Chapter 12

Purification of Cytoskeletal Proteins by Fast Protein Liquid Chromatography (FPLC) Using an *ÄKTA Start System*

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Abstract

Actin, myosin, and tubulin are ubiquitous components of the fibrous network known as the cytoskeleton. Cytoskeletal proteins are involved in a plethora of intracellular processes such as maintenance of cellular organization, organelle translocation, and various nuclear roles including chromosome separation during mitosis. Early methods for protein extraction primarily relied on the salting-out method which was performed in conjunction with biochemical assays. Since the advent of recombinant molecular biology, protein tagging has been coupled with chromatography to obtain highly purified proteins required for sensitive assays. This chapter provides a general standard operating procedure (SOP) for using the *ÄKTA™ Start System* controlled by UNICORN software for fast protein liquid chromatography (FPLC) of 6× his-tagged cytoskeletal proteins. The protocol can readily be modified for affinity and non-affinity purification techniques using the various ÄKTA™ Chromatography Systems.

Key words Protein purification, Salting-out, Recombinant DNA technology, Affinity purification, UNICORN software, Cytiva Life Sciences

1 Introduction

Actin, myosin, and tubulin are ubiquitous components of the fibrous network known as the cytoskeleton. The importance of actin and myosin in cellular processes within non-muscle cells began to emerge with the identification of actin [1] and myosin [2] in non-muscle cells and the subsequent development of the concept of the cytoskeleton. Myosins are generally referred to as conventional if they are filament-forming and unconventional if they are non-filament-forming. Both conventional and unconventional myosins are ubiquitous in eukaryotic cells, and conventional myosin is an integral fibrous component of the cytoskeleton. Eukaryotic cells contain multiple unconventional myosins that are

Ray H. Gavin (ed.), *Cytoskeleton: Methods and Protocols*, Methods in Molecular Biology, vol. 2364,
https://doi.org/10.1007/978-1-0716-1661-1_12,

involved with diverse organelle function. Some protists are known to contain a family of unconventional myosins [3]. The actomyosin complex is important in a plethora of functions such as muscle contraction, cell organization and division, and organelle trafficking [4–10]. Tubulin is the major constituent of microtubules, which are better appreciated for their involvement in chromosome segregation [11, 12] but may also be involved in cellular processes such as osmoregulation [13]. Diverse proteins form 10-nm-diameter cytoskeleton fibers known as intermediate filaments, which are well noted for their role in nuclear envelope stabilization and other nuclear processes [14].

Traditionally, cytoskeletal proteins were primarily purified by the salting-out method [15, 16] or in conjunction with ATPase activity assays [1] (*see* **Note 1**). Such purification methods can be useful for identification of cytoskeletal proteins but may be too crude for more detailed characterization [17] as required in X-ray crystallography [18], biochemical assays [19], or microscopy studies that can visualize single molecules [20]. Antibody-based purification [21, 22], which relies on an antibody to bind to a specific epitope, can be used to purify cytoskeletal proteins [9, 21, 23]. However, the aforementioned technique may not be practical due to the inhibitory cost of antibody production for large-scale purification. With the advent of modern recombinant technology, the field of protein purification has been greatly advanced by the ability to clone a purification tag(s) specifically for a protein of interest [19, 24] such as polyhistidine [25] or maltose-binding protein tag, the latter of which may even aid in proper folding of target proteins [26]. Traditional salting-out protein purification can be further clarified using chromatography, such as ion-exchange chromatography, as performed to purify the first unconventional myosin in *Acanthamoeba castellanii* [2]. Chromatography in general offers many advantages for protein purification such as compatibility with both affinity and non-affinity purification techniques, reusability of columns, enhancement of purity, and scalability [27, 28].

This chapter provides standard operating procedures (SOP) for 6× his-tagged protein purification using the *ÄKTA Start* Fast Protein Liquid Chromatography System controlled by UNICORN software (*see* **Notes 2** and **3**). The protocols are useful for purification of proteins by chromatography through HisTrap HP column installed on an *ÄKTA Start System* and can readily be readily be adapted to the purification method of choice. The ÄKTA™ Chromatography System has been used to purify cytoskeletal proteins [20, 29, 30] and analyze cytoskeleton dynamics [31, 32], as well as purification of proteins that interact with the cytoskeletal system [33–35].

2 Materials and Equipment

1. *ÄKTA Start System* (Fig. 1).
2. Column (e.g., HisTrap HP).
3. Glass bottles and flasks (1 L).
4. Filtration glassware.
5. Membrane filters (0.2 μm).
6. Syringes (5, 50 mL).
7. Tubes (1.5, 15, 50 mL).
8. Ultrapure water.
9. 20% ethanol.
10. 10% (w/v) sodium azide.
11. 0.5 M disodium phosphate.
12. 1 M monosodium phosphate.
13. Sodium chloride.
14. Imidazole.
15. 1 M hydrogen chloride.
16. 1 M sodium hydroxide.
17. 0.5 M EDTA.

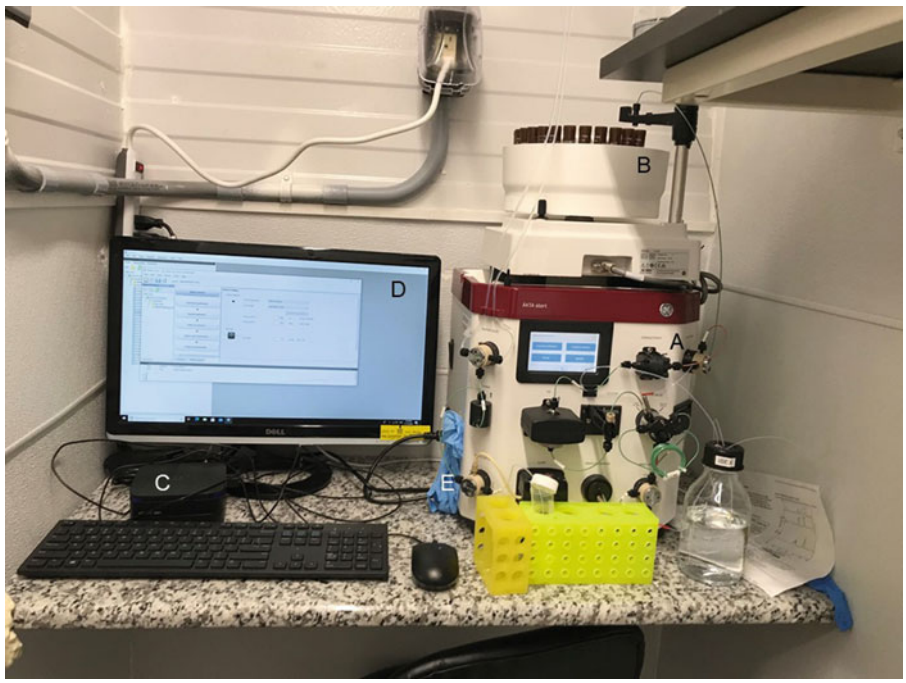

Fig. 1 FPLC setup (**a**–**e**): (**a**) ÄKTA Start, (**b**) fraction collector, (**c**) computer, (**d**) control screen, (**e**) gloves; *see* **Note 4**

3 Methods

3.1 Prepare Reagents (See Notes 4 and 5)

1. Make 1 L of 0.02% sodium azide solution in a glass bottle by diluting 2 mL of 10% sodium azide stock with 998 mL of ultrapure water.
2. Make Buffer A (lysis buffer) and Buffer B (elution buffer) in 1 L glass bottles according to compositions shown in Tables 1 and 2.
3. Prepare a suitable HisTrap HP Affinity Column (*see* **Note 7**).

3.2 Engage the ÄKTA Start Instrument (See Note 4)

1. Place Buffer A and Buffer B on top of the ÄKTA Start instrument (Fig. 2).
2. Locate inlet tubing A and B (Fig. 2). Transfer tubing A to Buffer A bottle and tubing B to Buffer B bottle.
3. Turn on the PC, and then the ÄKTA Start machine, and wait a few minutes for the instrument to initialize.
4. Double click the UNICORN start icon on the desktop to open the software. Select the following modules: *System Control*, *Method Editor*, and *Evaluation*. *System Control* is used to start, view, and control a run. *Method Editor* provides flexibility to create or edit the chromatography methods. *Evaluation* module is used to manage and evaluate results from chromatography runs.
5. In *System Control*, click "Connect" to connect the instrument to the PC.
6. The top pane of the window shows the current state of the instrument, and the bottom pane shows the real-time flow path and manual controls.

Table 1
Buffer A (lysis buffer)

Component	Formula	Stock solution or reagent	Amount of stock or reagent	Final buffer concentration
Disodium phosphate	Na_2HPO_4	0.5 M	94 mL	47 mM
Monosodium phosphate	NaH_2PO_4	1 M	3 mL	3 mM
Sodium chloride	NaCl	58.44 g/mol	17.53 g	300 mM
Sodium azide	NaN_3	10%	2 mL	0.02%
Ultrapure water	H_2O	–	Top up to 1 L	–
Total	–	–	*1 L*	

Table 2
Buffer B (elution buffer; *see* Note 6)

Component	Formula	Stock solution or reagent	Amount of stock solution or reagent	Final buffer concentration
Disodium phosphate	Na_2HPO_4	0.5 M	94 mL	47 mM
Monosodium phosphate	NaH_2PO_4	1 M	3 mL	3 mM
Sodium chloride	NaCl	58.44 g/mol	17.53 g	300 mM
Imidazole	$C_3H_4N_2$	68.08 g/mol	34.04 g	500 mM
Sodium azide	NaN_3	10%	2 mL	0.02%
Ultrapure water	H_2O	–	Top up to 1 L	–
Hydrogen chloride	HCl	1 M	A few drops	–
Total	–	–	*1 L*	

Fig. 2 Instrument modules with annotated parts (**a**–**m**): (**a**) Bottle A; (**b**) Bottle B; (**c**) Bottle A attachment to buffer valve; (**d**) Bottle B attachment to buffer valve; (**e**) Mixer; (**f**) Sample valve; (**g**) Sample valve attachment port; (**h**) Pump; (**i**) UV monitor; (**j**) Injection valve; (**k**) Column attachment; *see* **Note 16**; (**l**) Outlet valve; (**m**) Waste bottle

3.3 Priming the Pump

1. Place all waste tubing, labeled as W1, W2, and W3, in a 500 mL glass bottle.
2. In *System Control*, select "Manual Run." Set the flow rate at 0.5 mL/min.
3. Set %B as 50% at the mixer and click the wash valve to initiate flow of 50% Buffer A and 50% Buffer B at 0.5 mL/min through the system.
4. Wait until the pump is primed with 10 mL of the buffers.
5. Set %B as 0% at the mixer to initiate 10 mL flow of 100% Buffer A at 0.5 mL/min through the system.

3.4 Install/Change a Chromatography Column on the ÄKTA Start System (See Note 8)

1. Remove the column stoppers; mount the column on the union connector if the column type requires a union.
2. Remove the tube connector from the injection valve part 1 by unscrewing the knurled fastener.
3. As a droplet emerges from the tubing, touch the tubing to the column head and begin to thread the fitting in. Leave a slight looseness of threads, which allows liquid to escape around the fitting and prevents pressure buildup in the column.
4. Wait for a few seconds until a droplet comes out from the column bottom. Screw the column into the UV detector inlet.
5. Tighten the column inlet and outlet fitting just enough to prevent leaking. Do not overtighten when connecting columns. Overtightening might break the connectors or squeeze the tubing and thereby obstructing the flow (*see* **Note 9**).

3.5 Equilibrate the Column

1. Follow the protocol in Subheading 3.3 to initiate flow of 100% Buffer A at 0.5 mL/min through the column for 10 column volume (CV).
2. Set %B as 100% at the mixer to initiate flow of 100% Buffer B at 0.5 mL/min through the column for 10 CV.
3. Repeat this procedure beginning with **step 1** in order to equilibrate the column with another 10 CV of 100% Buffer A.

3.6 Prime the Sample Tubing Using the Pump

1. Transfer 5 mL of Buffer A into a 15 mL conical tube.
2. Immerse the end of the sample valve inlet tubing in the tube.
3. Click the sample valve and waste valve to divert the flow.
4. Click "End" after the priming with required volume of buffer has been completed.
5. Carefully transfer the sample valve inlet tubing from the buffer tube to the sample tube. Make sure no air bubbles enter the tubing (*see* **Note 10**).

3.7 Prime the Sample Loop Before Injecting Sample

1. Fill a syringe with 5 mL of Buffer A.
2. Tap the syringe with a finger to move air bubbles to the top. Then push gently on the plunger to push out air bubbles.
3. Make sure that the injection valve is set to position: Load Sample.
4. Connect the syringe to the injection valve port 3.
5. Load 1.5 mL of the buffer into the sample loop.
6. Repeat all steps in Subheading 3.7 using at least 5× the loop volume before loading the sample (*see* **Note 11**).

3.8 Write Methods in UNICORN Start (See Note 12)

1. In the UNICORN software, navigate to the *Method Editor* window.
2. Under the *File* menu, select "New Method."
3. In the pop-up window, select a pre-defined method (affinity, anion exchange, cation exchange, desalting, gel filtration) for the desired type of chromatography, and then click OK.
4. Select "Method Settings" in the left pane.
5. If a GE Life Sciences prepacked column is used, find and select the column in the Column Type dropdown menu. Properties of the column will be automatically displayed.
6. If the column is not listed, select "user desired column" in the Column Type dropdown menu. Manually enter the column volume, pressure limit, and flow rate, as specified by the manufacturer.
7. Select "Prime and Equilibration" in the left pane.
8. Check the prime-the-pump box.
9. Set the equilibration volume as 5 CV.
10. In *Advanced Options*, select "Use the same flow rate as in Method Settings" to equilibrate the column with the default flow rate.
11. Set the start concentration of Buffer "B" as 0%.
12. Select "reset UV monitor (Auto Zero)" to zero the detector before loading the sample.
13. Select "Sample Application" in the left pane.
14. Select either "loop/super loop or pump" for applying the sample.
15. Enter the sample volume. If the pump will be used to apply the sample, enter a volume at least 5 mL less than the actual volume to prevent the introduction of air into the system.
16. In *Advanced Options*, enter the desired flow rate. Capture methods such as ion exchange or affinity separations usually achieve a higher binding efficiency at lower flow rates.

17. Select either "fractions" or "no fractions" for collecting the flow through.
18. Enter the desired volume per fraction if fractionation is desired.
19. The flow through will be collected in the "W3" waste tubing if fractionation is not selected.
20. Select "Wash out unbound" in the left pane.
21. Enter the desired wash volume.
22. Enter the desired flow rate in *Advanced Options*.
23. Set the start concentration of Buffer "B" as 0%.
24. Select either "fractions" or "no fractions" for collecting the wash.
25. Enter the desired volume per fraction if enabling fractionations.
26. The wash will be collected in the W3 waste tubing if fractionation is not selected.
27. Select "Elution and Fractionation" from the left pane.
28. Select either "isocratic elution" (constant elution strength) or "gradient elution" (increasing elution strength).
29. If using isocratic elution, enter the target concentration of Buffer B and desired elution volume.
30. If using gradient elution, enter the start and target concentration of Buffer B and the desired gradient volume.
31. Select "Add more steps" to add step segment(s) during the gradient elution.
32. Select "Enable Fractionation" to collect eluates in 1.5 or 15 mL tubes in the fraction collector carousel. Set the fractionation type as "fixed volume fractionation." Enter the desired fractionation volume.
33. In *Advanced Options*, enter the desired flow rate.
34. Select "Prime and Equilibration" from the left pane.
35. Set the equilibration volume as 5 CV.
36. In *Advanced Options*, select "Use the same flow rate as in Method Settings" to equilibrate the column with the default flow rate.
37. Set the start concentration of Buffer B as 0%.
38. Select "reset UV monitor" (Auto Zero) to zero the detector for subsequent runs.
39. Under the *File* menu, select "Save" and name the method. Save the method in a designated folder.

40. In *System Control*, click "Method Run" and select the saved method to perform the purification.
41. Make sure enough tubes are placed on the fraction collector carousel and the drip outlet is aligned with the tube before starting the run.

3.9 Equipment Shutdown and Short-Term Storage (See Note 13)

1. After completion of the final run of the day, transfer inlet tubing A and B to a flask of degassed 0.02% sodium azide solution.
2. In *System Control*, select "Manual Run." Set the flow rate at 0.5 mL/min.
3. Set %B as 50% at the mixer and click the wash valve to initiate flow of the sodium azide solution at 0.5 mL/min through the system.
4. Pump 20 mL of the sodium azide solution through the system.
5. End the run and leave the system filled with the sodium azide solution during the storage period.
6. Close UNICORN start software. Turn off the ÄKTA Start instrument and then the PC.

3.10 Equipment Shutdown and Long-Term Storage (See Note 14)

1. After completion of the System Short-Term Storage method (*see* Subheading 3.9), transfer inlet tubing A and B to a flask of degassed 20% ethanol.
2. Follow the protocol in Subheading 3.9 (**steps 2–5**) and start a manual run. Pump 20 mL of 20% ethanol through the system. Use the same run parameters recommended for the short-term storage procedure.
3. Pause the run when 10 mL of the ethanol has been pumped through the system.
4. Disconnect the tubing from the column.
5. Use the union mounted on the tubing connected to the UV flow cell to reconnect the flow path between the injection valve and the UV monitor (Fig. 2, i).
6. Screw the column stoppers back onto the column.
7. Continue the run until 20 mL of 20% ethanol is pumped through the system.
8. End the run and leave the system filled with 20% ethanol during the storage period.
9. Close UNICORN start software. Turn off the *ÄKTA Start* instrument, and then turn off the PC (*see* **Note 15**).
10. Store the column filled with 20% ethanol at 4 °C.

4 Notes

1. Salting-out refers to the use of ammonium sulfate precipitation which can gently precipitate proteins of interest from a cytosolic mixture using gentle fractionation techniques. Although it is considered crude, ammonium sulfate precipitation does significantly reduce downstream burdens in purification processes.
2. Cytiva Life Sciences, formerly part of General Electric, has recently been acquired by Danaher and rebranded as a standalone operating company within the Life Sciences. Cytiva offers several options for UNICORN software-controlled protein purification under the ÄKTA™ Chromatography Systems label. This chapter is based on the manual for the *ÄKTA™ Start System*, which is our recommendation for beginners. Additional information about the various options can be found on Cytiva's website (last accessed 10.23.2020): https://www.cytivalifesciences.com/en/us/shop/chromatography/chromatography-systems/akta-start-p-05773.
3. The type of column (such as size exclusion, hydrophobicity, and affinity-based) and/or tag used for purification is outside the realm of this chapter and should be worked out empirically. Although this chapter describes the procedure for a 6× His-tag purification, more helpful information regarding tag(s) selection can be found in refs. 24–28.
4. Always wear gloves during preparation of reagents as well as operation and maintenance of *ÄKTA Start*.
5. Prepare fresh reagents shortly before use. Solutions made from the highest-grade reagents and ultrapure water should be passed through a 0.2 μm filter and degassed thoroughly in an ultrasonic bath shortly before their use.
6. Buffer B serves to elute the trapped His-tag proteins from the Ni-NTA (HisTrap HP) column. Prior to using Buffer B, it must be pH balanced by adding a few drops of HCl.
7. HisTrap HP columns can be purchased directly from Cytiva Life Sciences; we recommend starting with Cat. # 17524701 which provides five (×) 1 mL HisTrap HP columns.
8. In order to avoid introducing air bubbles into the column and flow path, make a drop-to-drop connection prior to inserting the threaded fitting into its position.
9. Avoid applying excessive forces when connecting plastic fasteners in order to prevent damaging the threads of the fitting.

10. Avoid air bubbles in the flow path in order to prevent spurious readouts from the UV detector and damage to the column and pumps.
11. Samples should be clarified by centrifugation and filter sterilization immediately before introducing them into the flow path.
12. To better control the *ÄKTA Start instrument*, assemble all programmed steps into a method. Steps vary for a given column and protein of interest but typically include *Prime and Equilibration*, *Sample Application*, *Wash Out Unbound*, and *Elution and Fractionation*.
13. If the system will be unused for 1–4 days, prepare for short-term storage by filling the system with 20% ethanol or 0.02% sodium azide to prevent bacterial growth.
14. If the system will be unused for more than 4 days, prepare the system for long-term storage.
15. Keep the pump cover open when not using the system to enhance the lifetime of the pump tubing.
16. Figure 2 shows a 1 mL HisTrap HP column attached to the column holder located immediately below the letter K.

Acknowledgments

Protocols in this chapter are based on research performed at Ibex Biosciences, LLC, and was partially funded by a 2020 PSC-CUNY Adjunct/CET Professional Development Fund Award granted to M.G. The authors would like to thank the amazing CEOs at Ibex Biosciences, LLC, Michael J. Karlin, Esq, and Murat Croci, for their support in writing this manuscript.

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