

## Rapid Method Development With the BioLogic DuoFlow™ Chromatography System for the Purification of His-Tagged Proteins

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### Introduction

Immobilized metal affinity chromatography (IMAC) is a powerful technique that can be used for the efficient purification of recombinant histidine-tagged (His-tagged) proteins from a variety of expression systems.

The synthesis of IMAC resins begins with the derivatization of an appropriate wide-pore base resin with metal chelating groups, such as iminodiacetic acid (IDA) or nitrilotriacetic acid (NTA). Specific transition metal ions — usually  $\text{Cu}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Ca}^{2+}$ , or  $\text{Zn}^{2+}$  — are then immobilized to form a charged support. IMAC resins exhibit high affinity and metal-dependent selectivity for His-tagged proteins, as well as naturally occurring proteins that are rich in histidine, cysteine, aspartic acid, or glutamic acid residues.

To obtain highly purified proteins from an IMAC system, a series of method development experiments is typically required. These experiments involve optimization of the mobile phase pH, buffer system, flow rate, concentration of the competitive eluting agents, and a number of other potential parameters that may also be examined, depending on purity and recovery requirements. Usually, these would require a series of tedious and repetitive operations by the researcher, including the loading and recharging of the column with fresh metal ions after every run in order to ensure reproducible results.

Bio-Rad has developed an extremely rapid and efficient method using the BioLogic DuoFlow high-resolution chromatography system to purify a 75 kD His-tagged protein from crude *E. coli* lysate on a Profinity™ IMAC  $\text{Ni}^{2+}$ -charged resin. In the present study, the concentration of a competitive eluting agent, imidazole, was optimized using the BioLogic DuoFlow system by executing multiple repetitive chromatography runs with a nine-buffer system. The plumbing setup of the system included an SV5-4 select valve (5-port, 4-position) and an AVR9-8 select valve (9-port, 8-position), which were both designed for flexible automated operation. This multiple inlet port configuration, coupled with the buffer scouting feature of the BioLogic DuoFlow software, allowed us to complete this experiment with a single linked scouting run and virtually no user intervention.

### Methods

#### Setup

The column, UV detector, conductivity monitor, BioFrac™ fraction collector, SV5-4 select valve, AVR7-3 automated sample injection valve, and AVR9-8 select valve were connected to the workstation and controller as illustrated in Figure 1. All nine buffers, with the following compositions, were filtered and connected to the indicated SV5-4 and AVR9-8 valve ports:

- A1: loading or equilibration buffer (50 mM potassium phosphate, 50 mM EDTA, 300 mM NaCl, pH 7.5)
- A2: cleaning buffer (50 mM potassium acetate, 300 mM NaCl, pH 4.0)
- A3: charging buffer (100 mM  $\text{NiSO}_4$ , 300 mM NaCl, pH 4.3)
- A4: loading buffer (50 mM potassium phosphate, 300 mM NaCl, pH 8.0)
- B1: elution buffer 1 (loading buffer + 75 mM imidazole)
- B2: elution buffer 2 (loading buffer + 100 mM imidazole)
- B3: elution buffer 3 (loading buffer + 250 mM imidazole)
- B6: elution buffer 4 (loading buffer + 500 mM imidazole)
- B7: elution buffer 5 (loading buffer + 750 mM imidazole)

#### Experimental

A 1 ml Econo-Column® chromatography column (0.5 cm ID x 5 cm) was packed manually with Profinity IMAC  $\text{Ni}^{2+}$ -charged resin (60  $\mu\text{m}$ ). Five separate runs were conducted, with either 75, 100, 250, 500, or 750 mM imidazole in the elution buffer. Following elution and a subsequent wash step, the column was regenerated using the loading and charging buffers listed under Setup. These five successive runs were linked through the buffer scouting function of the BioLogic DuoFlow software. For each run, the column was preequilibrated with 10 column volumes (CV) of loading buffer at a flow rate of 1.5 ml/min. Crude *E. coli* lysate (250  $\mu\text{l}$ ) containing a 75 kD His-tagged protein was loaded into a 3 ml injection loop and injected into the column through an AVR7-3 valve. Host protein contaminants, which were not retained by the Profinity IMAC resin (Figure 2), were removed by washing the column with 10 CV of the loading buffer. The 75 kD His-tagged protein was then eluted with 5 CV of imidazole elution buffer, followed by an additional wash with 10 CV of the loading buffer to eliminate residual proteins from the column. The column was then regenerated as detailed in the following steps using

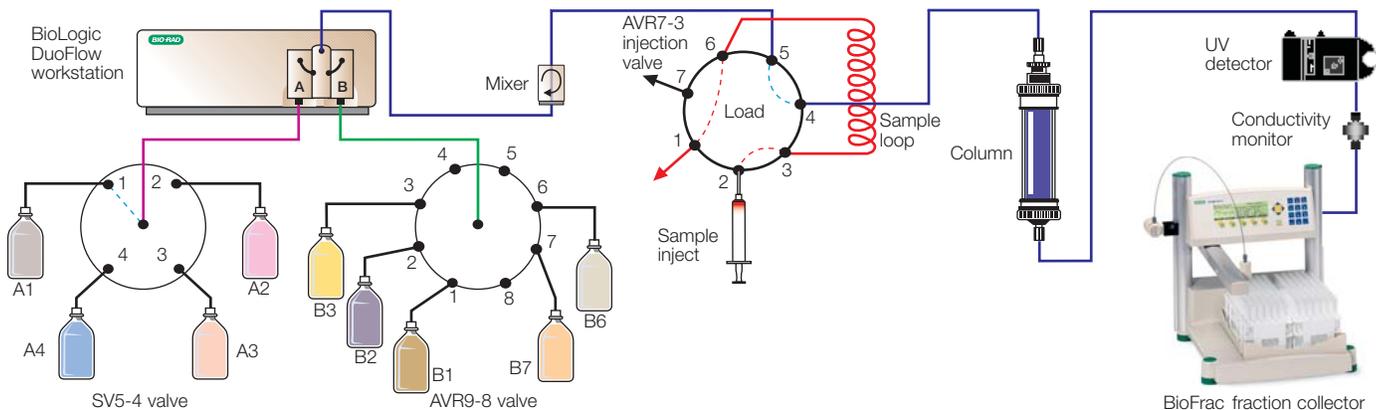


Fig. 1. System plumbing diagram.

10 CV of each buffer: metal removal with the discharging buffer; washing with the cleaning buffer; recharging of the metal ion with the charging buffer; removal of excess metal ions with the cleaning buffer; and finally, reequilibration with the loading buffer.

In order to confirm the separation of the target protein from other *E. coli* proteins in the crude sample, the peak fractions were analyzed by electrophoresis on a Criterion™ Tris-HCl 4–15% gradient polyacrylamide gel.

## Results

The elution profile during each run was monitored at 280 nm, and a series of 1 ml fractions was collected using a BioFrac fraction collector.

The resulting chromatograms obtained with the 75, 100, 250, 500, and 750 mM imidazole elution buffers were superimposed using the Trace Compare feature provided within the BioLogic DuoFlow software; these are illustrated in Figure 3.

The data in Figures 2 and 3 indicate that optimal results were obtained with an elution buffer consisting of 250 mM imidazole, 50 mM potassium phosphate, 300 mM NaCl, pH 8.0. This elution buffer provided the most effective separation and removal of the impurities (Figure 2, lanes 5 and 6, respectively).

## Conclusions

The development of rapid and efficient methods for the chromatographic isolation and characterization of biomolecules requires a chromatography system that is capable of advanced automated operation with minimal user intervention. This study demonstrates that the sophisticated valve configuration, high degree of flexibility,

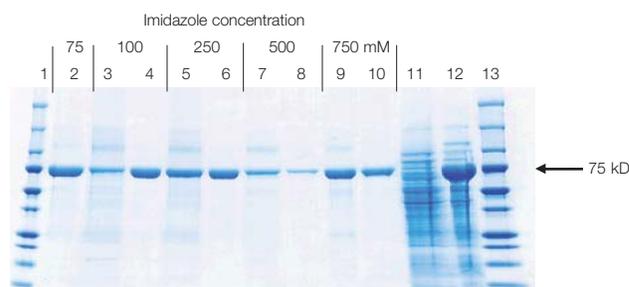


Fig. 2. Analysis of protein fractions purified using Profinity IMAC resin.

Fractions were run on a Tris-HCl 4–15% gel and stained with Coomassie Blue. Lanes 1 and 13, molecular weight markers; lanes 3, 5, 7, 9, peak 1; lanes 2, 4, 6, 8, 10, peak 2; lane 11, unbound protein; lane 12, crude *E. coli* lysate. Arrow indicates location of 75 kD His-tagged protein. Peak 1 contained impurities and the 75 kD His-tagged protein. Peak 2 contained the 75 kD His-tagged protein.

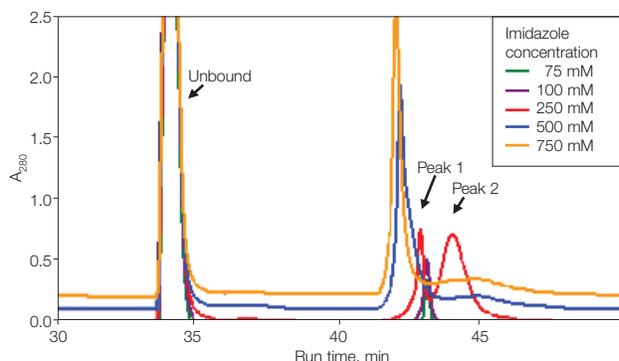


Fig. 3. Effect of imidazole concentration on resolution of the protein complex.

The 75 mM sample is aligned to the y-axis; the traces for other concentrations are offset to facilitate comparison.

and versatile software of the BioLogic DuoFlow system make it ideal for meeting the demands of a modern method development laboratory.

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