

# Multistep purification of recombinant tagged proteins for SPR studies using ÄKTExpress™

L. Pearce<sup>1</sup>, R. J. Hosse<sup>2</sup>, J. Simon<sup>3</sup>, and O. Dolezal<sup>1</sup>

<sup>1</sup> CSIRO Molecular Science and Engineering, Melbourne, Australia

<sup>2</sup> Roche Glycart AG, Schlieren, Switzerland

<sup>3</sup> GE Healthcare, Uppsala, Sweden

Recombinant tagged DNaseE7 and NC10 scFv-Im7, used for the development of a novel protein capture assay based on surface plasmon resonance (SPR) technology, were purified on ÄKTExpress system from *E. coli* lysates.

Single-step methods were replaced by unattended multistep methods, incorporating multiple chromatography techniques: Affinity (IMAC or a customized affinity chromatography [AC] column), desalting (DS), ion exchange chromatography (IEX), and gel filtration (GF). An overnight dialysis step into a low pH buffer was successfully replaced by a DS step into the same buffer, automating the purification. These methods, incorporating operator predefined UV<sub>280</sub> peak collection parameters, have enabled us to rapidly and reliably purify the recombinant tagged proteins.

## Introduction

The nuclease domain of the bacterial toxin colicin E7 (DNaseE7) and its cognate partner, the immunity protein 7 (Im7), exhibit an extremely high binding affinity of close to 10<sup>-14</sup> M (1).

We have exploited the natural high affinity interaction between Im7 and DNaseE7 to develop an SPR-based system for capture of protein-Im7 fusions (2). The DNaseE7 was immobilized onto the biosensor chip surface by standard amine coupling, thus providing a stable surface for the oriented capture of Im7 protein fusions (Fig 1). In this scenario, the Im7 moiety of NC10 scFv-Im7 tightly binds DNaseE7 and orients the scFv for specific binding to its antigen (3-2G12 Fab: anti-NC10 idiotype Fab fragment). The captured scFv-Im7 is easily removed, with the overall capture/antigen binding assay highly reproducible with very little loss of capturing capacity over more than 200 cycles (2).

This article describes the use of ÄKTExpress for a streamlined multistep purification of DNaseE7 and scFv-Im7 proteins.

## Methods

### Recombinant plasmids

The DNA sequence encoding the nuclease domain of the bacterial ColicinE7 (residues 447–573 of DNaseE7) with an N-terminal hexahistidine tag and Im7 protein were cloned into pQE30 as described previously (3) and expressed in *E. coli* M15 (REP4). The DNA sequence encoding NC10 scFv-Im7 in pGC (2) was modified to include tandem C-terminal FLAG™ (Sigma-Aldrich) and hexahistidine affinity purification tags. The resulting construct was expressed in *E. coli* TOP10F' (Invitrogen).

### *E. coli* lysate preparation

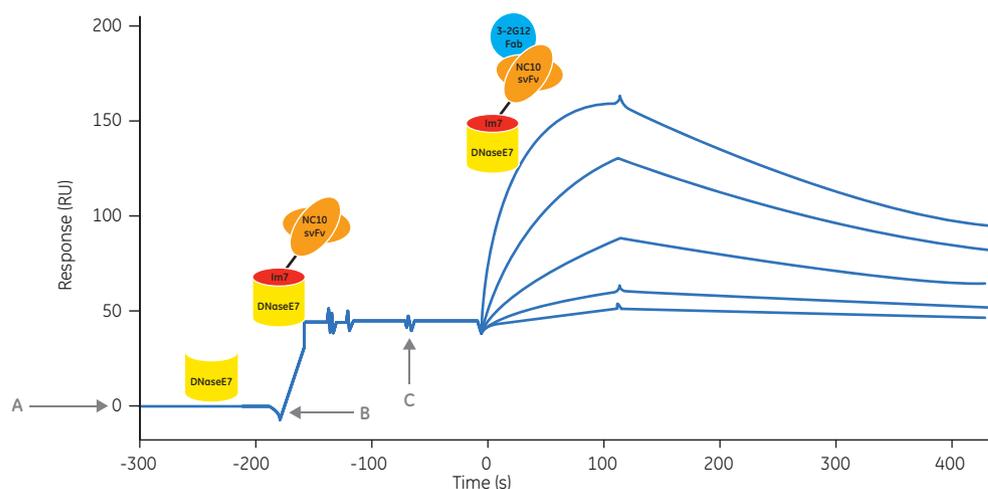
Bacterial cell pellets, harvested by centrifugation, were resuspended in 10 ml/g wet pellet weight of cellular extraction buffer: 20 mM sodium phosphate, 500 mM NaCl, 10 mM imidazole, 2 mM MgCl<sub>2</sub>, pH 7.4. To facilitate cellular lysis and reduce viscosity prior to cell crushing, we added 0.25 mg/ml of lysozyme and 0.0025 U/μl benzonase (Novagen). The lysate was then passed three times through an EmulsiFlex™-C5 cell crusher (Avestin, 15 000 psi/4°C) and centrifuged at 48 300 × g for 15 min at 4°C. The clarified lysate was filtered through a 5 μm syringe filter prior to sample loading.

### Customized affinity column

Previously described (4) anti-FLAG (DYKDDDDK) monoclonal antibody (mouse IgG1/κ, 41 mg, 8.25 mg/ml) was coupled to a 5 ml HiTrap™ NHS-activated HP column at room temperature for 50 min by recirculation with a syringe according to the manufacturer's instructions.

### Purification of tagged proteins

Utilizing the air sensor enabled loading feature, clarified lysate from an *E. coli* culture was purified on ÄKTExpress. (His)<sub>6</sub>-DNaseE7 was purified using a three-step method (IMAC, DS, and IEX, Fig 2). NC10 scFv-Im7-FLAG-(His)<sub>6</sub> was purified in a four-step method (IMAC, DS, AC, and GF, Fig 3).



**Fig 1.** High affinity capture assay of NC10 scFv-Im7 fusions on DNaseE7 biosensor surface. Overlaid and partially processed SPR binding sensorgrams consisting of: **A)** Biosensor chip surface containing amine-coupled DNaseE7 (3651 RU); **B)** Injection and capture of NC10 scFv-Im7 fusion protein resulting in an oriented immobilization of antibody fragments; **C)** injection and binding of 3-2G12 Fab (NC10 anti-idiotype Fab fragment, at 0.333, 1, 3, 9 and 27 nM) to the captured scFv. DNaseE7 capture surface was regenerated with 10 mM glycine, pH 2.0 (not shown here) as previously described (2).

## Results and discussion

### Three-step purification of tagged DNaseE7

The soluble lysate from *E. coli* cells expressing hexahistidine-tagged DNaseE7 in complex with Im7, was purified on ÄKTExpress preprogrammed with a three-step chromatography method (Fig 2). The sample was initially passed over a HisTrap™ HP 1 ml column to quickly capture the tagged protein complex from contaminating *E. coli* proteins. The captured protein was eluted with a step gradient of 250 mM imidazole. A peak (peak 1,  $UV_{280} > 50$  mAU) of 3.9 ml was collected into an intermediate capillary loop.

In previous reports (3), an overnight dialysis step (20 mM glycine, pH 3) was used to break the tight interaction between DNaseE7 and Im7. To automate this purification, we substituted the dialysis step with a low pH desalting step, and both proteins of the DNaseE7 and Im7 complex were desalted as a single peak (peak 2,  $UV_{280} > 25$  mAU) of 13.4 ml, collected into intermediate capillary loops.

The desalted DNaseE7 (pI 10.91) was separated from Im7 (pI 4.21) by automatic injection of the denatured complex onto a HiTrap SP HP 1 ml IEX column. Fractions ( $UV_{280} > 30$  mAU, peak 3) were collected, pooled, and analyzed by SDS-PAGE for purity (Fig 2, insert); the yield was determined to be 13.7 mg of DNaseE7 in ~12 ml. The Im7 domain remained bound to the IEX column, and could be eluted manually with sodium phosphate buffer at neutral pH (data not shown).

#### A) IMAC

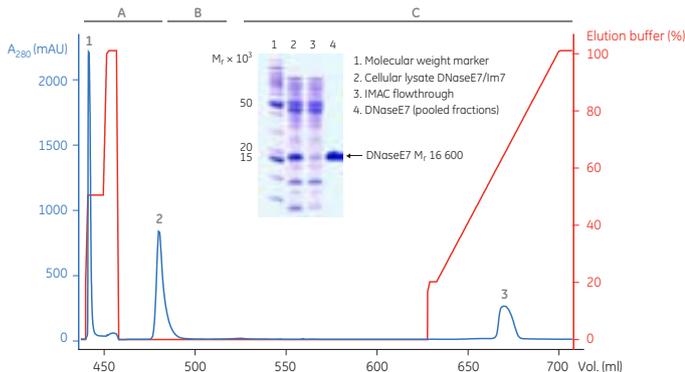
**Column:** HisTrap HP, 1 ml  
**Binding buffer:** 20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.4  
**Elution buffer:** 20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4  
**Flow rate:** 1 ml/min  
**Gradient:** Step gradient 50% elution buffer in 10 CV

#### B) DS

**Column:** HiPrep™ 26/10 Desalting, 53 ml  
**Binding buffer:** 20 mM glycine, pH 3.0  
**Flow rate:** 10 ml/min

#### C) IEX

**Column:** HiTrap SP HP, 1 ml  
**Binding buffer:** 20 mM glycine, pH 3.0  
**Elution buffer:** 20 mM glycine, 2 M NaCl, pH 3.0  
**Flow rate:** 1 ml/min  
**Gradient:** Linear gradient, 20% to 100% elution buffer in 70 CV



**Fig 2.** Three-step, automated purification of DNaseE7. Peak numbers are shown and are described in the text. Insert shows SDS-PAGE result.

### Unattended four-step purification of dual-tagged NC10 scFv-Im7

The clarified lysate containing NC10 scFv-Im7-FLAG-(His)<sub>6</sub> was purified on ÄKTExpress, utilizing four different chromatography techniques (Fig 3). The protein was initially captured on a 5 ml HisTrap FF column, and eluted with a step gradient of 250 mM imidazole. Peak 1, ( $UV_{280} > 100$  mAU) of the maximum allowable volume (7.5 ml) was collected into an intermediate capillary loop. This peak was automatically selected and injected onto a HiPrep 26/10 Desalting column, to exchange the protein into 1× PBS buffer. The desalted single peak (peak 2,  $UV_{280} > 50$  mAU) was collected into two intermediate capillary loops, then automatically injected onto the custom affinity column (anti-FLAG IgG coupled to HiTrap NHS-activated HP, 5 ml). Three minor peaks (peak region 3) were detected preceding the elution from the affinity column; these correspond to the HisTrap FF captured proteins that did not bind to the anti-FLAG IgG. A sharp peak (peak 4,  $UV_{280} > 100$  mAU) was eluted from the affinity column, and collected into the capillary loop. The affinity column was immediately neutralized by washing with 5 CV of 1× PBS buffer.

#### A) IMAC

**Column:** HisTrap FF, 5 ml  
**Binding buffer:** 20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.4  
**Elution buffer:** 20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4  
**Flow rate:** 5 ml/min  
**Gradient:** Step gradient, 50% elution buffer in 10 CV

#### B) DS

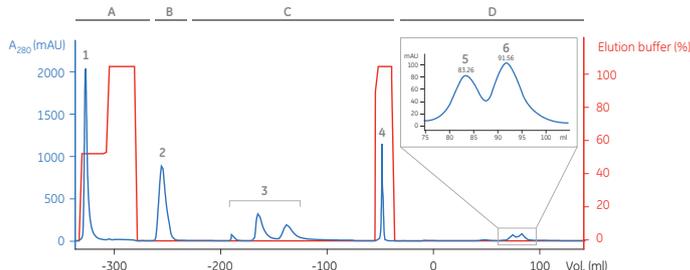
**Column:** HiPrep 26/10 Desalting, 53 ml  
**Binding buffer:** 1× PBS, pH 7.5  
**Flow rate:** 10 ml/min

#### C) AC

**Column:** HiTrap NHS-activated HP, 5 ml with anti-FLAG IgG coupled  
**Binding buffer:** 1× PBS, pH 7.5  
**Elution buffer:** 20 mM glycine, pH 2.5  
**Flow rate:** 5 ml/min  
**Gradient 1:** 100% elution buffer, 5 CV  
**Gradient 2:** 0% elution buffer, 5 CV to reneutralize antibody column

#### D) GF

**Column:** HiLoad™ 16/600 Superdex™ 200 pg, 120 ml  
**Elution buffer:** 1× PBS, pH 7.5  
**Flow rate:** 1.5 ml/min



**Fig 3.** Four-step, automated purification of dual-tagged NC10 scFv-Im7. Peak numbers are shown and are described in the text. Inset: enlarged peaks (5 and 6) show dimeric and monomeric forms of the protein, respectively.

The peak collected from the AC step was purified by GF. Two major peaks (peak 5 and 6,  $UV_{280} > 5$  mAU) corresponding to dimeric and monomeric forms of NC10 scFv-Im7 were collected. Fractions were analyzed by SDS-PAGE to assess purity (data not shown) and the yields were estimated. The dimer (peak 5) yielded 1.1 mg, the monomer (peak 6) 1.6 mg of protein.

## Conclusions

Using ÄKTExpress, single-step protein purification methods were successfully replaced by unattended multistep methods. To purify DNaseE7 from Im7 in the DNaseE7/Im7 complex, we successfully substituted a low pH dialysis step with a DS step, reducing the purification time from three days to one, whilst enabling the automation of purification. To purify dual-tagged NC10 scFv-Im7, a customized AC step rather than an IEX step was employed, which enabled automated purification of both monomeric and dimeric forms of scFv. The use of an IEX step here would have restricted us to the selection of only one of these peaks for the final GF step.

The proteins purified in this study were used to demonstrate a novel protein capture system for SPR-based studies, demonstrating high specificity, reproducible capture (> 200 cycles), oriented immobilization, stable baseline, mild regeneration conditions, and low reagent cost (2).

## References

1. Cramer, W. A. *et al.* The best offense is a good defense. *Nat. Struct. Biol.* **6**, 295–297 (1999).
2. Hosse, R. J. *et al.* Kinetic screening of antibody-Im7 conjugates by capture on a colicin E7 DNase domain using optical biosensors. *Anal. Biochem.* **385**, 346–357 (2009).
3. Ko, T. P. *et al.* The crystal structure of the DNase domain of colicin E7 in complex with its inhibitor Im7 protein. *Structure* **7**, 91–102 (1999).
4. Nuttall, S. D. *et al.* Utilization of the *Streptoalloteichus hindustanus* resistance determinant ShBle as a protein framework: Effect of mutation upon ShBle dimerization and interaction of C-terminal displayed peptide epitopes. *J. Pr. Ch.* **18(8)**, 813–821 (1999).

### Ordering information

Product	Code number
ÄKTExpress Single chromatography system	28-9089-36
HisTrap HP, 5 × 1 ml	17-5247-01
HisTrap FF, 5 × 5 ml	17-5255-01
HiPrep 26/10 Desalting column, 1 × 53 ml	17-5087-01
HiTrap SP HP, 5 × 1 ml	17-1151-01
HiTrap NHS-activated HP, 1 × 5 ml	17-0717-01
HiLoad 16/600 Superdex 200 pg, 1 × 120 ml	28-9893-35

For more information on purification of tagged proteins using ÄKTExpress system, visit [www.gelifesciences.com/akta](http://www.gelifesciences.com/akta) or [www.gelifesciences.com/protein-purification](http://www.gelifesciences.com/protein-purification)

