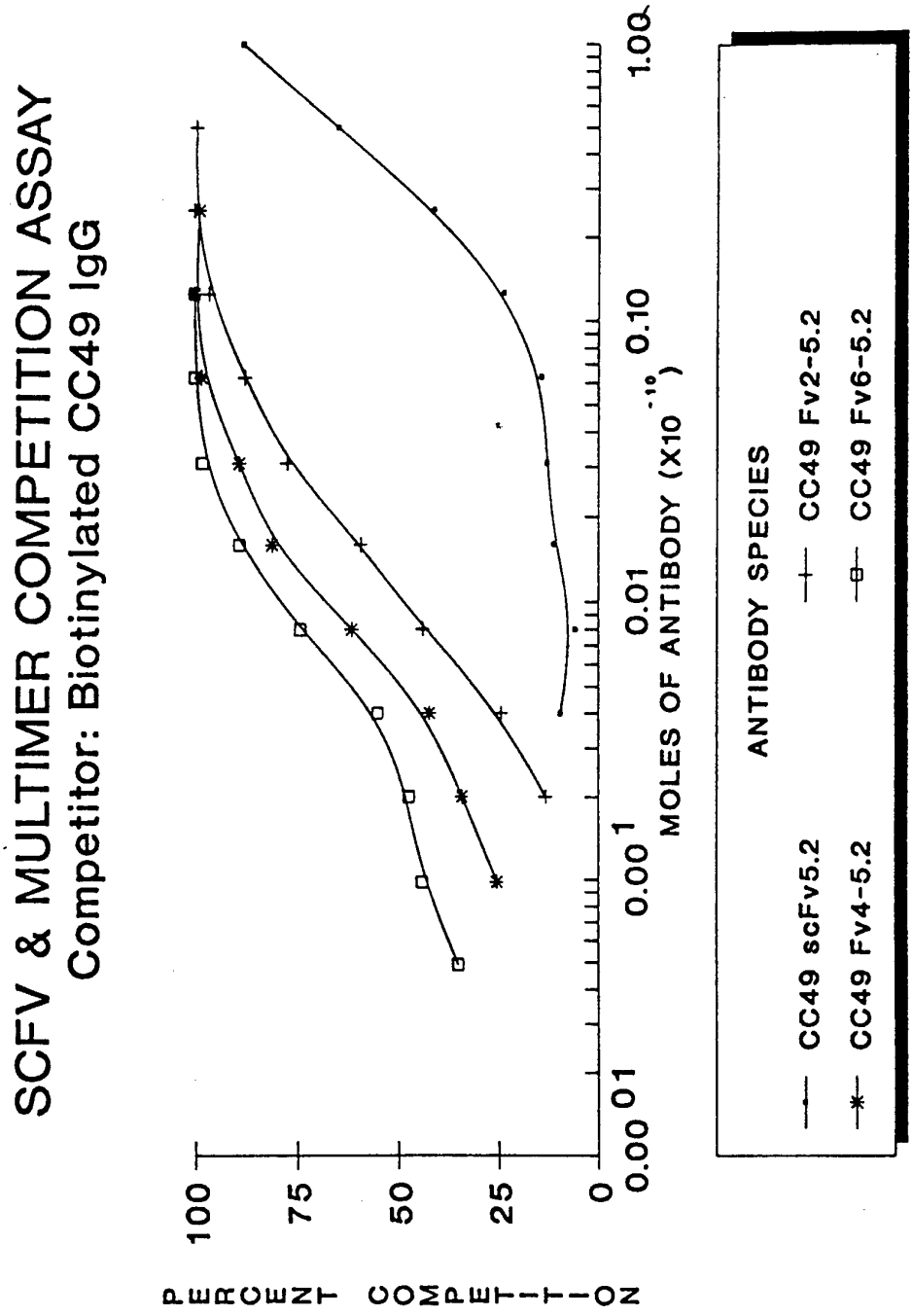

SCFV & MULTIMER COMPETITION ASSAY
Competitor: Biotinylated CC49 IgG
PERCENT COMPETITION
100
75
50
25
0
0.00 01
0.00 1
0.01
0.10
1.00
MOLES OF ANTIBODY (X10 -10)
ANTIBODY SPECIES
CC49 scFv5.2
CC49 Fv2-5.2
CC49 Fv4-5.2
CC49 Fv6-5.2

FIGURE 21A

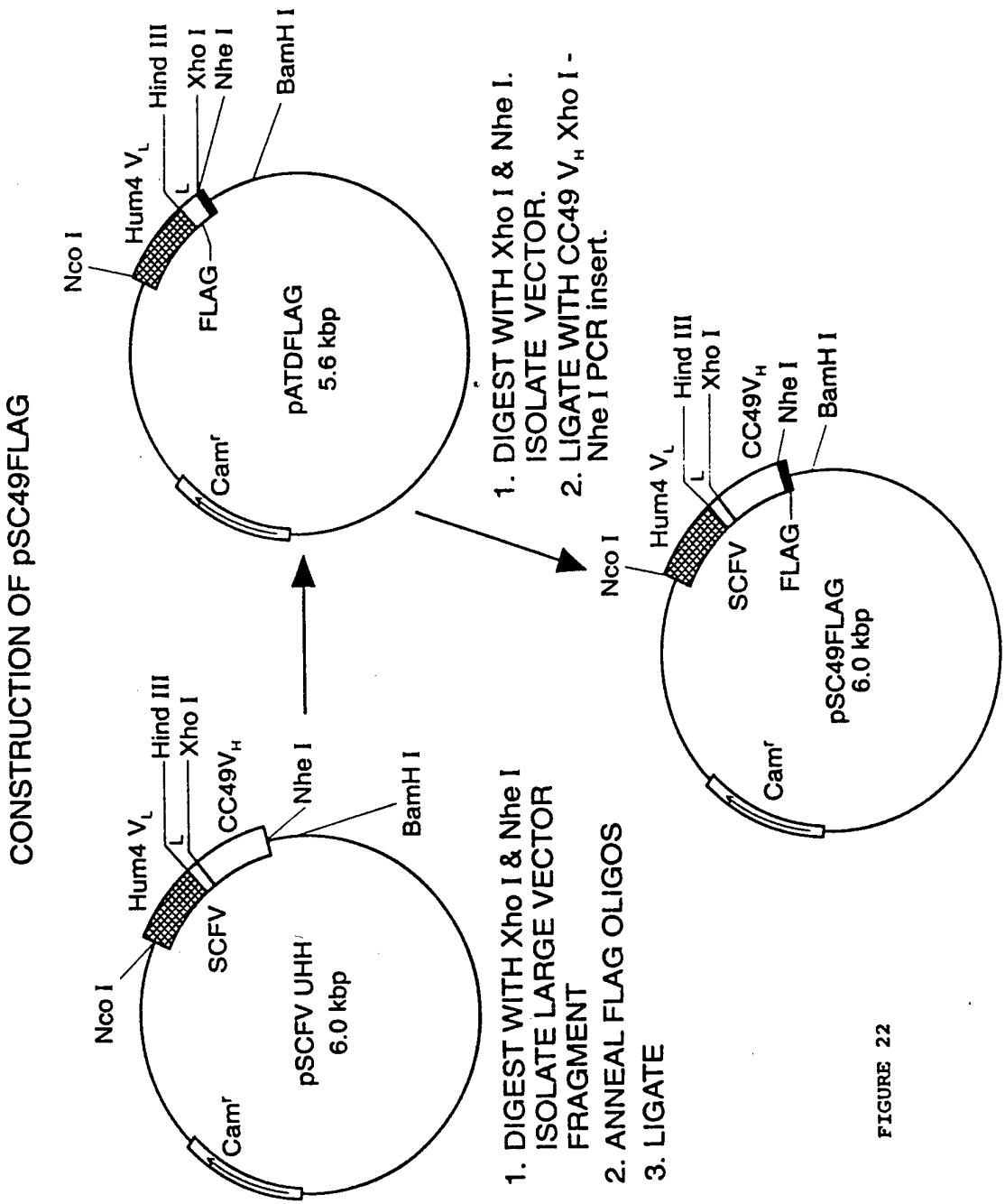

CONSTRUCTION OF pSC49FLAG
Nco I
Hum4 V_L
Hind III
Xho I
Nhe I
BamH I
FLAG
pATDFLAG
5.6 kbp
Cam^r
1. DIGEST WITH Xho I & Nhe I.
ISOLATE VECTOR.
2. LIGATE WITH CC49 V_H Xho I -
Nhe I PCR insert.
SCFV
CC49V_H
pSC49FLAG
6.0 kbp
pSCFV UHH
6.0 kbp
1. DIGEST WITH Xho I & Nhe I
ISOLATE LARGE VECTOR
FRAGMENT
2. ANNEAL FLAG OLIGOS
3. LIGATE

FIGURE 22

FIGURE 23

DNA AND AMINO ACID SEQUENCE IN pATDFLAG

```
                                    Cla I   EcoR I
5'-C TCA TGT TTG ACA GCT TAT CAT CGA TGA ATT CCA TCA CTT CCC TCC     46

GTT CAT TTG TCC CCG GTG GAA ACG AGG TCA TCA TTT CCT TCC GAA AAA     94

ACG GTT GCA TTT AAA TCT TAC ATA TAT AAT ACT TTC AAA GAC TAC ATT    142

TGT AAG ATT TGA TGT TTG AGT CGG CTG AAA GAT CGT ACG TAC CAA TTA    190

TTG TTT CGT GAT TGT TCA AGC CAT AAC ACT GTA GGG ATA GTG GAA AGA    238

GTG CTT CAT CTG GTT ACG ATC AAT CAA ATA TTC AAA CGG AGG GAG ACG    286

        -22
        Met Lys Tyr Leu Leu Pro Thr Ala Ala Ala Gly Leu Leu Leu
ATT TTG ATG AAA TAC CTA TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA    334

                        Nco I       +1
Leu Ala Ala Gln Pro Ala Met Ala Asp Ile Val Met Thr Gln Ser Pro
CTC GCT GCC CAA CCA GCC ATG GCC GAC ATC GTG ATG ACC CAG TCT CCA    382

     10                                          20
Asp Ser Leu Ala Val Ser Leu Gly Glu Arg Ala Thr Ile Asn Cys Lys
GAC TCC CTG GCT GTG TCT CTG GGC GAG AGG GCC ACC ATC AAC TGC AAG    430

                    30                                          40
Ser Ser Gln Ser Val Leu Tyr Ser Ser Asn Asn Lys Asn Tyr Leu Ala
TCC AGC CAG AGT GTT TTA TAC AGC TCC AAC AAT AAG AAC TAC TTA GCT    478

                                    50
Trp Tyr Gln Gln Lys Pro Gly Gln Pro Pro Lys Leu Leu Ile Tyr Trp
TGG TAC CAG CAG AAA CCA GGA CAG CCT CCT AAG CTG CTC ATT TAC TGG    526

              60                                        70
Ala Ser Thr Arg Glu Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly
GCA TCT ACC CGG GAA TCC GGG GTC CCT GAC CGA TTC AGT GGC AGC GGG    574

                                80
Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Ala Glu Asp
TCT GGG ACA GAT TTC ACT CTC ACC ATC AGC AGC CTG CAG GCT GAA GAT    622

     90                                         100
Ala Ala Val Tyr Tyr Cys Gln Gln Tyr Tyr Ser Tyr Pro Leu Thr Phe
GCG GCA GTT TAT TAC TGT CAG CAA TAT TAT AGT TAT CCT CTC ACT TTC    670

                    110          Hind III                   120
Gly Gly Gly Thr Lys Val Val Ile Lys Leu Ser Ala Asp Asp Ala Lys
GGC GGA GGG ACC AAG GTG GTG ATC AAG CTT AGT GCG GAC GAT GCG AAA    718
```

FIGURE 23 Continued

```
                                                    130
Lys Asp Ala Ala Lys Lys Asp Asp Ala Lys Lys Asp Asp Ala Lys Lys
AAG GAT GCT GCG AAG AAG GAT GAC GCT AAG AAA GAC GAT GCT AAA AAG    766

    Xho I                Nhe I
Asp Leu Glu              Ala Ser Asp Tyr Lys Asp Asp Asp Asp Lys ***
GAC CTC GAG ACAATGTCGCT AGC GAC TAC AAG GAC GAT GAT GAC AAA TAA    816

AAA CCT AGC GAT GAA TCC GTC AAA ACA TCA TCT TAC ATA AAG TCA CTT    864
            PENPTSEQ: ← G TTT TGT AGT AGA ATG TAT TTC

GGT GAT CAA GCT CAT ATC ATT GTC CGG CAA TGG TGT GGG CTT TTT TTG    912

TTT TCT ATC TTT AAA GAT CAT GTG AAG AAA AAC GGG AAA ATC GGT CTG    960

CGG GAA AGG ACC GGG TTT TTG TCG AAA TCA TAG GCG AAT GGG TTG GAT   1008

                 BamH I
TGT GAC AAA ATT CGG ATC C-3'                                     1027
```

FIGURE 24

DNA AND AMINO ACID SEQUENCE OF pSC49FLAG

```
                                    Cla I   EcoR I
5'-C TCA TGT TTG ACA GCT TAT CAT CGA TGA ATT CCA TCA CTT CCC TCC     46

GTT CAT TTG TCC CCG GTG GAA ACG AGG TCA TCA TTT CCT TCC GAA AAA     94

ACG GTT GCA TTT AAA TCT TAC ATA TAT AAT ACT TTC AAA GAC TAC ATT    142

TGT AAG ATT TGA TGT TTG AGT CGG CTG AAA GAT CGT ACG TAC CAA TTA    190

                          PENPR1: AAC ACT GTA GGG ATA GTG GAA →
TTG TTT CGT GAT TGT TCA AGC CAT AAC ACT GTA GGG ATA GTG GAA AGA    238

GTG CTT CAT CTG GTT ACG ATC AAT CAA ATA TTC AAA CGG AGG GAG ACG    286
                          PENPR2: ← TAT AAG TTT GCC TCC CTC TG

        -22
        Met Lys Tyr Leu Leu Pro Thr Ala Ala Ala Gly Leu Leu Leu
ATT TTG ATG AAA TAC CTA TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA    334

                      Nco I       +1
Leu Ala Ala Gln Pro Ala Met Ala Asp Ile Val Met Thr Gln Ser Pro
CTC GCT GCC CAA CCA GCC ATG GCC GAC ATC GTG ATG ACC CAG TCT CCA    382

     10                                                  20
Asp Ser Leu Ala Val Ser Leu Gly Glu Arg Ala Thr Ile Asn Cys Lys
GAC TCC CTG GCT GTG TCT CTG GGC GAG AGG GCC ACC ATC AAC TGC AAG    430

                         30                                      40
Pro Ser Gln Ser Val Leu Tyr Ser Ser Asn Asn Lys Asn Tyr Leu Ala
CCC AGC CAG AGT GTT TTA TAC AGC TCC AAC AAT AAG AAC TAC TTA GCT    478

TAGVLFR2:
TGG TAC CAG CAG AAA CCA GG →          50
Trp Tyr Gln Gln Lys Pro Gly Gln Pro Pro Lys Leu Leu Ile Tyr Trp
TGG TAC CAG CAG AAA CCA GGA CAG CCT CCT AAG CTG CTC ATT TAC TGG    526

               60                                          70
Ala Ser Thr Arg Glu Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly
GCA TCT ACC CGG GAA TCC GGG GTC CCT GAC CGA TTC AGT GGC AGC GGG    574

                                  80
Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Ala Glu Asp
TCT GGG ACA GAT TTC ACT CTC ACC ATC AGC AGC CTG CAG GCT GAA GAT    622

     90                                             100
Ala Ala Val Tyr Tyr Cys Gln Gln Tyr Tyr Ser Tyr Pro Leu Thr Phe
GCG GCA GTT TAT TAC TGT CAG CAA TAT TAT AGT TAT CCT CTC ACT TTC    670

                        110          Hind III                   120
Gly Gly Gly Thr Lys Val Val Ile Lys Leu Ser Ala Asp Asp Ala Lys
GGC GGA GGG ACC AAG GTG GTG ATC AAG CTT AGT GCG GAC GAT GCG AAA    718
```

FIGURE 24 Continued

```
                UHVHSEQ:
    GAT GCT GCG AAG AAG GAT GAC G → 130
Lys Asp Ala Ala Lys Lys Asp Asp Ala Lys Lys Asp Asp Ala Lys Lys
AAG GAT GCT GCG AAG AAG GAT GAC GCT AAG AAA GAC GAT GCT AAA AAG    766

    Xho I   140                                         150
Asp Leu Glu Val Gln Leu Gln Gln Ser Asp Ala Glu Leu Val Lys Pro
GAC CTC GAG GTT CAG TTG CAG CAG TCT GAC GCT GAG TTG GTG AAA CCT    814

                            160
Gly Ala Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr
GGG GCT TCA GTG AAG ATT TCC TGC AAG GCT TCT GGC TAC ACC TTC ACT    862

    170                                         180    TAGVHCDR2: A
Asp His Ala Ile His Trp Val Lys Gln Asn Pro Glu Gln Gly Leu Glu
GAC CAT GCA ATT CAC TGG GTG AAA CAG AAC CCT GAA CAG GGC CTG GAA    910

TGG ATT GGA TAT TTT TCT CC →                                   200
Trp Ile Gly Tyr Phe Ser Pro Gly Asn Asp Asp Phe Lys Tyr Asn Glu
TGG ATT GGA TAT TTT TCT CCC GGA AAT GAT GAT TTT AAA TAC AAT GAG    958

                            210
Arg Phe Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr
AGG TTC AAG GGC AAG GCC ACA CTG ACT GCA GAC AAA TCC TCC AGC ACT   1006

            220                                         230
Ala Tyr Val Gln Leu Asn Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr
GCC TAC GTG CAG CTC AAC AGC CTG ACA TCT GAG GAT TCT GCA GTG TAT   1054

                        TAGVHFR4: AC TGG GGT CAA GGA ACC TCA
Phe Cys Thr Arg Ser Leu Asn Met Ala Tyr Trp Gly Gln Gly Thr Ser
TTC TGT ACA AGA TCC CTG AAT ATG GCC TAC TGG GGT CAA GGA ACC TCA   1102

G → 250             Nhe I                   260
Val Thr Val Ser Ser Ala Ser Asp Tyr Lys Asp Asp Asp Asp Lys ***
GTC ACC GTC TCC TCA GCT AGC GAC TAC AAG GAC GAT GAT GAC AAA TAA   1150

AAA CCT AGC GAT GAA TCC GTC AAA ACA TCA TCT TAC ATA AAG TCA CTT   1198

GGT GAT CAA GCT CAT ATC ATT GTC CGG CAA TGG TGT GGG CTT TTT TTG   1246

TTT TCT ATC TTT AAA GAT CAT GTG AAG AAA AAC GGG AAA ATC GGT CTG   1294

CGG GAA AGG ACC GGG TTT TTG TCG AAA TCA TAG GCG AAT GGG TTG GAT   1342

                BamH I
TGT GAC AAA ATT CGG ATC C-3'                                    1361
```

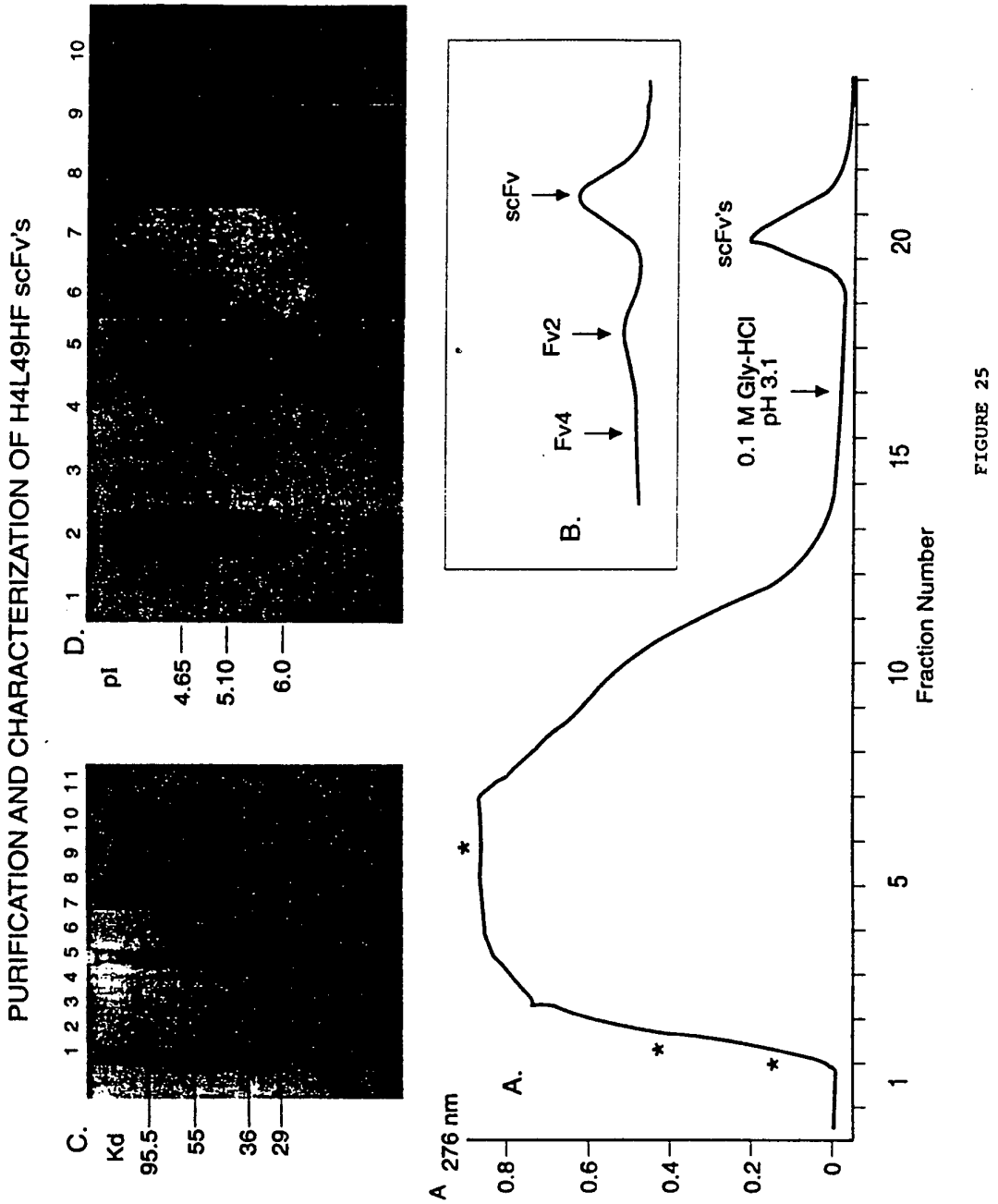

PURIFICATION AND CHARACTERIZATION OF H4L49HF scFv's
A.
A 276 nm
0.8
0.6
0.4
0.2
0
1
5
10
15
20
Fraction Number
0.1 M Gly-HCl pH 3.1
scFv's
B.
Fv4
Fv2
scFv
C.
Kd
95.5
55
36
29
1 2 3 4 5 6 7 8 9 10 11
D.
pI
4.65
5.10
6.0
1 2 3 4 5 6 7 8 9 10

FIGURE 25

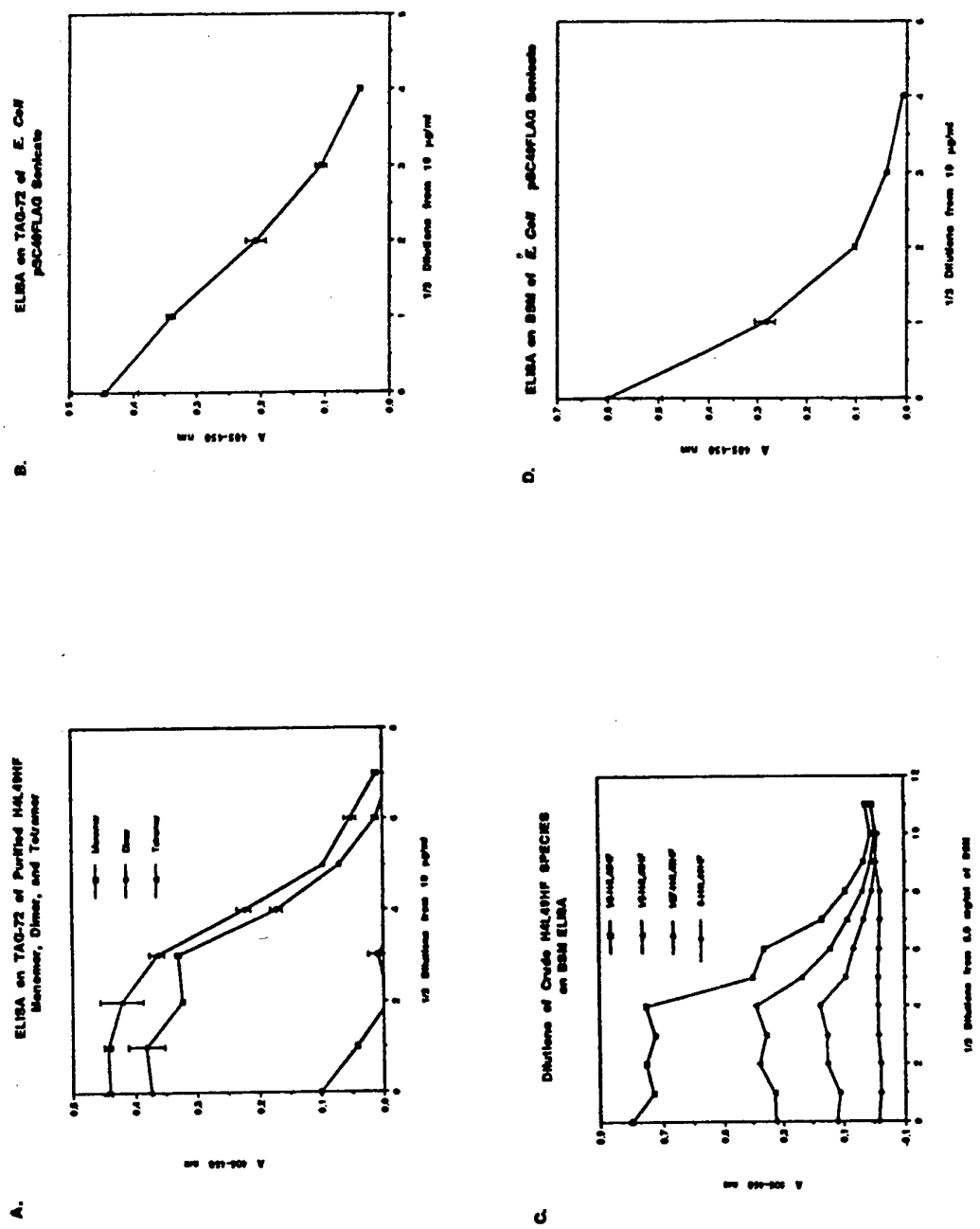


FIGURE 26

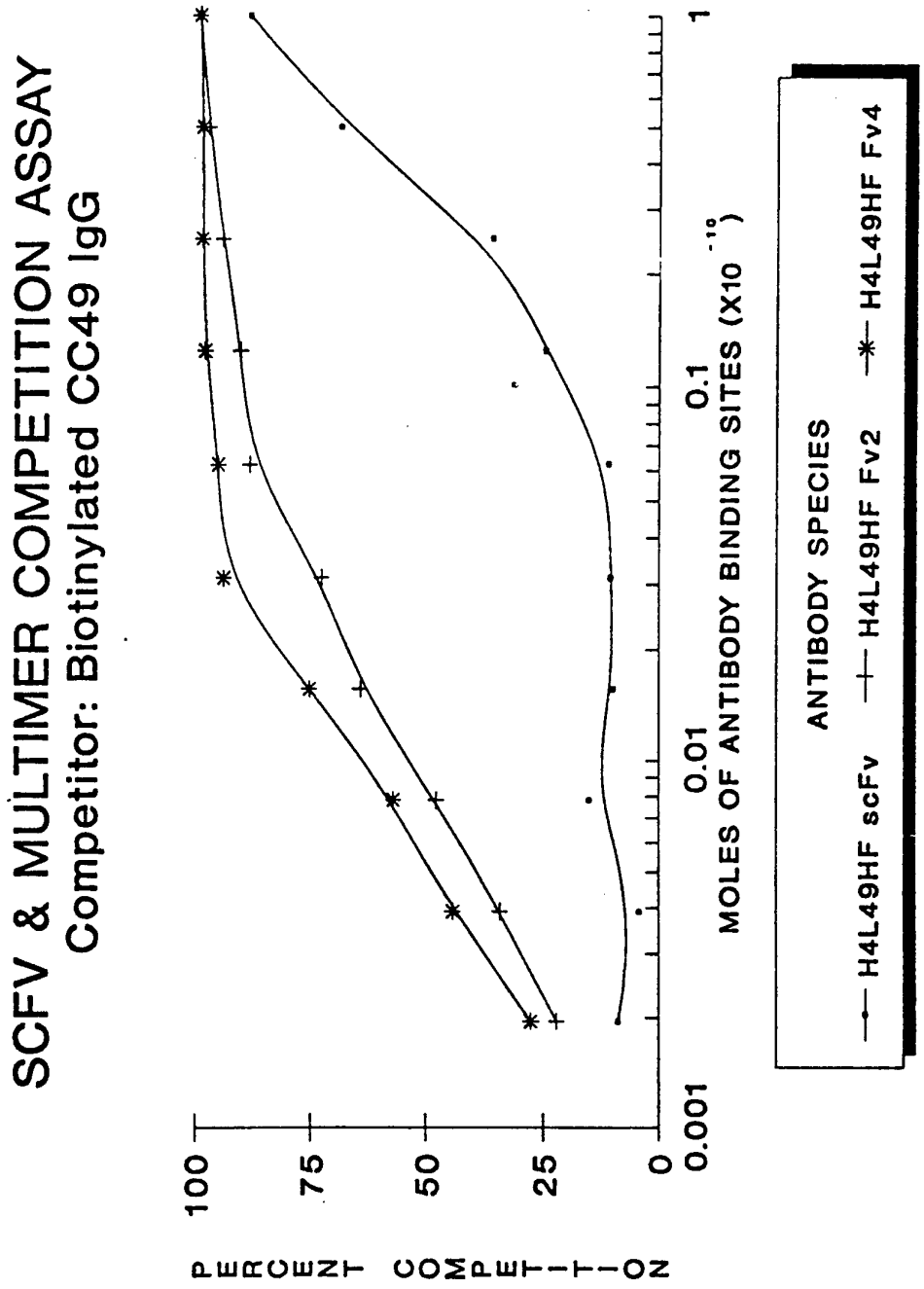

SCFV & MULTIMER COMPETITION ASSAY
Competitor: Biotinylated CC49 IgG
PERCENT COMPETITION
100
75
50
25
0
0.001
0.01
0.1
1
MOLES OF ANTIBODY BINDING SITES (X10 -10)
ANTIBODY SPECIES
H4L49HF scFv
H4L49HF Fv2
H4L49HF Fv4

FIGURE 27

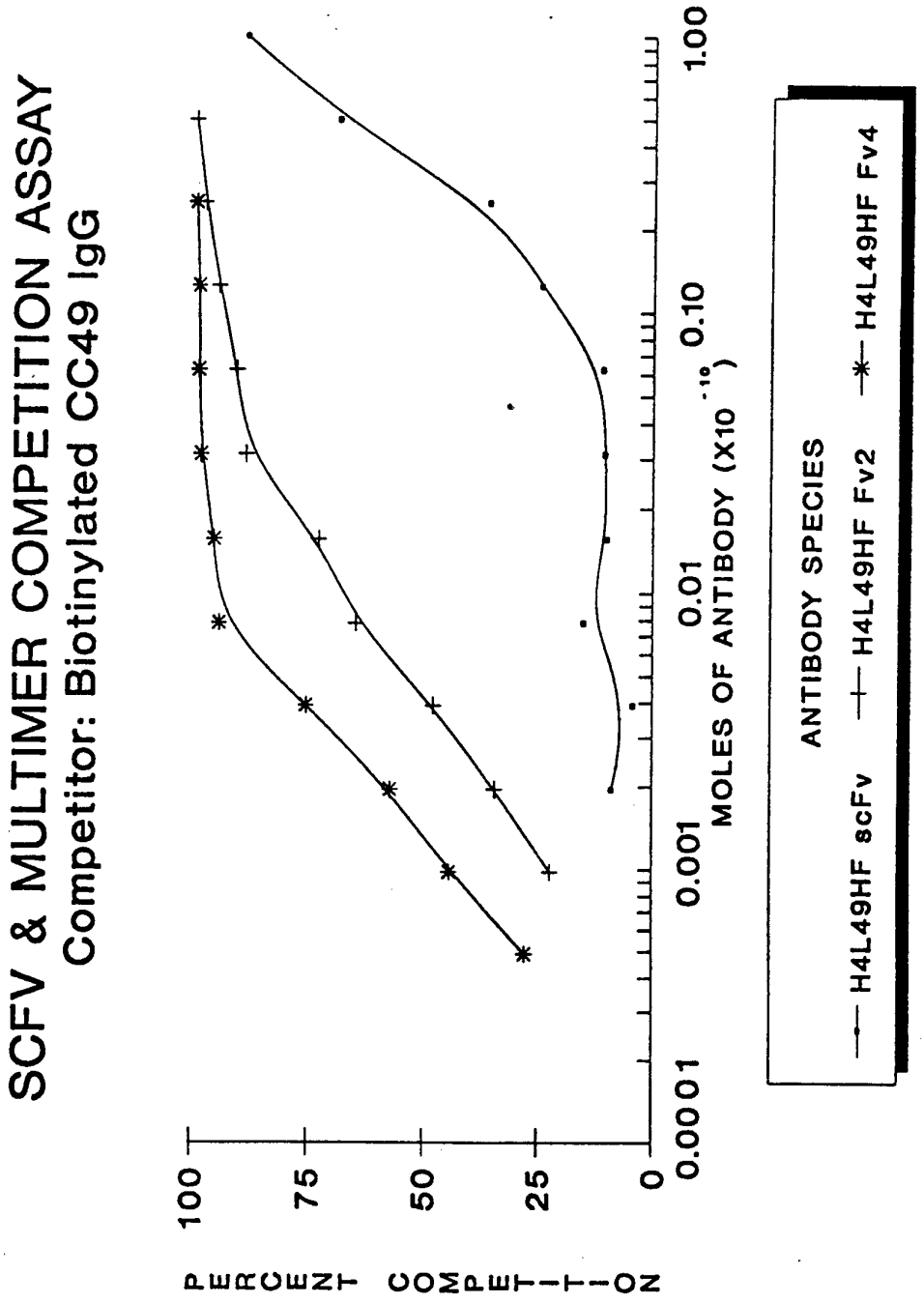

SCFV & MULTIMER COMPETITION ASSAY
Competitor: Biotinylated CC49 IgG
PERCENT COMPETITION
100
75
50
25
0
0.0001
0.001
0.01
0.10
1.00
MOLES OF ANTIBODY (X10 -10)
ANTIBODY SPECIES
H4L49HF scFv
H4L49HF Fv2
H4L49HF Fv4

FIGURE 28