RPAS Purification Module: A New Tool for the Purification of Recombinant E-tagged ScFv Antibodies

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Abstract

Selection of recombinant antibodies by the phage display technique and expression of soluble functional fragments in *E.coli* has become a new tool for many scientists. Purification of the expressed protein can be achieved by a number of methods, affinity chromatography being especially powerful.

The RPAS Purification Module is a part of Recombinant Phage Antibody System (RPAS). Soluble, functional mouse single chain fragments variable (ScFv) antibodies are produced in *Escherichia coli* using the pCANTAB 5 E expression vector. The ScFv carries a 13 amino acid peptide tag (E-tag) which is recognized by an Anti-E Tag affinity column. The ScFv binds to the Anti-E Tag column at neutral pH and is easily eluted from the column by a decrease in pH.

The work presented shows the purification of two different recombinant E-tag ScFv antibodies directly from *E. coli* periplasmic extracts. The purified material was characterised by SDS-PAGE, ELISA and Western Blot.

All purified ScFv fragments were immunologically active and >95% pure.

The purifications were performed within 10-15 minutes without using complicated instrumentation, just a simple syringe.

The influence of flow rate, sample concentration and sample volume on the yield of ScFv is presented. The results show that the binding capacity for the column, HiTrap®Anti-E Tag (Anti E-Tag Sepharose® High Performance) included in RPAS Purification Module, is approximately constant, even under different loading conditions.

The stability of the HiTrap Anti-E Tag column is high, giving a lifetime of over 20 purification cycles.

Introduction

The Recombinant Phage Antibody System (RPAS) is an integrated modular system designed for the cloning and expression of recombinant mouse antibody fragments in bacteria, and the purification of the expressed single chain fragments variable (ScFv) antibodies.

The technology, developed in collaboration with Cambridge Antibody Technology Ltd., UK, is based on a phage-display system in which antibody fragments are expressed as fusion proteins displayed on the phage surface.

When antibody genes are cloned in the phagemid vector pCANTAB 5 E, soluble recombinant antibodies can be produced for use as immunological reagents. The ScFv carries a 13 amino acid peptide tag (E-tag) which is recognized by a mouse monoclonal Anti-E tag antibody.

The system is comprised of four integrated modules:

- Mouse ScFv Module
- Expression Module
- Detection Module
- Purification Module

The work presented here will show the performance of the Purification Module.

Affinity Chromatography using mouse monoclonal Anti-E tag as a ligand, coupled to Sepharose High Performance, offers a very specific purification method for E-tagged ScFv antibodies.

This convenient, one-step purification method results in > 95% pure and active E-tagged ScFv antibodies in 10-15 minutes using pre-packed HiTrap Anti-E Tag column, a syringe and the pre-made buffers. No other special equipment is needed.

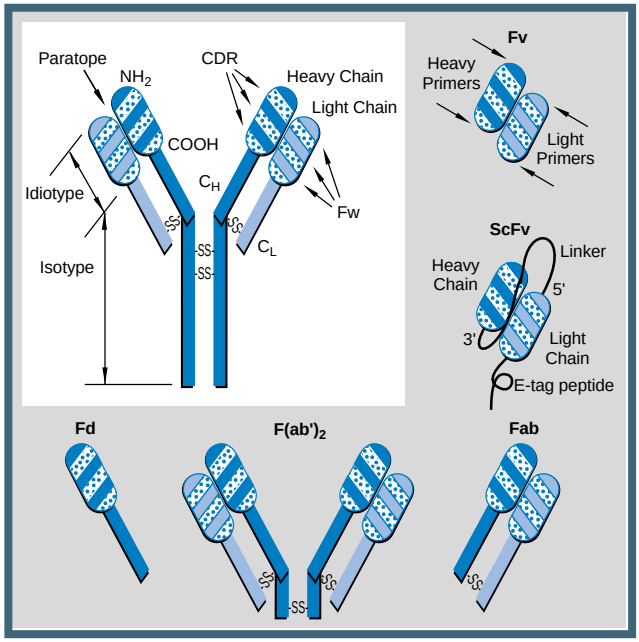

Fig. Antibody model showing subunit composition. Fragments generated by proteolytic cleavage and/or recombinant technology appear in the shaded area.

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Material & Methods

Column:	HiTrap Anti-E Tag, 5 ml
Samples:	*E.coli* periplasmic extracts, pH 7.4 containing anti-lysozyme ScFv (E-tagged), ≈11 µg/ml **or** anti-rabbit IgG ScFv (E-tagged), ≈1.5 µg/ml
Sample pretreatment:	Filtered 0.45 µm
10 x Binding Buffer:	0.2 M phosphate buffer, pH 7.0
10 x Elution Buffer:	1 M glycine, pH 3.0
Buffer pretreatment:	Dilution 1:10 of stock solutions of Binding Buffer and Elution Buffer
Fraction treatment:	100 µl Neutralisation Buffer/ml collected eluted fraction

Characteristics of HiTrap Anti E-Tag column

Column dimension:	16 x 25 mm
Bed volume:	5 ml
Ligand:	Mouse monoclonal, Anti-E tag specific
Binding capacity:	≈0.7 mg ScFv/column
Mean particle size:	≈34 µm
Bead structure:	Highly cross-linked spherical agarose
Recommended flow rate:	1-5 ml/min, 30-150 cm/h
Maximum flow rate:	20 ml/min
Maximum back pressure:	3 bar, 0.3 MPa, 44 psi
pH stability, short term:	pH 2.8-10.5

Analysis

Concentration determination

Absorbance measurement at 280 nm using Ultrospec® Plus, Amersham Biosciences

$A_{280}^{0.1\%}$ = 2.0 (according to amino acid analysis)

Purity check

SDS-PAGE electrophoresis

Gel:	PhastGel® Gradient 10-15
Sample pretreatment:	Dilution 1:6 with 15% SDS, 60 mM Tris, 6 mM EDTA, 0.06% Bromophenol Blue, pH 8.0. Heating, 3 min., 90ºC
Sample volume:	4 µl
Molecular weight standard:	Low Molecular Weight Calibration Kit (LMW), Amersham Biosciences
Staining:	Silver, according to the manufacturer's standard protocol
Instrumentation:	PhastSystem™, Amersham Biosciences

Activity and Identity check

Western Blot

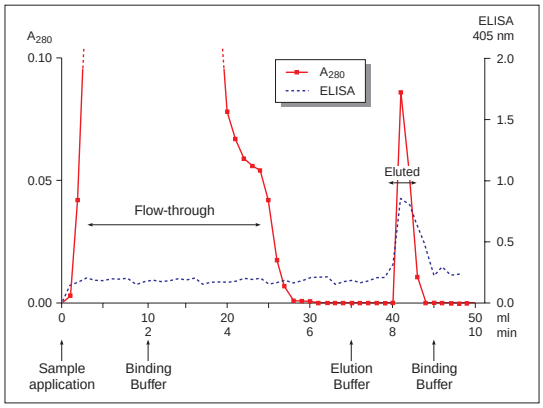
Gel:	PhastGel Gradient 10-15 (SDS-PAGE, 60Vh)
Nitrocellulose paper:	Schleicher & Schnell, BA85
Transfer buffer:	25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3
Blotting:	Electroblotting according to the manufacturer's instructions, 6 Vh, 20 min
Washing buffer:	PBS, 0.05% Tween 20, pH 7.4
Blocking buffer:	15 g non-fat dry milk in 500 ml PBS, pH 7.4
Primary antibody:	Mouse monoclonal Anti-E tag antibody, Amersham Biosciences
Secondary antibody:	Goat anti-mouse IgG alkaline phosphatase, Sigma A-7434
Substrate:	BCIP/NBT, Sigma B-5655
Instrumentation:	PhastSystem with PhastTransfer™

ELISA

Microtiter plate:	Polyvinyl microtiter plate, Falcon 3912
Coating buffer:	0.05 M sodium carbonate, pH 9.6
Washing buffer:	PBS, 0.05% Tween 20, pH 7.4
Blocking buffer:	15 g non-fat dry milk in 500 ml PBS, pH 7.4
Antigen:	Turkey egg white lysozyme, Sigma L-6255
Primary antibody:	Mouse monoclonal Anti-E tag antibody, Amersham Biosciences
Secondary antibody:	Goat anti-mouse IgG alkaline phosphatase, Sigma A-7434
Substrate:	pNPP, Sigma N-2770
Assay:	1. Coating with the antigen, 10 µg/ml in coating buffer, 200 µl/well, over-night at room temperature 2. Wash the microtiter plate, 3 x blocking buffer 3. Add 50 µl sample + 150 µl blocking buffer/well, 1 h, room temperature 4. Wash, 6 x 250 µl blocking buffer 5. Add 150 µl primary antibody/well, 1 µg/ml in blocking buffer, 1 h, room temperature 6. Wash, 6 x 250 µl blocking buffer 7. Add 150 µl secondary antibody/well, dilution 1:1000, 1 h, room temperature 8. Wash, 6 x 250 µl blocking buffer 9. Add substrate, 150 µl/well, prepared according to the manufacturer's protocol 10. Detect at 405 nm

Applications

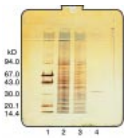
Purification of anti-lysozyme ScFv (E-tagged) from a periplasmic extract



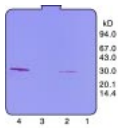
Sample volume: 10 ml periplasmic extract, anti-lysozyme ScFv (E-tagged) ≈11 µg/ml
Flow rate: ≈5 ml/min, 150 cm/h
Instrumentation: 10 ml syringe
Chromatographic procedure:
1) Conditioning of the column:
25 ml Elution Buffer and 25 ml Binding Buffer (to waste)
2) Sample application:
10 ml sample (collection of 1 ml fractions starts)
3) Wash: 25 ml Binding Buffer
4) Elution: 10 ml Elution Buffer
5) Reconditioning: 25 ml Binding Buffer (to waste)

Result:

Eluted ScFv, 3.3 ml, A_{280}: 0.058
Total amount: 96 µg
Yield: 87%

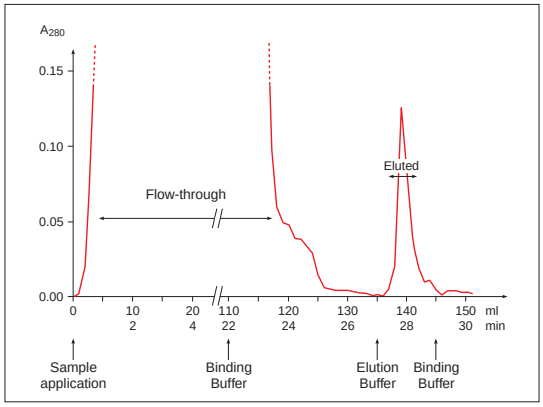


SDS-PAGE
Lane 1: Low Molecular Weight Calibration Kit
Lane 2: Starting material, periplasmic extract, dil. 1:3
Lane 3: Flow-through, dil. 1:2
Lane 4: Eluted ScFv, anti-lysozyme



Western blot
Lane 1: Low Molecular Weight Calibration Kit
Lane 2: Starting material, periplasmic extract, dil. 1:3
Lane 3: Flow-through, dil. 1:2
Lane 4: Eluted ScFv, anti-lysozyme

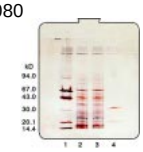
Purification of anti-rabbit IgG ScFv (E-tagged) from a periplasmic extract



Sample: 110 ml, periplasmic extract, anti-rabbit IgG ScFv (E-tagged) ≈1.5 µg/ml
Flow rate: ≈5 ml/min, 150 cm/h
Instrumentation: 10 ml syringe
Chromatographic procedure:
1) Conditioning of the column:
25 ml Elution Buffer and 25 ml Binding Buffer (to waste)
2) Sample application: 110 ml sample (collection in batch)
3) Wash: 25 ml Binding Buffer
4) Elution: 10 ml Elution Buffer (collection of 1 ml fractions starts)
5) Reconditioning: 25 ml Binding Buffer (to waste)

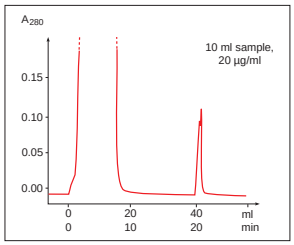
Result:

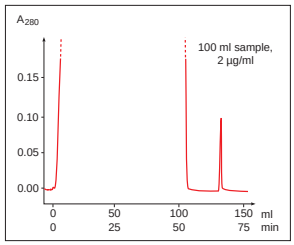
Eluted ScFv, 3.3 ml, A_{280}: 0.080
Total amount: 132 µg
Yield: 80%

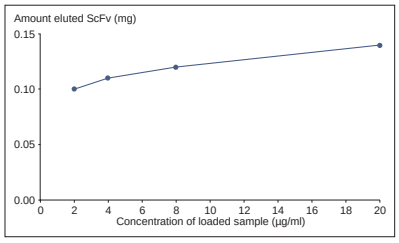


SDS-PAGE
Lane 1: Low Molecular Weight Calibration Kit
Lane 2: Starting material, periplasmic extract
Lane 3: Flow-through
Lane 4: Eluted ScFv, anti-rabbit IgG

Dependence of sample concentration for binding







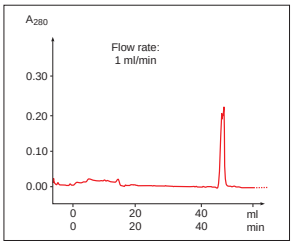
Samples: 10 ml periplasmic extract containing E-tagged ScFv diluted to different loading concentrations with PBS, 1 mM EDTA, pH 7.4
1) 10 ml, 20 µg/ml
2) 25 ml, 8 µg/ml (not shown)
3) 50 ml, 4 µg/ml (not shown)
4) 100 ml, 2 µg/ml
Total amount ScFv loaded/run: 200 µg
Flow rate: 2 ml/min, 60 cm/h
Instrumentation: FPLC® System with FPLCdirector™

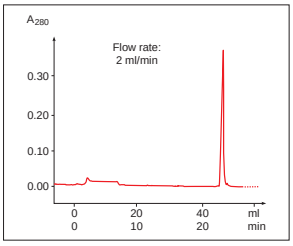
Results:

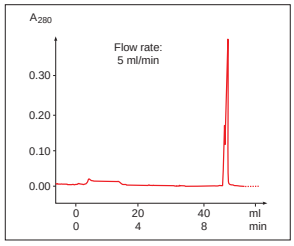
Loading sample volume ml	Loading sample conc µg/ml	Eluted ml	Eluted A_{280}	Eluted Tot. mg
10	20	3.3	0.084	0.14
25	8	4.4	0.056	0.12
50	4	3.3	0.069	0.11
100	2	3.3	0.061	0.10

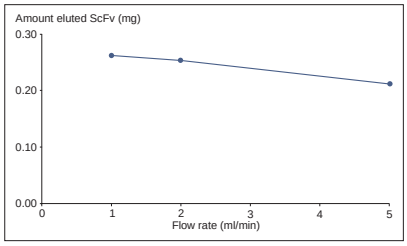
The binding of E-tagged ScFv to HiTrap Anti-E Tag column is relatively insensitive to the ScFv concentration in the sample.

Dependence of flow rate for binding









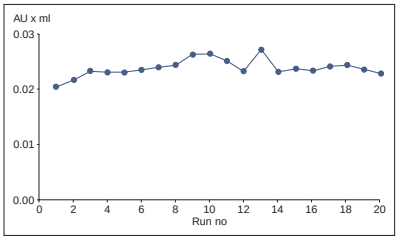
Samples: 10 ml **pure** E-tagged ScFv/run
Flow rate: 1) 1 ml/min, 30 cm/h
2) 2 ml/min, 60 cm/h
3) 5 ml/min, 150 cm/h
Instrumentation: FPLCSystem with FPLCdirector

Results:

Flow rate		Eluted material	
ml/min	ml	A_{280}	Tot. mg
1	2.2	0.232	0.26
2	2.2	0.224	0.25
5	2.2	0.195	0.21

Binding capacity is relatively insensitive to flow rate in the range 1-5 ml/min.

Stability of HiTrap Anti-E Tag column



20 repeated purifications of E-tagged ScFv from a periplasmic extract.

Column: HiTrap Anti-E Tag, 1 ml
Sample volumes: 2.5 ml/run, periplasmic extract containing anti-lysozyme ScFv (E-tagged)
Flow rate: 0.5 ml/min, 78 cm/h
Instrumentation: FPLCSystem with FPLCdirector
Analysis: Evaluation of peak area using FPLCdirector

Results:

Run no	Peak area, AU x ml	Run no	Peak area, AU x ml
1	0.0204	11	0.0251
2	0.0216	12	0.0233
3	0.0232	13	0.0271
4	0.0230	14	0.0232
5	0.0230	15	0.0238
6	0.0236	16	0.0234
7	0.0239	17	0.0242
8	0.0244	18	0.0244
9	0.0263	19	0.0236
10	0.0264	20	0.0229

Min peak area: 0.0204 AU x ml Mean value, area: 0.0239 AU x ml
Max peak area: 0.0271 AU x ml Standard Deviation: 0.0015

This experiment shows that the column can be used for at least 20 purifications without any loss in binding capacity. No trends can be seen in the analysed data.

Discussion

Affinity chromatography is a powerful tool for a large number of specific purifications. The one step purification method makes it easy to use without recourse to complicated equipment. This work shows that with the use of a simple syringe attached to the HiTrap Anti-E Tag column, >95% pure and functional E-tagged ScFv can be obtained.

The elution is performed by a decrease in pH to pH 3.0. Addition of Neutralisation Buffer to the eluted material quickly restores the pH of the purified ScFv to a safe value after elution. This is shown by the ELISA and Western Blot results.

But as ScFv's are individuals (as are monoclonal antibodies) the ScFv can be more or less sensitive to low pH and some ScFv may be eluted at a higher pH than 3.0.

It is also shown in this work that the column can be used repeatedly for at least 20 purifications without any loss in binding capacity. The RPAS Purification Module contains buffers for 10 purifications, to enable easy and convenient start-up. Buffer recipes are also included in the kit, and more buffers can easily be prepared if necessary.

Since the kinetics and binding of the E-tag to the Anti-E Tag coupled to the column are fast and the dissociation constant is low, the binding capacity is not appreciably affected by increasing the flow rate 5 times, from 1 ml/min to 5 ml/min.

The concentration of the expressed E-tagged protein in the periplasmic extract is not critical for binding to the HiTrap Anti-E Tag column.

Conclusion

The RPAS Purification Module is a part of the Recombinant Phage Antibody System (RPAS) designed for the cloning and expression of recombinant mouse antibody fragments in bacteria. The Purification Module includes a HiTrap Anti-E Tag 5 ml column, pre-made buffers, connectors and a syringe, and makes it fast and easy to purify E-tagged ScFv antibodies of various specificity.

Greater than 95% pure and active E-tagged ScFv are purified with the use of a simple syringe in 10-15 min.

This work also shows that the binding capacity of the column is not appreciably affected by either repeated runs, increased flow rates or binding of E-tagged ScFv from low expression systems.

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