RPAS Purification Module

INSTRUCTIONS

Introduction

The RPAS Purification Module is an integral part of Amersham Biosciences' Recombinant Phage Antibody System (RPAS). Soluble, functional mouse single chain fragments variable (ScFv) antibodies are produced in *E. coli* using the pCANTAB 5E expression vector. The ScFv carries a C-terminal 13 amino acid peptide tag (E-Tag) which is recognized by an Anti-E Tag monoclonal antibody. The RPAS Purification Module contains a 5 ml HiTrap Anti-E Tag affinity column for fast purification of the E-tagged mouse ScFv's. The ScFv binds to the Anti-E Tag column at neutral pH and is easily eluted from the column with a decrease in pH.

The RPAS Purification Module contains all of the reagents necessary to perform a complete one step purification, without using complicated equipment. It also eliminates time consuming processes such as precipitation, dialysis of the sample, buffer preparation and column packing. The Anti-E Tag column can be operated with a simple syringe or a peristaltic pump, e.g. Pump P-1 Perastaltic Pump. The RPAS Purification Module contains enough reagents to perform up to 10 purification's when operated with a syringe.

71-5000-87

Edition AB

Amersham Biosciences

Kit Contents

Designation	Code no.	No. supplied
RPAS Purification Module	17-1362-01	1
HiTrap Anti-E Tag column, 5 ml		1
Binding Buffer, 50 ml, 10 x concentrate containing 20% ethanol as a preservative		2
Elution Buffer, 40 ml, 10 x concentrate		1
Neutralizing Buffer, 25 ml, containing 20% ethanol as a preservative		1
Connectors		
luerlock, female / M6 male		1
luerlock female / M6 female		1
tubing connector flangeless / M6 male		1
tubing connector flangeless / M6 female		1
Syringe, 10 ml		1
Domed nut		1
Instructions		1

Description

Column

The HiTrap column is made of medical grade polypropylene. The column is delivered with a stopper on the inlet and a twist-off end on the outlet. Both ends have M6 connections (6 mm metric threads). The column can not be opened or refilled. A set of connectors is supplied to make it easy to connect the column to a syringe or different chromatography equipment. Operation is easy, either using the syringe (see last page) or, alternatively, a laboratory pump, e.g. P-1 Perastaltic Pump. The necessary connectors are described below.

System/equipment	Connector
Syringe	Luer connector female/M6 male
FPLC, GradiFrac systems.	Flangeless/M6 male
Low pressure system with capillary tubing	Flangeless/M6 female
Systems with luer connections	Luer connector female/M6 male Luer connector female/M6 female

Matrix

The Anti-E Tag media is produced by coupling a mouse monoclonal antibody, specific for the 13 amino acid E-Tag, to N-hydroxysuccinimide activated Sepharose High Performance. The coupling method is optimized to give a stable covalent binding of the ligand, ensuring long column life. The mouse antibody has been carefully selected to minimize non-specific cross reactions with *E. coli* proteins. Under the recommended conditions, the column can be re-used in excess of 20 times.

The maximum binding capacity is ≈ 0.7 mg ScFv at saturation. The capacity depends however on factors such as ScFv concentration and sample application flow rate. Samples with concentrations down to 2 µg ScFv/ml have been purified at 2 ml/min with good yields. The characteristics of the HiTrap Anti-E Tag column are summarised in Table 1.

Table 1. HiTrap Anti - E Tag characteristics.

Property	HiTrap Anti - E Tag
Column dimension, d x h	16 x 25 mm
Bed volume	5 ml
Ligand	E-tag specific mouse monoclonal
Binding capacity	≈ 0.7 mg ScFv/column
Mean bead size	34 µm
Bead structure	Highly cross-linked spherical agarose
Maximum back pressure*	3 bar, 0.3 MPa, 44 psi
Maximum flow rate	20 ml/min
Recommended flow rate	1–5 ml/min
pH stability: Short term	2.8–10.5
Storage buffer	12 mM phosphate, 140 mM chloride, 0,15% Kathon CG/ICP Biocide**, pH 7.4

*Maximum pressure without damage to the column packing.

**Kathon is a registered trademark owned by Rhom and Haas Inc.

Buffers

Buffers have been prepared using the highest quality salts and water and have been filtered through a 0.45 µm membrane.

Sample preparation

Soluble ScFv can be obtained from either *E. coli* cell culture medium, a cell periplasmic extract or from a whole cell extract. The cell culture medium and periplasmic extract generally contain functional antibody, with the highest concentration of antibody generally being found in the periplasmic extract. Antibodies obtained from the cytoplasmic portion of a whole cell extract are not always functional since the antibody's intra-chain disulphide bonds are not usually formed in the cell cytoplasm. Since the expression level for some ScFv's may be low, purify the ScFv from periplasmic extract obtained from 1 liter or more of cell culture. It should be noted that the optimal conditions for generating ScFv will vary depending upon the ScFv.

Note: Optimal ScFv production can be achieved by varying cell growth conditions. These conditions can be ascertained by varying the concentration of the medium's components and the concentration of various additives (salts, e.g. NaCl, detergents, e.g. Tween-20, alternative carbon or sugar sources) that may be included in the medium's formulation.

The incubation time and temperature used to grow the cells and type of container (glass vs. plastic) in which the cells are grown can also influence the level of ScFv production. The level of ScFv production should be monitored during optimization through the use of a functional assay such as an enzyme-linked immunosorbent assay (ELISA). Additionally, some ScFv genes may not be stable in *E. coli*. If the level of ScFv production drops over time, streak out the stock culture on medium containing ampicillin and 2% glucose, pick isolated colonies and evaluate each colony for antibody expression. Prepare new stock cultures from positive colonies and use the new cultures to produce ScFv.

The Anti-E Tag column is known to be resistant to the following detergents: 0.5% (w/v) Tween-20*, 0.5% (w/v) Triton X-100**, 0.5% (w/v) CHAPS. It can also be used with 1 M NaCl or 1.0 M Urea.

Do not use the column together with SDS or deoxycholate. These detergents irreversible destroy the antibody structure necessary for the binding to its antigen. The presence of reducing agents in the sample, such as DTT or Mercaptoethanol, can reduce intra-molecular disulphide bounds on the Anti-E Tag antibody, leading to loss of binding capacity, and should therefore be avoided. Structure forming salts at high concentration, such as guanidine-HCl should also be avoided. Removal of some of these compounds may be done by desalting on Sephadex G-25 or by dialysis.

Periplasmic extract

1. Pellet the *E. coli* cells by centrifugation at 1,000–1,500 x g for 10 minutes.

2. Carefully pour off the cell culture medium without disturbing the cell pellet.

3. Re-suspend the *E. coli* cell pellet in ice-cold extraction buffer (e.g. 1 X TES or equivalent). Use 20 ml of 1 X TES solution for each liter of cell culture.

4. Add 33 ml of 1/5 X TES for every liter of cell culture and mix. Incubate the re-suspended cells on ice while agitating for 30 minutes.

5. Pellet the cells by centrifugation as above.

6. Carefully transfer the supernatant (periplasmic extract) containing the soluble ScFv to a polypropylene container and store at –20 °C to –70 °C.

7. Before applying the periplasmic extract to the Anti-E Tag column, filter the solution through a 0.45 µm filter to prevent the column from being clogged with cell debris or other particulates.

*Tween 20 is a registered trademark owned by Atlas Chemicals Ind. Inc.

**Triton X-100 is a registered trademark owned by Rohm and Haas Inc.

Note: The stability of ScFv's varies. Some unpurified ScFv are stable for months when stored under these conditions while others are stable for only a few days.

Cell culture medium

The concentration of ScFv in the cell culture medium is often very low, compared to the periplasmic extract, and the volume of medium needed to purify an adequate amount of ScFv may be too large to be practical. It may therefore be advantageous to concentrate the cell culture medium by ultra filtration using a filter with a 10 kDa cut off. The ScFv antibody fragment has a molecular weight off ≈ 30 kDa. Other concentration techniques such as ammonium sulphate precipitation are also possible. Do not concentrate using trichloroacetic acid (TCA) since antibodies will not retain functionality.

1. Pellet the *E. coli* cell culture by centrifugation as above.
2. Carefully remove the supernatant which contains the extra cellular ScFv. To reduce sample volume, concentrate the supernatant if necessary.
3. Filter the solution through 0.45 µm filter to prevent the column from being clogged with cell debris or other particulates.

Syringe operation

The following protocol can be used to purify soluble functional ScFv from the cell periplasm or cell culture medium.

1. Perform an ELISA to insure that the sample contains a sufficient amount of biologically active ScFv.
2. Remove the RPAS Purification Module from 4 °C storage and allow the kit to warm to room temperature.
3. Filter the sample containing the E-tagged ScFv through a 0.45 µm filter.

Note: In some cases the sample may be to viscous to filter through a 0.45 µm filter due to chromosomal DNA/RNA content. Dilution with binding buffer, mechanical sheering, sonication or addition of DNase can be used to reduce viscosity.

Tube preparation

Prepare collection tubes by adding 100 µl of Neutralizing Buffer per ml of fraction to be collected from the Anti-E Tag column. This allows for immediate pH adjustment of the purified antibody to maintain the activity.

Note: The purified ScFv will be eluted from the Anti-E Tag column in ≈ 5 ml of Elution Buffer. If all of the purified ScFv will be collected in a single collection tube, add 500 µl of Neutralizing Buffer to the single collection tube. For sensitive ScFv and to reduce losses of material during storage of the purified material (some ScFv may be hydrophobic), Tween 20 can be added to a final concentration of 0.5% in the Neutralising Buffer before use, i.e. to give a concentration of 0.05% in the collected tubes.

Buffer preparation

1. Dilute the 10 x Binding Buffer: Add 10 ml of the 10 x Binding Buffer to 90 ml of distilled water.

2. Dilute the 10 x Elution Buffer: Add 3.0 ml of Elution Buffer to 27 ml of distilled water.

Regeneration and equilibration

When the column has been stored for a long time or is new, it is advisable to perform a blank run to regenerate the column before use.

1. Fill the syringe with the Elution Buffer.

2. Remove the stopper on the top of the Anti-E Tag column. Add a drop of Elution Buffer to the top of the column and connect the syringe to the column with the Luer adapter provided. Make the connection ”drop to drop” to avoid introducing air into the column.

Note: The column can also be connected to a chromatography system, e.g. FPLC or GradiFrac systems using the supplied connectors.

3. Remove the twist-off end.

4. Wash the column with 15 ml of Elution Buffer at ~5 ml/min (1–2 drop/second) followed immideately by 25 ml Binding Buffer.

The column is now ready for use.

Binding and elution of the ScFv

1. Adjust the pH of the periplasmic extract containing the ScFv to pH 7–8.

2. Apply the sample using the syringe at a flow rate of ~5 ml/min.

Note: At very low sample concentration, (< 1 µg/ml) all of the ScFv may not bind. Multiple passes over the column or using a lower flow rate will improve the yield.

3. Wash the column with 25 ml Binding Buffer at ~5 ml/min to remove excess unbound *E. coli* proteins.

4. Elute the bound ScFv from the Anti-E Tag column with Elution Buffer. Discard the first 4.5 ml eluted. The first 4.5 ml of material eluted from the column, generally, will not contain a significant amount of ScFv and can be discarded to waste.

5. Collect the following 5 ml (contains the purified E-tagged ScFv antibody) either in one or several fractions.

6. Immediately re-equilibrate the column with 25 ml Binding Buffer. Too long exposure of the anti-E Tag antibody at low pH destroys the anti-E Tag antibody on the column.

The column is now ready for a new sample.

Note: If ScFv's, specific for different antigens, are purified on the same column, there is always a risk for cross-contamination. To avoid this, use different anti-E Tag columns for each individual ScFv.

If necessary, the purified ScFv (approx. 30 kDa) fragment can be buffer exchanged using HiTrap Desalting columns or futher purified by gel filtration on a Superdex 75 HR 10/30 size-exclusion column to remove any dimers/polymers.

As there is a possibility of dimer formation when stored frozen in the elution buffer we recommend buffer exchange to for e.g. phosphate buffer.

Pump operation

1. Connect the column to the pump or chromatography system using the supplied connectors. The column can be connected directly to the monitor inlet of a Amersham Biosciences UV monitor, to minimize dead volumes.

2. Wash the column with 15 ml of Elution Buffer followed by 25 ml Binding Buffer at ~5 ml/min.

Note: The volume of the buffers supplied are adapted for syringe use only.

3. Apply the sample using a flow rate of ~5 ml/min.

Note: At very low sample concentration, (< 1 µg/ml) all of the ScFv may not bind. Multiple passes over the column or using a lower flow rate will improve the yield.

4. Wash the column with 25 ml Binding Buffer at ~5 ml/min to remove excess unbound *E. coli* proteins.

5. Elute the bound ScFv from the Anti-E Tag column with 15 ml of Elution Buffer at ~5 ml/min. Collect fractions prepared with Neutralizing Buffer (100 µl/ml fraction).

6. Re-equilibrate the column with 25 ml Binding Buffer.

Storage

Store the entire Purification Module refrigerated. When not in use, the HiTrap Anti-E Tag column should be washed with 25 ml Binding Buffer containing a bacteriostat e.g. 0.05 % NaN_3. Close the column with the stopper and the domed nut to avoid dehydration.

Function testing

The HiTrap anti-E Tag column is tested by affinity chromatography and binds at least 20 nmol E-Tag peptide/column.

Applications

Purification of a ScFv antibody against lysozyme using the Purification Module and syringe operation.

Purification procedure (all buffers prepared according to “buffer preparation”)

Column:	HiTrap Anti-E Tag, 5 ml
Sample:	10 ml *E. coli* periplasmic extract, pH 7.4. Filtered 0.45 µm
Flow rate:	~5 ml/min
Instrumentation:	10 ml syringe
Equilibration:	25 ml Binding Buffer (to waste)
Washing:	25 ml Binding Buffer, pH 7.0
Elution:	10 ml Elution Buffer, pH 3.0
Neutralisation:	100 µl Neutralizing Buffer per ml fraction

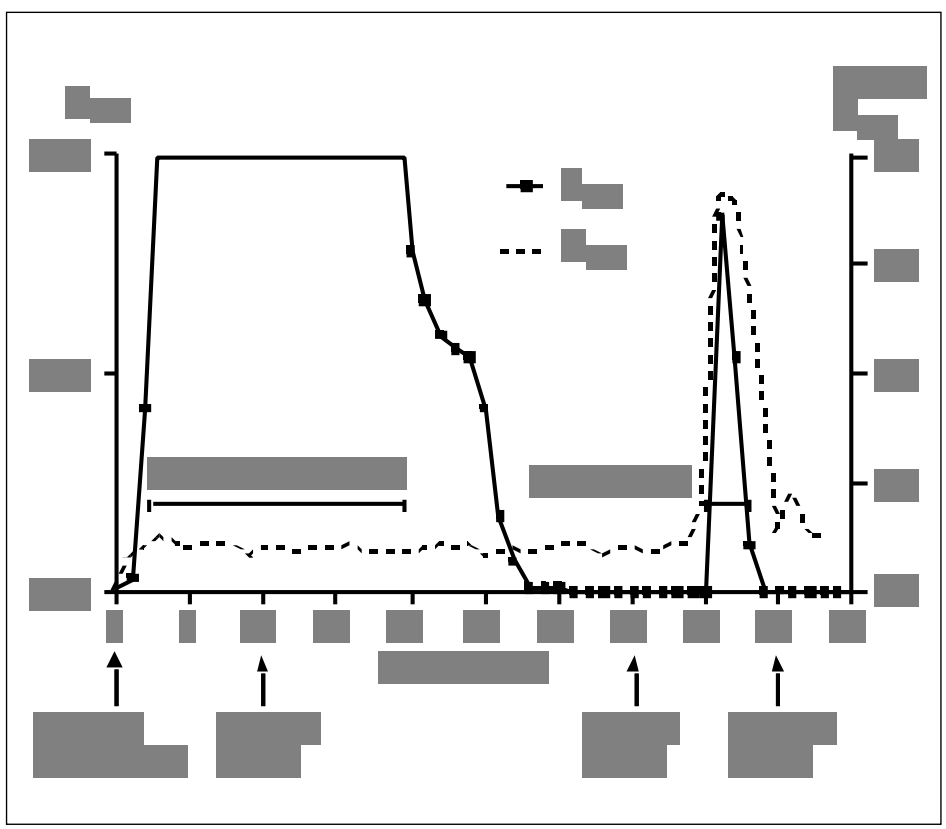


Figure.1. Purification of anti-lysozyme ScFv from a *E. coli* periplasmic extract using syringe operation. Activity of eluted material was determined with ELISA.

Results:
Operating the column with a syringe resulted in a pool of 3.3 ml with an A_{280} = 0.058 (0.14 mg) mouse ScFv. Analysis with SDS PAGE (silver staining) revealed a purity greater then 98%.

Table 2. Amount of ScFv purified from a perplasmic extract at different starting concentrations. The extract was diluted with extration buffer and pH adjusted to pH 7.0.

Sample concentration µg ScFv/ml	Sample volume ml	Eluted ml	Eluted mg ScFv	Yield %
20	10	3.3	0.198	100
8	25	4.4	0.176	89
4	50	3.3	0.163	82
2	100	3.3	0.144	73

Trouble shooting guide

No ScFv was obtained after Anti-E Tag column purification.
Perform an ELISA and Western blot on the crude periplasmic extract using the un-conjugated mouse Anti-E Tag antibody and an enzyme-labelled secondary antibody conjugate or the Anti-E-HRP conjugate to determine if biologically active ScFv was present in the sample. If biologically active ScFv is not present in the sample, the ScFv may have degraded prior to purification. Re-isolate ScFv using freshly prepared sample.

Biologically active ScFv was present in the sample but did not bind to the Anti-E Tag column.
Check the pH of the periplasmic extract. Adjust if necessary to a pH ~7–8. Then re-purify the sample according to the instructions.

Check to make sure that the 10 x Binding and Elution Buffers were diluted appropriately. Also check that the column was re-equilibrated in diluted Binding Buffer prior to sample application. If the column was regenerated with

diluted Elution Buffer and not re-equilibrated with diluted Binding Buffer, the pH of the column will be to low to allow the ScFv to interact with the Anti-E Tag monoclonal antibody bound to the solid support.

The yield of the ScFv after Anti-E Tag column affinity purification was unexpectedly low.

The concentration of the ScFv in the sample may be very low. Pass the sample through the column, collect the flow-through and re-pass the flow-through, through the column again. Repeat this process if necessary. Additionally, it may be helpful to pass the sample through the column using a lower flow rate.

The purified material contained dimers/aggregates of ScFv.

Some ScFv (MW: ≈30 kDa) have a tendency to form multimers. Generally, ScFv multimers are biologically active, however, in some immunoassays, aggregates of ScFv can enhance or reduce the signal obtained in the assays. Aggregates of ScFv may influence the outcome of other assays (e.g. viral neutralization assays) through steric effects. Additionally, the affinity of an ScFv can be difficult to determine if the ScFv is a part of an aggregate. If this is the case, the ScFv will have to be further purified by size-exclusion chromatography under non-denaturating conditions (e.g. on Superdex 75).

Purified ScFv appears degraded.

Some ScFv can be degraded by enzymes present in the *E. coli* extract prior to purification. The Anti-E Tag column will affinity purify all proteins (intact or degraded) that display the E-Tag. The addition of protease inhibitors to freshly prepared periplasmic extract may alleviate this problem. Additionally, changes in the *E. coli* cell growing conditions (e.g. media, time and temperature of the expression and extraction process, etc.) may also prevent degradation problems.

Buffers

10 x Binding Buffer: 0.20 M phosphate buffer, 0.05 % NaN_3 pH 7.0

To 13.8 g of NaH_2PO_4 x H_20 (137.99 g/mol), 17.8 g Na_2HPO_4 x 2 H_20 (177.99 g/mol), 0.5 g NaN_3 (65.01 g/mol) add distilled water to 1000 ml. Adjust pH if necessary to 7.0. Filter through a 0.22 µm filter.

10 x Elution Buffer: 1.0 M glycine pH 3.0

To 75.1 g glycine (75.07 g/mol) add distilled water to 900 ml, adjust pH with conc. HCl to pH 3.0. Finally dilute the solution to 1000 ml. Filter through a 0.22 µm filter.

1 x Neutralizing Buffer: 1 M Tris , 0.05 % NaN_3 pH 8.2

To 12.1 g Tris-base (121.14 g/mol), 0.05 g NaN_3 (65.01 g/mol) add 80 ml distilled water. Adjust pH to 8.2 with conc. HCl. Dilute to 100 ml. Filter through a 0.22 µm filter.

Storage solution: 12 mM phosphate, 140 mM chloride, 0.05% NaN_3, pH 7,4

To 8 g of NaCl (58.44 g/mol), 0.2 g of KCl (74.56 g/mol), 1.44 g of Na_2HPO_4 (141.96 g/mol), 0.24 g KH_2PO_4 (136.09 g/mol) 0.5 g NaN_3 (65.01 g/mol), add distilled water to 900 ml, adjust pH to 7.4 with NaOH or HCl. Finally dilute the solution to 1000 ml. Filter through a 0.22 µm filter.

E. coli **extraction buffer:** 0.2 M Tris-HCl, 0.5 mM EDTA, 0.5 M sucrose pH 8.0, **(**1 x TES).

To 24.23 g Tris-base (121.14 g/mol), 0.19 g EDTA x 2 H_20 (372.24 g/mol), 171.2 g sucrose (342.30 g/mol) add distilled water to 900 ml, adjust pH with conc. HCl to 8.0. Finally dilute the solution to 1000 ml. Filter, sterilize and store at 4 °C. To prepare 1/5 X TES Buffer, add 1 volume 1 X TES Buffer to 4 volumes of distilled water.

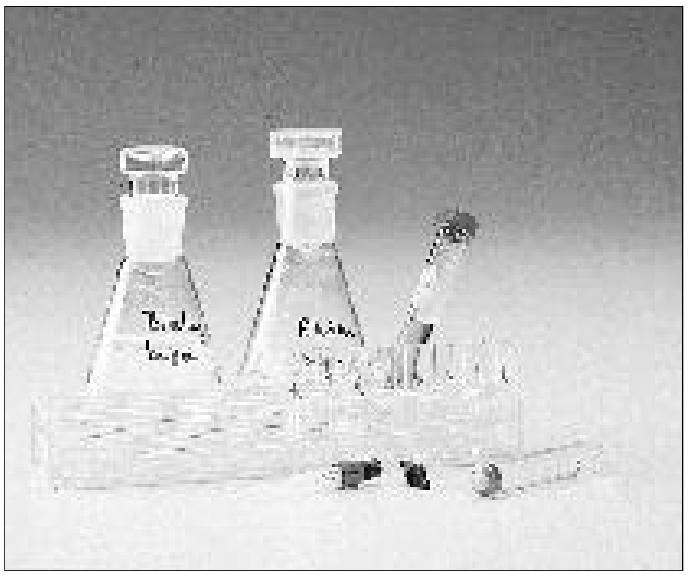
Ordering Information:

Product	Code No.	Quantity
Purification Module	17-1362-01	1 kit, 10 purifications
Companion Products		
Mouse ScFv Module	27-9400-01	5 reactions†
Expression Module	27-9401-01	1 kit†
Detection Module	27-9402-01	20 micro titre plates†
Anti-E Tag Antibody	27-9412-01	1 mg†
Anti-E Tag Antibody	27-9412-02	5 mg†
Anti-E Tag Antibody–HRP Conjugate	27-9413-01	0.5 mg
Anti-M 13 Antibody	27-9410-01	1 mg†
Anti-M 13 Antibody	27-9410-02	5 mg†
Anti-M 13 Antibody–HRP Conjugate	27-9411-01	200 micro titre plates†
pCANTAB 5 Gene Rescue Primers	27-1581-01	1 nmol each†
pCANTAB 5 Sequencing Primer Set	27-1585-01	250 pmol each†
Column Accessories		
Domed nut	18-2450-01	4
Union Luerlock		
female/M6 female	18-1027-12	2
female/M6 male	18-1027-62	2
Tubing connector		
flangeless/M6 male	18-1017-98	2
flangeless/M6 female	18-1003-68	2
M6 female/M6 female	19-2143-01	5
Pump P-1, 110/120 V AC	19-4611-02	1
Pump P-1, 210/220 V AC	19-4610-02	1
HiTrap Desalting	17-1408-01	5
Superdex 75 HR 10 /30	17-1047-01	1

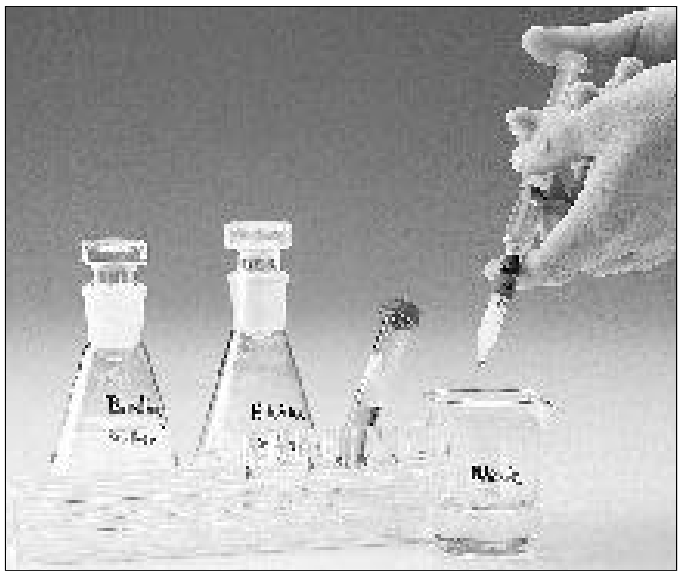
† Product must be shipped cold. There is an extra charge for insulated container and refrigerant.

Use with a syringe...

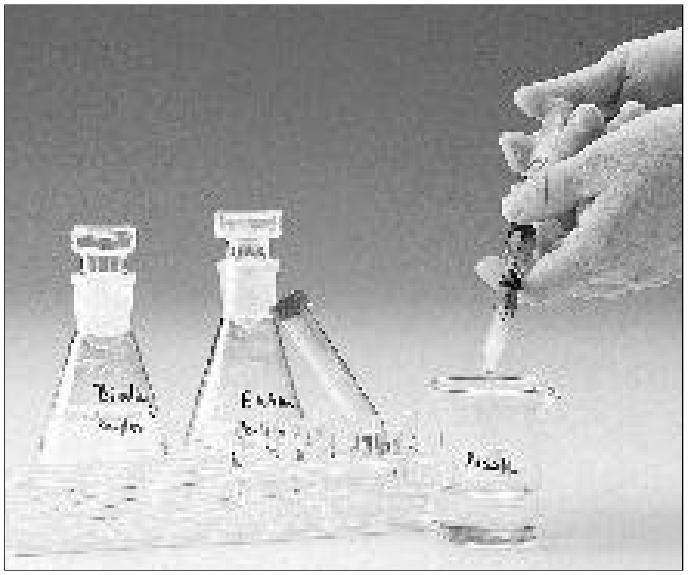
Purification of mouse ScFv with RPAS Purification Module.



1. Dilute concentrated buffers. Prepare sample.



2. Equilibrate with 25 ml Binding Buffer.



3. Load sample. Wash with 25 ml Binding Buffer. Begin collecting fractions.

4. Elute with 10 ml Elution Buffer. Continue collecting fractions.

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