Preface

Immunotoxins represent a new class of human therapeutics that have widespread applications and a potential that has not yet been fully recognized since they were first conceived of by Paul Ehrlich in 1906. The majority of advances in the development and implementation of immunotoxins has occurred over the last 20 years. The reasons for this use of immunotoxins in basic science and clinical research are the powerful concurrent advances in genetic engineering and receptor physiology. Recombinant technology has allowed investigators to produce sufficient quantities of a homogeneous compound that allows clinical trials to be performed. The identification of specific receptors on malignant cell types has enabled scientists to generate immunotoxins that have had positive results in clinical trials. As more cellular targets are identified in coming years, additional trials will be conducted in different disease states affecting still larger patient populations. Modulation of the immune system to decrease the humoral response to immunotoxins may improve their overall efficacy. As increasingly more effective compounds are generated, it will be necessary to decrease the local and systemic toxicity associated with these agents, and methods for doing so are presently being developed.

The work presented in *Immunotoxin Methods and Protocols* focuses on three specific areas of immunotoxin investigation that are being conducted by experts throughout the world. The first section describes the construction and development of a variety of immunotoxins. The conjugates that have been created by these investigators contain entire monoclonal antibodies, single-chain antibody fragments, and cytokines as their carrier ligands. The toxic portion of these agents can include such conventional chemotherapeutics as doxorubicin or such ribosome-inactivating proteins as ricin A chain or saporin. Additional considerations that must be addressed when developing immunotoxins, and are included in this section, are the use of such potentiating agents as the carboxylic ionophore monensin for plant-derived toxins, receptor expression modulation for immunotoxin targeting using such modalities as irradiation, and the quantification of the number of immunotoxin molecules that are necessary for a complete therapeutic response. The section ends with an evaluation of the very important potential side effect, vascular leak syndrome, which can occur with any of these molecules.

Because of the unparalleled toxicity of these compounds, it is no surprise that the area of their greatest application has been in the field of oncology, which supports the focus of the second section of this work on these investigations. The central nervous system provides an ideal environment for the administration of immunotoxins because of the relative impermeability of the blood–brain barrier. Circumventing the blood–brain barrier by directly infusing these compounds into primary malignant brain tumors, makes it possible to achieve high local concentrations; these have resulted in complete therapeutic responses in some cases. The central nervous system is also of considerable clinical interest for bacterial-based immunotoxins because of the encouraging therapeutic responses that are described in this section.

The last section of *Immunotoxin Methods and Protocols* investigates alternative applications of immunotoxins for generating selective lesions within the brain by targeting neural antigens and their receptors. By creating lesions in specific areas, investigators hope to better understand and localize such critical functions as memory and learning. Since the onset of the acquired immunodeficiency syndrome epidemic, there has been little progress toward finding a cure, although the overall survival for patients has been extended. Targeting the virus with immunotoxins represents a reasonable concept, provided that a unique receptor can be identified on the human immunodeficiency virus. In reflecting on the present state of the wide variety of immunotoxin research compiled in this text, it is hoped that the present contributors and other investigators who read *Immunotoxin Methods and Protocols* will be stimulated to consider and to create new applications for this exciting class of compounds.

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CD7-Specific Single Chain Fv Immunotoxins

Design and Expression

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1. Introduction

This chapter summarizes our experience with the design and expression of single-chain Fv (sFv) immunotoxins (sFv-IT) specific for CD7, a differentiation antigen found on human peripheral T and NK cells, and on subsets of committed myeloid and lymphoid progenitors in the bone marrow ***(1–4)***. In vitro studies have shown that CD7 participates in the activation and adhesion of mature T and NK cells, but the exact role it plays in the development and function of these cells in vivo remains obscure ***(5,6)***. A recent analysis of CD7-deficient mice revealed an association between resistance to endotoxin-induced shock and selective deficiencies of liver $NK1.1^+/CD3^+$ T-cell numbers and serum interferon-γ (IFN-γ) levels ***(7)***. These data suggest that CD7 participates in the development or migration of murine $NK1.1^+$ T-cells in the liver, and that reduced numbers of these cells results in decreased cytokine production elicited by bacterial lipopolysaccharides.

Regardless of its function, CD7 expression in disease has important clinical ramifications. It is one of the most useful markers for T-cell acute lymphoblastic leukemia (T-ALL), since most T-ALL cells are $CD7^+$ in >95% of cases ***(8–10)***. CD7 is expressed at high densities (approx 60,000 mol/cell) on T-cells and is rapidly internalized, even when bound by monovalent antibody fragments ***(11)***. It therefore makes an ideal target antigen for immunotoxin-mediated therapy of T-cell disease. This was evidenced in a recent phase I clinical trial of a CD7-specific immunotoxin called DA7 ***(12)***. DA7 was synthesized at the University of Minnesota by biochemically linking deglycosylated ricin toxin A-chain (RTA) to a CD7-specific monoclonal antibody ***(13)***. DA7 achieved

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objective clinical responses at its maximal tolerated dose despite being limited by instability and vascular toxicity.

Based on this trial, we reasoned that a recombinant sFv-IT derived from DA7 might have a larger "therapeutic window" due to enhanced stability and tissue penetration. Immunoglobulin sFv fragments consist of heavy- and light-chain variable regions (V_H and V_L) linked by small, flexible peptides; as such, sFv fragments retain the specificity of the antibodies from which they are derived ***(14,15)***. The major advantages of recombinant sFv-IT over biochemically synthesized immunotoxins are that the former are homogenous, genetically modifiable, have enhanced tissue penetration, and the site and manner in which the sFv and toxin moieties are joined can be exactly controlled.

To construct the *sFv* genes, we first used a commercial kit (RPAS; Amersham Pharmacia Biotech) to clone the V_H and kappa light chain variable (V_κ) region genes expressed by several CD7-specific hybridomas by reverse-transcriptase polymerase chain reaction (RT-PCR; ***11***). One of these was 3A1e, the hybridoma used to produce the antibody moiety in DA7. A carboxy-terminal cysteine residue was genetically added to each sFv fragment. This provided an exact site for the sFv to be disulfide-linked to RTA to yield an sFv-IT.

For reasons that remain unclear, we were unable to obtain a functional 3A1e sFv fragment following denaturation and refolding of the protein when it was expressed as an insoluble inclusion body. Because we successfully refolded the other sFv fragments, we suspected that the 3A1e sFv was "disulfide restricted" (i.e., a protein prone to inappropriate disulfide bond formation during refolding in a redox buffer; ***16,17***). To circumvent this and other problems inherent to protein refolding, we switched to expressing all sFv fragments solubly in bacteria and yeast ***(11,18,19)***. We found that the yields of soluble and functional sFv fragments were approx 100-fold greater in yeast than in bacteria; therefore, we routinely used the yeast system for sFv expression ***(18,19)***. The methods presented below include sFv cloning and assembly, sFv expression in yeast, and construction and purification of the sFv–RTA conjugates.

2. Materials

2.1. sFv Cloning

1. 3A1a, 3A1d, 3A1e, and 3A1f are murine hybridomas that secrete cross-blocking antibodies specific for human CD7 ***(20,21)***. 3A1d, e, and f hybridomas were generously provided by Dr. Barton Haynes' laboratory (Duke University). 3A1a was purchased from the American Type Culture Collection (ATCC; Rockville, MD). The *E. coli* bacterial strain INVαF' was purchased from Invitrogen (Carlsbad, CA).
2. Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum, 50 U of penicillin G, and 50 μg/mL streptomycin sulfate (all were purchased from Celox, Hopkins, MN).

3. FastTrack mRNA isolation kit (Invitrogen); diethyl pyrocarbonate (DEPC; Sigma; St. Louis, MO).
4. Random hexamer primers for first-strand cDNA synthesis (Boehringer Mannheim; Indianapolis, IN); dithiothreitol (DTT; Pierce; Rockford, IL); deoxynucleotide triphosphates (dNTPs; Boehringer Mannheim); 5X first-strand cDNA buffer (250 m*M* Tris-HCl (pH 8.3), 15 m*M* $MgCl_2$, 375 m*M* KCl; BRL Life Technologies; Gaithersburg, MD); Moloney murine leukemia virus reverse transcriptase (BRL Life Technologies) and recombinant RNasin (Promega Corp.; Madison, WI)
5. RPAS mouse scFv module (Amersham Pharmacia Biotech) kit contains oligonucleotide primers specific for the conserved 5' and 3' ends of immunoglobulin *V* genes, as well as primers that link a pair of amplified V_H and V_κ genes together to construct an sFv. The 5' and 3' primers anneal to conserved nucleotides that encode the amino-terminal portion of the first framework (FR) region and the *J*-gene–encoded carboxy-terminal portion of the fourth FR region, respectively. The sequences of these oligonucleotides are proprietary but can be gleaned from work published by Winter and colleagues ***(22)***. The two complementary linker primers that join a V_H and V_κ gene pair encode the flexible $(Gly_4\text{-}Ser)_3$ peptide commonly used in sFv constructs ***(16)***. The $(Gly_4\text{-}Ser)_3$ codons are flanked by nucleotides designed to anneal to the 3' ends of J_H genes and the 5' ends of V_κ genes. Correctly assembled V_H–linker–V_κ constructs are amplified by splice overlap extension using 5' V_H and 3' V_κ primers modified to include *Sfi*I and *Not*I restriction endonuclease sites, respectively.
6. All oligonucleotide primers were synthesized by Oligos Etc. (Bethel, ME) and were ordered at their 50 n*M* scale as "primer grade" (i.e., the oligonucleotides underwent organic extraction, precipitation, and two vacuum desiccation steps to remove small contaminants). The lyophilized oligonucleotides were dissolved to a final concentration of 500 pmol/μL in TE (10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA), aliquoted (10 μL), and stored at –85˚C. Single aliquots of each primer were stored at 4˚C and the required amount was diluted to 5 pmol/μL in TE just prior to use.
7. Cloned *Pfu* polymerase and 10X *Pfu* buffer (100 m*M* KCl, 100 m*M* $[NH_4]_2SO_4$, 200 m*M* Tris-HCl [pH 8.75], 20 m*M* $MgSO_4$, 1% Triton X-100, 1 mg/mL bovine serum albumin) (Stratagene; La Jolla, CA); *Taq* polymerase in buffer B and 10X reaction buffer without $MgCl_2$ (500 m*M* KCl, 100 m*M* Tris-HCl [pH 9.0] and 1% Triton X-100; Promega; Madison, WI). The enzymes were stored at –20˚C in a Stratacooler II benchtop cooler (Stratagene).
8. Agarose (BRL Life Technologies); 6X loading dye (0.25% bromophenol blue, 30% glycerol in water); 0.5X TBE running buffer (for 5X TBE, add 54 g Tris-base, 27.5 g boric acid, 20 mL 0.5 *M* EDTA [pH 8.0], and distilled water to bring to 1 L final volume); ethidium bromide (10 mg/mL in distilled water); 100 bp ladder (BRL Life Technologies).
9. Qiagen II gel extraction kit (Qiagen; Valencia, CA); PRISM sequencing kit (PE Applied Biosystems, Foster City, CA).
10. Vectors: pCRII and pPIC9K (Invitrogen).

2.2. sFv Expression

1. GS115 *his4*-deficient *Pichia pastoris* strain (Invitrogen).
2. Electroporator and 0.2 cm electroporation cuvets (Eppendorf; Madison, WI).
3. Recipes for the following solutions can be downloaded as a PDF file from Invitrogen (http://www.invitrogen.com/manuals.html): YPD (1% yeast extract, 2% peptone, 2% dextrose); BMMY (1% yeast extract, 2% peptone, 100 m*M* potassium phosphate [pH 6.0], 1.34% yeast nitrogen base, 4×10^{-5}% biotin, 0.5% methanol); MM and MD agar plates (1.5% agar, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, and either 0.5% methanol or 2% dextrose, respectively).
4. BA85 0.45 μm nitrocellulose filters, 82 mm diameter (Schleicher & Schuell; Keene, NH); Whatman 3MM chromatography paper cut in 82 mm diameter circles (Whatman; Clifton, NJ); Penta-His tag mouse monoclonal antibody (Qiagen); goat antimouse immunoglobulin–horseradish peroxidase (HRP) conjugate (Promega); ECL kit (Amersham Pharmacia Biotech); TBS (20 m*M* Tris-HCl [pH 7.5], 137 m*M* NaCl) TBST (TBS with 0.1% Tween-20), solution A (TBST with 10% w/v nonfat dry milk).
5. Laemmli minigel apparatus and power supply for SDS-PAGE; GelCode blue stain reagent (Pierce).
6. Centricon-3 and -10 concentrators (Millipore; Bedford, MA).

2.3. sFv-RTA Conjugate Formation and Purification

1. Deglycosylated RTA (Inland Laboratories; Austin, TX); SPDP, DTT, 5-5'-dithiobis-(2-nitrobenzoic acid) (DTNB/Ellman's reagent) (Pierce).
2. 5-mL Chelating–high trap, PD-10, Superdex 75 HR10/30 columns (Amersham Pharmacia Biotech).

3. Methods

3.1. V Gene Amplification and sFv Assembly

1. Culture the CD7-specific hybridomas in tissue culture medium at 37˚C in a humidified atmosphere containing 5% CO_2. The cells should be at a density of $0.5–1 \times 10^6$ cells/mL and >90% viable, as determined by trypan blue exclusion when harvested for mRNA isolation. Always wear gloves (and change frequently) in this and subsequent steps to prevent contamination of nucleic acid preparations.
2. Centrifuge 100 mL of cells for 5 min at 1000*g* and decant the supernatant. To purify mRNA from a pellet of $0.5–1 \times 10^8$ hybridoma cells, use the mRNA isolation kit exactly according to the manufacturer's protocol. Resuspend the purified mRNA in DEPC-treated distilled water (*see* **Note 1**) and store in aliquots at –85˚C. To minimize sample cross-contamination, use positive displacement pipets or pipet tips with aerosol blocks for all subsequent manipulations. Use each tip once.
3. Use RT-PCR to amplify the expressed *V* genes by initially synthesizing first-strand cDNA as follows. In a sterile 1.5-mL Eppendorf tube, combine 5 μg mRNA with 0.5 μg random hexamer primers, 10 m*M* DTT, 0.5 m*M* of dNTP, 20 μL of

5X first-strand cDNA buffer, and bring to a final volume of 100 µL with DEPC-treated water (all reagents should be prepared and diluted in DEPC-treated water). Include a negative control that contains DEPC-treated water in place of the mRNA. Place the tubes in heating block filled with water preheated to 68°C. Allow the mRNA to denature at 68°C for 10 min, then remove the block with the tubes from the heating element and place it on the bench top. Once the block has cooled to 37°C, add 4 U Moloney murine leukemia virus reverse transcriptase and 0.8 U recombinant RNasin to each tube. Place the tubes in a 37°C water bath for 1 h to allow for first-strand cDNA synthesis.

4. The V_H and V_κ genes are individually amplified from separate cDNA aliquots using the 5' and 3' V_H and V_κ primer sets supplied in the RPAS kit according to the manufacture's directions. (What follows are our adaptations of these directions. If we followed the directions exactly, then this is noted.) Add 33 µL from the first-strand cDNA synthesis tube, 2 µL of the V_κ light-chain primer mix, and 64 µL of sterile distilled water. To a second tube, add 33 µL from the first-strand cDNA synthesis tube, 2 µL each of V_H primers 1 and 2, and 62 µL of sterile distilled water. For negative controls, substitute 33 µL from the water control tube for the cDNA mix added to each primer pair. Overlay each sample with 100 µL mineral oil, tightly close the tubes, and place them in a thermal cycler. Denature the cDNA by heating at 95°C for 5 min, then add 1 µL of *Pfu* polymerase beneath the mineral oil using a fresh pipet tip every time. Amplify the *V* genes for 30 cycles as follows: 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min.
5. To determine if the amplifications were successful, add 5 µL of each reaction mix to 5 µL of 0.5X TBE buffer and 2 µL of 6X agarose gel-loading dye. Prepare a 1.5% agarose gel in 0.5X TBE buffer with 0.5 µg/mL ethidium bromide (added after the agarose is melted and cooled and before the gel is poured). Submerge the gel in a horizontal electrophoresis box containing 0.5X TBE. Load each sample into a separate well and load an additional well with a molecular weight marker (such as a 100 bp ladder). Apply a constant voltage between 50–100 V until the bromophenol blue dye front has migrated about two-thirds the length of the gel. Place the gel on a UV light box to visualize the ethidium bromide-stained DNA fragments. Expect to see bands of approx 340 and 325 bp resulting from the V_H and V_κ primer sets, respectively. No bands should be visible in the negative control lanes. Clone the amplified V_H and V_κ genes into pCRII and transform *E. coli* strain INVFα' according to the manufacturer's protocol. Pool all recombinant (white) colonies from an individual *V* gene transformation and sequence using an automated DNA sequencer.
6. To construct an *sF*v gene, the V_H and V_κ genes amplified from a given hybridoma are linked in a second PCR. The amplified *V* genes are gel purified by first adding 85 µL of each PCR mix to 18 µL of the 6X agarose gel loading dye, and then by loading the samples on a 1% agarose gel. Prepare the gel in 0.5X TBE buffer with 0.5 µg/mL ethidium bromide as described above. To make wells large enough to hold approx 100 µL, use a small piece of autoclave tape to join 3–4 teeth on the comb prior to inserting it into the molten agarose. Electrophorese the DNA as

previously described, but place a cardboard box over the gel box to minimize light-induced nicking of DNA in the presence of ethidium bromide. Visualize the ethidium bromide-stained DNA fragments using a hand-held long-wave UV light source, again to minimize DNA damage. Excise each DNA fragment by cutting it out of the gel with a separate razor blade. Remove excess gel from around the DNA fragment and then dice the gel slice into small pieces prior to extraction. Extract DNA using the gel extraction kit according to its protocol.

7. Determine the amount of each gel-purified PCR product relative to the linker–primer DNA supplied in the RPAS kit. The goal is to add equimolar amounts of the two PCR products and linker–primer DNA in a second PCR. Mix 5 μL of each PCR mix with 1 μL 6X agarose gel-loading dye. Mix 1 μL of the linker–primer DNA with 1 μL 6X agarose gel-loading dye and 4 μL distilled water. Run the three samples on a 1.5% agarose gel as described earlier. For equimolar concentrations, the ethidium bromide staining intensity of the two PCR products should be equivalent and about 3–4 times greater than the linker–primer DNA. Adjust the concentrations of the PCR products accordingly before proceeding to the next step.
8. The sFv assembly and fill-in reactions are performed as described by the manufacturer. Briefly, equimolar amounts of the amplified *V* gene PCR products and the linker–primer DNA (1–3 μL) are added to 2.5 μL 10X reaction buffer without $MgCl_2$, 12.5 μL dNTP mix (each at 2 m*M*), 4 μL 25 m*M* $MgCl_2$, 1 μL *Taq* polymerase (*see* **Note 2**), and sterile distilled water to a final volume of 25 μL. Overlay with 25 μL mineral oil, place in a thermocycler, and run seven cycles at: 94°C for 1 min, 63°C for 4 min. This procedure should append the linker–primer to the 3' and 5' ends of the amplified V_H and V_κ genes, respectively.
9. To link the modified V_H and V_κ genes and simultaneously append 5' and 3' restriction endonuclease sites, add the following components beneath the mineral oil to the 25 μL assembly mix: 13 μL sterile distilled water, 2.5 μL 10X reaction buffer without $MgCl_2$, 5 μL dNTP mix (each at 2 m*M*), 1 μL *Taq* polymerase, 1.5 μL 25 m*M* $MgCl_2$, and 2 μL restriction-site primer mix (these primers contain *Sfi*I or *Not*I restriction sites and anneal to the 5' end of the V_H [*Sfi*I site] and/or the 3' end of the V_κ gene [*Not*I site]). Overlay with an additional 25 μL mineral oil, place in a thermocycler, and run 30 cycles at: 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min.
10. Analyze the reactions by 1.5% agarose gel electrophoresis as described above. A band of approx 750 bp is expected, along with some heavy- and light-chain monomers.
11. Clone the PCR products into the pCRII vector and transform *E. coli* strain INVFα' (*see* **Note 3**). Pool all recombinant (white) colonies from an individual transformation, isolate plasmid DNA, and digest with *Sfi*I and *Not*I. DNA fragments of the size predicted for full-length *sFv* genes (approx 750 bp) are gel purified (see above), ligated to the pCANTAB 5E phagemid vector, and used to transform *E. coli* strain HB2151 as described in the RPAS manual.
12. Directly sequence the *sFv* genes in at least three 3 clones derived from a given hybridoma to identify ones that lack *Taq* polymerase-induced mutations. These

mutations are identified by comparing the *sFv V*-gene sequences to the originally amplified *V*-gene sequences (as previously discussed). First, amplify 0.5 μg of plasmid DNA from each clone using 25 p*M* of each S1 and R2 primer in a buffer containing 1.5 m*M* $MgCl_2$, 2.5 U *Taq* DNA polymerase, 100 m*M* Tris-HCl (pH 8.3) and 500 m*M* KCl. The S1 and R2 primers anneal to pCANTAB 5E vector-encoded nucleotides that flank the sFv insert. Incubate the reaction mixtures at 94°C for 5 min, then add 10 μL dNTPs (at 2 m*M* each). Amplify the mixtures for 1 min at 94°C, 2 min at 55°C, and 2 min at 72°C for 30 cycles. An additional extension time of 10 min at 72°C completes the amplification reaction. Gel purify the approx 875 bp product for direct sequencing. Sequencing is performed using the S1 and R2 primers and 50–100 ng of gel-purified DNA with the PRISM sequencing kit according to the manufacturer's directions.

3.2. sFv Expression and Purification

1. The pCANTAB 5E vector encodes a leader sequence upstream of the sFv cloning site. This theoretically allows for the transport of the sFv fragment to the periplasmic space, where it can be isolated as a soluble protein. However, only two of the four sFv- pCANTAB 5E constructs (3A1e and 3A1f) were exported to the periplasm upon induction. The other two (3A1a and 3A1d) were expressed insolubly in the cytoplasm. Because of this and the consistently low yields (in the range of 200 μg/L) of purified sFv fragments from the periplasm, we suggest modifying the sFv constructs to allow for their expression as secreted proteins by the yeast *P. pastoris.* For example, we modified the 3A1f sFv by adding a cysteine residue followed by six histidines at the carboxy terminus. The cysteine provided a free thiol for disulfide conjugation to RTA, while the histidine tag allowed for identification with a specific monoclonal antibody and purification via immobilized metal-cation affinity chromatography. The modifications were made in one step by PCR amplification (30 cycles: 1 min at 95°C, 1.5 min at 55°C, 2 min at 74°C) from pCANTAB 5E-3A1f sFv template DNA using the primers 5'-CCGGAATTCATGGCCCAGGTCCAGCTGCAG-3' and 5'-CCGGAATTCCCGGGCGGCCGCTCAGTGGTGGTGGTGGTGGTGACACCG TTTGATTTCCAACTTTGTCCC-3'. The amplified 3A1fcysHis_6 sFv fragment was cloned into pCRII, sequenced, and subcloned into a *P. pastoris* expression vector, pPIC9, as an *Eco*RI/*Not*I restriction fragment.
2. pPIC9 is designed to integrate into the yeast genome via homologous recombination following transformation. To facilitate homologous recombination, the plasmid DNA is linearized prior to electroporation. Linearization with *Bgl*II allows the endogenous *AOX1* gene in the GS115 genome to be replaced with the *3A1fcysHis_6* and plasmid-encoded histidinol dehydrogenase *(HIS4)* genes upon homologous recombination. Prepare electrocompetent *P. pastoris* by inoculating 5 mL YPD in a 50 mL conical tube with frozen stock GS115 cells and incubating at 30°C overnight with vigorous shaking (220–250 rpm). Inoculate 500 mL of fresh YPD medium in a 2 L flask with 0.1–0.5 mL of the overnight culture. Cover the opening at the top of the flask loosely with sterile aluminum foil. Incubate at

Table 1

Electroporator	Charging voltage (V)	Capacitance (μF)	Resistance (Ω)
Invitrogen Electroporator II	1500	50	200
Bio-Rad Gene Pulser	1500	25	200
Eppendorf 2510	1500	10	600

30˚C with vigorous shaking until the OD_{600} = 1.3–1.5 (this typically takes an overnight incubation [12–15 h]). Centrifuge the cells at 1500*g* for 5 min at 4˚C. Suspend the cell pellet in 500 mL of ice-cold, sterile water. Recentrifuge and resuspend the cell pellet in 250 mL of ice-cold, sterile water. Centrifuge the cells again, then resuspend the cell pellet in 20 mL of ice-cold, sterile 1 *M* sorbitol. Centrifuge the cells once more and resuspend cell pellet in 0.5–1 mL of ice-cold, sterile 1 *M* sorbitol for a final volume of approx 3–4 mL (accounting for the volume of the cell pellet). Electrocompetent cells can be aliquoted and stored at –80˚C for up to 6 mo.

3. Mix 80 μL of ice-cold electrocompetent cells with 1–3 μg of plasmid DNA linearized with Bgl II. Transfer the DNA and cell mix to an ice-cold 0.2 cm electroporation cuvet and incubate on ice for 5 min. Wipe the outside of the cuvet with a Kimwipe to remove moisture and place the cuvet in the electroporation unit. Pulse the cells according to the parameters for yeast (*S. cerevisiae*) suggested by the manufacturer of the specific electroporation device being used (**Table 1**). Using a 0.2 cm cuvet, these parameters generate a pulse length of approx 5 msec with a field strength of approx 7500 V/cm (*see* **Note 4**). Immediately after electroporation, add 1 mL ice-cold, sterile 1 *M* sorbitol to the cells in the cuvet. Transfer the cells to a sterile microcentrifuge tube. Spread 200–600 mL aliquots on MD plates. Incubate the plates at 30˚C until colonies appear (this takes at least 2 d; 3–4 d is not unusual).
4. To screen for clones secreting $sFvcysHis_6$ proteins, probe colony lifts with an anti-His tag antibody as follows. Replica plate His^+ clones from the transformation plates by using sterile toothpicks first to streak isolated clones onto an MM plate and then onto the corresponding positions on an MD plate (*see* **Note 5**). Incubate 2–3 d at 30°C. Store the master MD plates at 4°C. Place a BA85 nitrocellulose filter over the colonies on the MM plate using gloved hands to grasp the opposite edges of the filter, bend the edges upwards, and touch the bottom of the filter to the plate. Gently lower the edges of the filter so that the filter wets from the center outwards. On top of this filter, place three circular pieces of Whatman 3MM chromatography paper cut to about 80 mm in diameter. Add several paper towels and weigh down the blotting pile with a 150 mL empty beaker. Incubate at 30°C for 1–3 h. Remove the nitrocellulose filter with forceps and wash in TBS (20 m*M* Tris–base, 137 m*M* NaCl, pH 7.6) to remove adherent yeast. Use 50 mL/wash and wash three times for 5 min each at room temperature with

constant shaking. Add positive controls by spotting 100 ng of each protein diluted in TBST on the filter. The positive controls are any purified His-tagged protein and the secondary goat antimouse–HRP conjugate (see below). After two minutes, block the filter with 3% BSA/TBS overnight at 4°C or for 2 h at 37°C. Incubate the filter with mouse anti-Penta-His tag antibody diluted 1:1000 in 3% BSA/TBS at room temperature with constant shaking for 1 h. Wash once for 15 min followed by three 5-min washes in TBST (50 mL/wash) with constant shaking at room temperature. Incubate membrane with goat antimouse immunoglobulin-HRP diluted 1:10,000 in solution A at room temperature with constant shaking for 1 h. Wash as above. Leave the filter in its final wash and go to the dark room. Pour off the final wash from the filter. Pipet 1 mL each of ECL developing reagents 1 and 2 onto the filter (use the same container in which the filter was washed). Incubate for 1 min. Place the filter in a film cassette, smooth some plastic wrap on top of the filter, turn the lights off, and place a piece of XAR-5 film (Kodak) on top of the plastic. Close the cassette, expose for 30–60 s initially, and develop the film. If there are no initial results, try a longer exposure time. The ECL mix should be effective for up to half an hour.

5. Select several positive clones to analyze their time course of protein secretion. Grow clones expressing the sFvcysHis$_6$ fragments in 100 mL of YPD medium in a sterile 1 L flask for 2 d at 30°C in a shaking (250 rpm) incubator. Centrifuge cells (10 min at 3000*g*), resuspend in 10 mL of BMMY in a sterile 100 mL flask (with foil loosely covering the top), and incubate for another 5 d at 30°C in a shaking (250 rpm) incubator. Take 500 µL aliquots of culture supernatants daily, store at –20°C, and examine after 5 d by reducing SDS-PAGE to determine the optimal time for expression. For SDS-PAGE, combine 5 µL 4X SDS loading dye, 1 µL 2-mercaptoethanol, and 14 µL of culture supernatant in a 1.5 mL centrifugation tube. Cap tightly and place in boiling water for 2–3 min. Cool on ice and quick-spin in an Eppendorf centrifuge to recover condensate. Load samples in wells of a 15% polyacrylamide Laemmli gel. Electrophorese for 40 min at a constant 200 V. Remove the gel from its glass plates and wash three times (200 mL per wash for 5 min at room temperature with agitation) to remove excess SDS. Stain proteins by adding just enough GelCode to cover the gel. Agitate gently at room temperature for 1 h, remove the GelCode, and destain with distilled water for at least 1 h at room temperature. Bands at about 28 kDa should be visible in the induced supernatants.
6. For large-scale expression, grow clones in 1 L of YPD medium in sterile 2 L baffle flasks (with foil covering their openings) for 3 d at 30°C in a shaking (250 rpm) incubator. Centrifuge cells (10 min at 3000*g*), resuspend in 50 mL of BMMY in a sterile 500 mL flask (with foil covering the opening), and incubate for the optimal number of days (e.g., 4 d) at 30°C in a shaking (250 rpm) incubator (*see* **Note 6**). Harvest the supernatants following centrifugation (10 min at 3000*g*). Clarify supernatants by centrifuging at 12,000*g* at 4°C for 20 min.
7. To purify the sFvcysHis$_6$ fragments, dialyze clarified supernatants from the induced culture against 50 m*M* sodium phosphate (pH 7.0) plus 100 m*M* NaCl. Filter

dialysates through a 0.8 μm filter syringe, followed by a 0.45 μm filter syringe, and run over a prepacked Chelating-High Trap 5 mL column using FPLC (*see* **Note 7**). Prior to loading sFv samples, wash the column with 25 mL distilled water (2 mL/min), activate it with 6 mL 100 m*M* $ZnCl_2$, and wash it again with 25 mL distilled water (2 mL/min) followed by 30 mL dialysis buffer (2 mL/min). Run dialysate over the column at 1 mL/min and save the flow through. Wash the column with 30 mL dialysis buffer (2 mL/min) and then elute bound fragments with a linear gradient of 0–100% 300 m*M* imidazole at 1 mL/min for 35 min. Collect 1 mL fractions and pool those fractions that absorb light at 280 nm. Determine the purified protein concentration with Coomassie Plus protein assay reagent (Pierce) using BSA as the standard.

3.3. sFv–RTA Conjugate Formation and Purification

1. Dilute the dgRTA chain to 0.5–1.0 mg/mL with PBS, 1 m*M* EDTA, pH 7.2, and reduce with 1–2 m*M* DTT for 1 h at room temperature. Remove excess DTT from the reduced dgRTA by gel filtration with a PD-10 column following the manufacturer's protocol.
2. Derivatize the reduced dgRTA (RTA-TNB) by adding DTNB (Ellman's reagent) for 1 h at room temperature. Derivatization prevents dgRTA homodimerization in subsequent steps. Use PD-10 gel filtration to remove excess DTNB from the RTA-TNB.
3. Concentrate each affinity-purified sFvcysHis_6 sample to 250–500 μg/mL using Centricon-3 concentrators and mildly reduce with 1–2 m*M* DTT for 1 h at room temperature. This does not reduce the intrachain disulfide bonds in the immunoglobulin domains, but it does yield free thiols at the carboxy-terminal cysteines. Remove excess DTT from the reduced sFvcysHis_6 fragments by gel filtration with a PD-10 column following the manufacturer's protocol, and mix the reduced sFvcysHis_6 with the RTA-TNB at a 1:1 molar ratio. Incubate the mixture for 1 h at room temperature and then overnight at 4°C. Purify conjugated protein (approx 58 kDa) by size-exclusion chromatography using a Superdex 75 HR 10/30 column and FPLC apparatus. Collect 1 mL fractions in PBS. Pool fractions that contain protein corresponding to approx 58–60 kDa. Concentrate protein with Centricon-10 concentrators, analyze by 10% nonreducing SDS-PAGE (do not add 2-mercaptoethanol to the sample), and determine the concentration, all as described above.

4. Notes

1. To prepare DEPC-treated water, add 500 mL of distilled water to a baked Pyrex bottle (250°C for 4 h). Add 0.5 mL DEPC and swirl to disperse. Cover with a sterile bottle cap and incubate for at least 12 h at 37°C. Autoclave to inactivate DEPC.
2. We initially tried to use the high-fidelity *Pfu* polymerase for sFv assembly but were unsuccessful despite repeated attempts under varying experimental conditions. Therefore, we used *Taq* polymerase for this step and had uniform success.

3. The RPAS manual recommends purifying the assembled sFv DNA, incubating it directly with *Sfi*I and *Not*I restriction endonucleases, and cloning the digested fragment into pCANTAB 5E. We found this step problematic because of the inability to monitor the efficiency of digestion. Therefore, we first cloned the amplified sFv constructs into pCRII. This linearized vector has single T-nucleotide 5' and 3' overhangs, allowing for the easy ligation of *Taq*-amplified DNA (due to the terminal transferase activity of *Taq,* and its inherent preference for adding single A nucleotides to 3' OH groups). The clones were pooled, digested with *Not*I and *Sfi*I, and the doubly digested DNA fragments were gel purified and ligated to pCANTAB 5E.
4. After a 90 min incubation at 37°C, inactivate *Bgl*II by heating the sample at 68°C for 10 min. It is important to ethanol-precipitate the DNA and wash it several times in 70% ethanol to remove salt prior to electroporation. Residual salt in the DNA preparation leads to high conductance (pulse lengths ≤ 2–3 ms) and poor transformation efficiencies due to excessive cell death.
5. These plates are overlaid on a template that has two 80 mm diameter circles divided into 52 numbered squares of equivalent size. A clone is first streaked diagonally across one square on the MM plate and then across the same numbered square on the MD plate.
6. Optimal protein expression requires good aeration, and therefore Invitrogen suggests the use of sterile cotton or gauze to plug the flasks. We and our colleagues have noted that these porous materials allow strong and unpleasant odors from the cultures to permeate the work area, and so we loosely cover our baffled flasks with sterile foil. This reduces the odor without adversely affecting protein yields.
7. All solutions used for FPLC are passed through 0.45 μm filters to prevent clogging the columns.

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References

1. Barcena, A., Muench, M. O., Roncarolo, M. G., and Spits, H. (1995) Tracing the expression of CD7 and other antigens during T- and myeloid-cell differentiation in the human fetal liver and thymus. *Leuk. Lymphoma* **17,** 1–11.
2. Nakase, K., Kita, K., Sekine, T., Otsuji, A., Shirakawa, S., Tsuji, K., et al. (1993) CD7, CD4 and myeloid antigen-positive acute lymphoblastic leukemia. *Internat. J. Hematol.* **59,** 41–46.
3. Del Poeta, G., Stasi, R., Venditti, A., Cox, C., Aronica, G., Masi, M., et al. (1995) CD7 expression in acute myeloid leukemia. *Leuk. Lymphoma* **17,** 111–119.
4. Nimgaonkar, M. T., Roscoe, R. A., Persichetti, J., Rybka, W. B., and Winkelstein, A. (1995) A unique population of $CD34^+$ cells in cord blood. *Stem Cells* **13,** 158–166.

5. Shimizu, Y., van Seventer, G. A., Ennis, E., Newman, W., Horgan, K. J., and Shaw, S. (1992) Crosslinking of the T cell-specific accessory molecules CD7 and CD28 modulates T cell adhesion. *J. Exp. Med.* **175,** 577–582.
6. Chan, A. S., Mobley, J. L., Fields, G. B., and Shimizu, Y. (1997) CD7-mediated regulation of integrin adhesiveness on human T cells involves tyrosine phosphorylation-dependent activation of phosphatidylinositol 3-kinase. *J. Immunol.* **159,** 934–942.
7. Sempowski, G. D., Lee, D. M., Scearce, R. M., Patel, D. D., and Haynes, B. F. (1999) Resistance of CD7-deficient mice to lipopolysaccharide-induced shock syndromes. *J. Exp. Med.* **189,** 1011–1016.
8. Haynes, B. F., Denning, S. M., Singer, K. H., and Kurtzberg, J. (1989) Ontogeny of T-cell precursors: a model for the initial stages of human T-cell development. *Immunol. Today* **10,** 87–91.
9. Janossy, G., Coustan-Smith, E., and Campana, D. (1989) The reliability of cytoplasmic CD3 and CD22 antigen expression in the immunodiagnosis of acute leukemia: a study of 500 cases. *Leukemia* **3,** 170–181.
10. Janossy, G., Campana, D., Burnett, A., Coustan-Smith, E., Timms, A., Bekassy, A. N., et al. (1988) Autologous bone marrow transplantation in acute lymphoblastic leukemia—preclinical immunologic studies. *Leukemia* **2,** 485–495.
11. Pauza, M. E., Doumbia, S. O., and Pennell, C. A. (1997) Construction and characterization of human CD7-specific single-chain Fv immunotoxins. *J. Immunol.* **158,** 3259–3269.
12. Frankel, A. E., Laver, J. H., Willingham, M. C., Burns, L. J., Kersey, J. H., and Vallera, D. A. (1997) Therapy of patients with T-cell lymphomas and leukemias using an anti-CD7 monoclonal antibody ricin A chain immunotoxin. *Leuk. Lymphoma* **26,** 287–298.
13. Vallera, D. A., Burns, L. J., Frankel, A. E., Sicheneder, A. R., Gunther, R., Gajl-Peczalska, K., et al. (1996) Laboratory preparation of a deglycosylated ricin toxin A chain containing immunotoxin directed against a CD7 T lineage differentiation antigen for phase I human clinical studies involving T cell malignancies. *J. Immunol. Methods* **197,** 69–83.
14. Huston, J. S., Levinson, D., Mudgett-Hunter, M., Tai, M.-S., Novotny, J., Margolies, M. N., et al. (1988) Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli. Proc. Natl. Acad. Sci. USA* **85,** 5879–5883.
15. Bird, R. E., Hardman, K. D., Jacobson, J. W., Johnson, S., Kaufman, B. M., Lee, S.-M., et al. (1988) Single-chain antigen-binding proteins. *Science* **242,** 1038–1041.
16. Huston, J. S., McCartney, J., Tai, M. S., Mottola-Hartshorn, C., Jin, D., Warren, F., et al. (1993) Medical applications of single-chain antibodies. *Internat. Rev. Immunol.***10,** 195–217.
17. Huston, J. S., Mudgett-Hunter, M., Tai, M. S., McCartney, J., Warren, F., Haber, E., et al. (1991) Protein engineering of single-chain Fv analogs and fusion proteins. *Methods Enzymol.* **203,** 46–88.

18. Eldin, P., Pauza, M. E., Hieda, Y., Lin, G., Murtaugh, M. P., Pentel, P. R., et al. (1997) High-level secretion of two antibody single chain Fv fragments by *Pichia pastoris. J. Immunol. Methods* **201,** 67–75.
19. Pennell, C. A. and Eldin, P. (1998) In vitro production of recombinant antibody fragments in *Pichia pastoris. Res. Immunol.* **149,** 599–603.
20. Palker, T. J., Scearce, R. M., Hensley, L. L., Ho, W., and Haynes, B. F. (1984) Comparison of the CD7 (3A1) group of T cell workshop antibodies, in *Leukocyte Typing II, Vol. I.* (Rheinherz, E. L., ed.), Springer-Verlag, New York, pp. 303–313.
21. Amlot, P. and Cammisuli, S. (1990) CD7 monoclonal antibodies, in *Therapeutic monoclonal antibodies* (Borrebaeck, C. A. K. and Larrick, J. W., eds.), Stockton Press, New York, pp. 287–302.
22. Orlandi, R., Gussow, D. H., Jones, P. T., and Winter, G. (1989) Cloning immunoglobulin variable domains for expression by the polymerase chain reaction. *Proc. Natl. Acad. Sci. USA* **86,** 3833–3837.