GE Healthcare

# Optimizing Protocols for Automated Multistep Purification of (histidine)$_6$–tagged Protein using ÄKTAxpress

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## Introduction

ÄKTAxpress™ is a chromatography system for automated multistep purification of large numbers of (histidine)$_6$- and Glutathione S-transferase (GST)-tagged proteins. Pre-optimized methods are easily created using the method wizard in UNICORN™ control software and can be further optimized using a special feature called Advanced Zone.

To increase the final purity of a target protein, parameters for each individual step can be changed. In this paper, two different optimization approaches purifying a (histidine)$_6$-tagged kinase are described.

- Increased imidazole concentration in the wash step prior to affinity elution
- Shallow gradient during ion exchange elution

**Fig 1.** ÄKTAxpress system containing 2 modules.

## ÄKTAxpress

- Automated multistep purification of affinity-tagged proteins
- Method wizard for easy creation of purification protocols
- Intelligent peak detection and collection in intermediate steps
- Optional on-column tag cleavage
- Up to four samples can be purified per module
- Up to twelve modules in parallel can be controlled from one computer

## Conclusions

The Advanced Zone allows further optimization of ÄKTAxpress purification protocols.

For a three-step protocol (Affinity – Desalting – Ion exchange),

- purifications performed with default values yielded pure test protein
- a wash with raised imidazole concentration, prior to elution in the affinity step, resulted in increased purity of the test protein
- higher resolution was obtained using a shallower gradient in the ion exchange step

ÄKTA™

All protocols start with affinity chromatography followed by different combinations of desalting, ion exchange chromatography, and gel filtration. The largest peak from each step is transferred to the next column.

Fig 2. Schematic chromatogram of an automated four-step protein purification protocol.

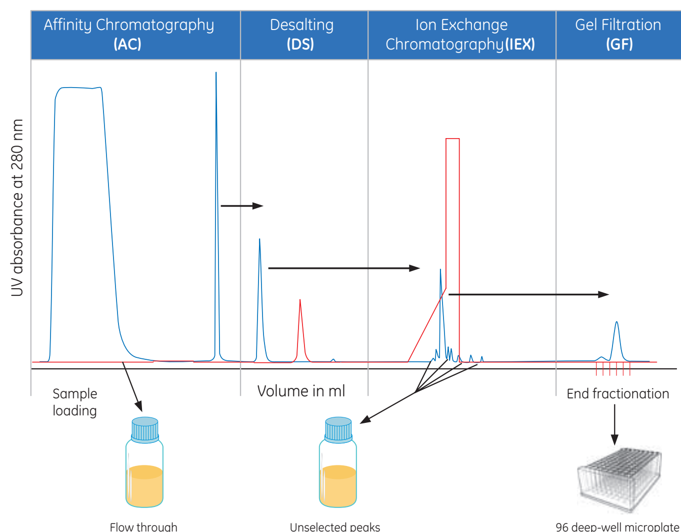



**Fig 2.** Schematic chromatogram of an automated four-step protein purification protocol.

**Table 1.** Descriptions of available multistep protocols supported by ÄKTAxpress

| Available multistep protocols | Effects from additional chromatographic steps |
|---|---|
| AC–DS | Buffer exchange |
| AC–GF | Separation from undesired aggregates and contaminants |
| AC–DS–IEX | Separation from other isoforms (e.g. heterogenously phosphorylated or glycosylated proteins) |
| AC–DS–IEX–DS | Separation from other isoforms on IEX and buffer exchange on DS |
| AC-DS-IEX-GF | Separation from other isoforms on IEX and removal of undesired aggregates and contaminants on GF |

# Material and methods

## *Sample*

A (histidine)$_6$-tagged kinase ($M_r$ 42.4x10$^3$, pI 5.75) expressed in *E. coli* was used in this study.

## *Buffers*

| | |
|---|---|
| *AC binding buffer:* | 50 mM Tris-HCl, 500 mM NaCl, 5 mM imidazole, 1 mM DTT, 10% glycerol, pH 8 |
| *AC elution buffer:* | 50 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole, 1 mM DTT, 10% glycerol, pH 8 |
| *DS and IEX binding buffer:* | 50 mM Tris-HCl, 25 mM NaCl, 1 mM DTT, 10% glycerol, pH 7.5 |
| *IEX elution buffer:* | 50 mM Tris-HCl, 1 M NaCl, 1 mM DTT, 10% glycerol, pH 7.5 |
| *GF buffer:* | 50 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, 10% glycerol, pH 7.5 |

## *Columns*

| | |
|---|---|
| *AC:* | HisTrap™ HP, 1 ml |
| *DS:* | HiPrep™ 26/10 Desalting |
| *IEX:* | Mono Q™ 5/50 GL, 1 ml |
| *GF:* | HiLoad™ 16/60 Superdex™ 75 pg |

## *Analysis*

Purity was analyzed on Coomassie™ stained ExcelGel™ 8–18 % SDS polyacrylamide gels under reduced conditions. Approximately 7.5 µg of protein was loaded per lane.

## Advanced Zone

When creating ÄKTAxpress methods, it is possible to view or edit parameters from the Advanced Zone in the method wizard. A selection of the parts of the method to be changed are made on the first page in Advanced Zone (Fig 3). In the following pages, the variables are shown and can be edited in a straightforward way.

There are many variables that can be changed in Advanced Zone; some of these are described below.

**Additional affinity wash:** By using harsher wash conditions (e.g. a raised concentration of imidazole) weak and nonspecifically bound proteins are removed. This can increase final purity.

**Ion exchange gradient:** To increase the resolution of an ion exchange column, elution flow rate can be decreased or the gradient parameters (length and target concentration) can be altered.

**Peak detection:** For peak detection, values are column dependent and can be altered when default settings do not give desired results.

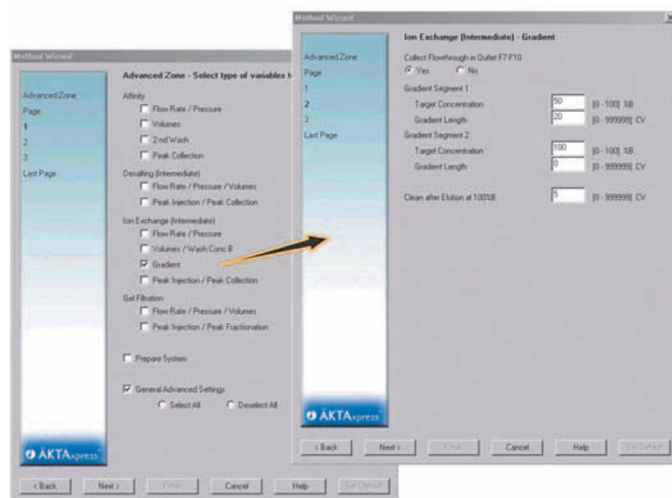


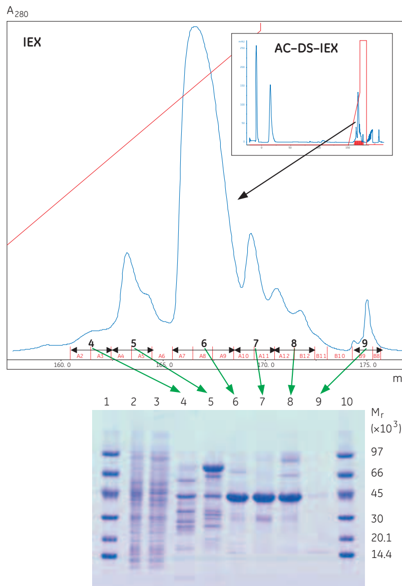

**Fig 3.** Two pages from the method wizard's Advanced Zone.

**Table 2.** Examples of variables used in a four-step protocol

| Technique | Variable | Default setting |
|---|---|---|
| Affinity (AC) | Additional wash<br>Parameters for peak detection | 0% B during 20 CV<br>Column dependent |
| Desalting (DS) | Parameters for peak detection | Column dependent |
| Ion exchange (IEX) | Flow rates<br>Gradient settings<br>Parameters for peak detection | Column dependent<br>0–50% B during 20 CV<br>Column dependent |
| Gel filtration (GF) | Parameters for peak fractionation | Column dependent |

## Optimization of the affinity and ion exchange steps

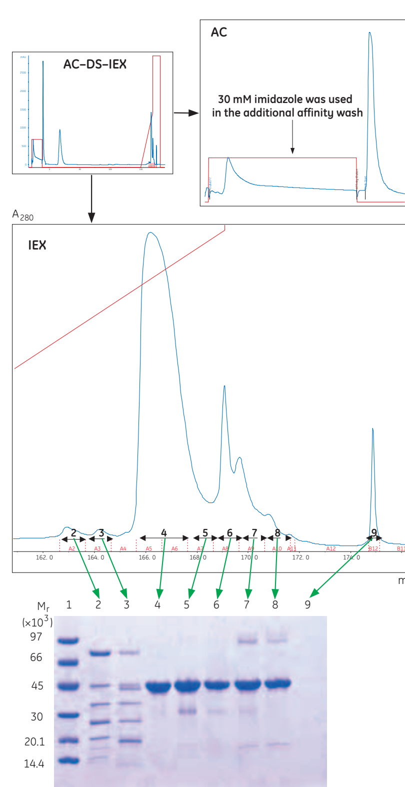
### AC–DS–IEX default




*Lane 1.* Low Molecular Weight marker
*Lane 2.* Start sample
*Lane 3.* Flow through
*Lane 4–9.* See corresponding fractions in chromatogram
*Lane 10.* Low Molecular Weight marker

**Fig 4.**

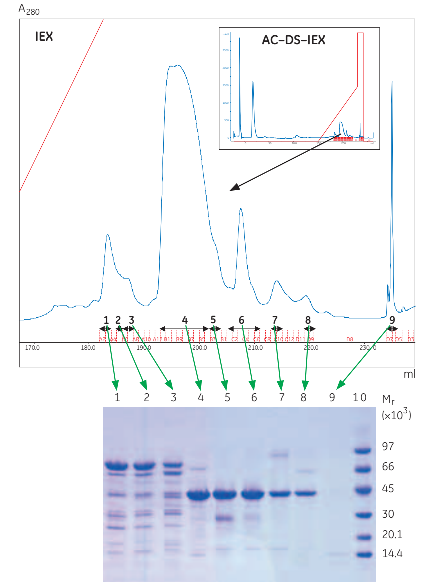
### AC–DS–IEX with additional wash




*Lane 1.* Low Molecular Weight marker
*Lane 2–9.* See corresponding fractions in chromatogram

**Fig 5.**

### AC–DS–IEX with 80 CV IEX gradient




*Lane 1–9.* See corresponding fractions in chromatogram
*Lane 10.* Low Molecular Weight marker

**Fig 6.**

## Discussion

- An additional wash prior affinity elution removed the weakly bound $M_r$ $66\times10^3$ protein and low molecular weight contaminants (compare the first peak in the chromatograms of Fig 4 and 5, as well as SDS-PAGE lane 4–6 in Fig 4 with lane 2–4 in Fig 5).
- The resolution was slightly increased using a shallower ion exchange elution gradient compared to when using the default gradient setting (Fig 6).
- By adding a gel filtration column as the fourth step, the protein was conditioned into final buffer and size homogeneity guaranteed. In this study however, the purity achieved after both three- and four-step purifications was very similar (results not shown).
- Another way to increase final purity would be to overload the affinity column. By this approach, strong binders such as Histidine-tagged proteins, will out-compete weak binding compounds during sample loading (results not shown).

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