The Amicon® Pro purification system: condensing the protein purification workflow into one centrifugal device

Introduction
Analyses of protein structure and function are critically dependent upon the reliable production of highly pure, functionally active, protein. Although protein production is often lengthy and consists of multiple steps, the use of fusion tags and complementary affinity resin matrices has greatly simplified the purification phase. Gravity-driven separation columns are well suited for handling large sample volumes; by contrast, most small-scale purifications employ centrifugation-based techniques.

Traditional centrifugation-based affinity purification presents certain challenges. Batch processing of resins directly in microcentrifuge tubes is subject to sample loss during aspiration. Spin columns alleviate this concern; however, due to their limited volume capacity (typically 0.5 mL), the protocol is tedious, requiring multiple spin cycles during the wash and elution steps. Moreover, while most purification workflows require additional sample neutralization, buffer exchange, and/or concentration prior to downstream analysis, no single traditional centrifugation device is capable of performing all steps in the purification process.

The Amicon® Pro purification system is an adaptable centrifugal device that couples affinity-based spin column purification with downstream sample concentration and buffer exchange. By condensing the protein preparation workflow, the Amicon® Pro system eliminates the need for multiple sample transfers, thereby minimizing protein loss.
The large exchange reservoir (up to 10 mL) accommodates a greater range of sample volumes, permitting rapid purification using up to 1 mL packed resin. The increased reservoir capacity also reduces the need for multiple centrifugation steps. Direct coupling to Amicon® Ultra 0.5 mL centrifugal filters provides simultaneous concentration during the elution phase for compatible sample sizes (200 µL packed resin). This simultaneous elution and concentration is particularly advantageous for downstream applications that benefit from concentrated samples, such as the depletion of abundant serum proteins from samples requiring proteomic analysis. The Amicon® Pro system was also specifically engineered for highly efficient diafiltration (>99.99%) in a single spin, further condensing the protein purification workflow.

The results presented in this report highlight the performance and workflow benefits outlined above.

**Materials and Methods**

**Affinity purification using the Amicon® Pro device.**

This protocol included the Bind-Wash-Elute-Concentrate and Buffer Exchange steps (following lower path in Figure 1). Each Amicon® Pro device (included in the Amicon® Pro Affinity Concentration Kit Ni-NTA, Cat. No. ACK5010NT) was assembled and His-CRP (C-reactive protein) was purified according to the user manual1,2. *E. coli* lysate (0.5 mL) containing His-CRP was incubated with 100 µL Ni-NTA HIS•Bind® Resin (Cat. No. ACR5000NT) in the device for 60 min at room temperature with gentle agitation. Unbound sample was cleared by centrifugation (at 1000 g for 1 min in a swinging bucket rotor). For the wash step, 1.5 mL Wash Buffer (Ni-NTA Buffer Kit, Cat. No. ACR5000NB) was added to the resin and centrifuged (1000 g for 1 min).

For simultaneous concentration with sample elution, an Amicon® Ultra 0.5 mL (10K NMWL) centrifugal filter was attached to the base of the exchange device. The resin was gently resuspended in 1 mL Elution Buffer (Ni-NTA Buffer Kit, Cat. No. ACR5000NB) and centrifuged at 4000 g for 15 min. In certain cases, the resulting sample...
was buffer-exchanged by centrifugation (4000 g for 15 min) using 1.5 mL phosphate-buffered saline (PBS). The concentrated protein sample (in an average volume of 45 µL) was recovered from the Amicon® Ultra 0.5 mL device by reverse spin in a microcentrifuge (1000 g for 2 min). Here and in all future references, “reverse spin” refers to the process of placing a collection tube upside-down over the Amicon® Ultra 0.5 mL device, inverting the assembly, and centrifuging.

Affinity purification using traditional 0.5 mL spin column.
His-CRP was purified using a Brand P Spin Column. Briefly, the pre-inserted small frit was replaced with a larger frit (included) to accommodate the 0.5 mL reaction size. *E. coli* lysate (0.5 mL) was incubated with 100 µL Ni-NTA HIS•Bind® Resin in the spin column for 60 min at room temperature with end-over-end mixing. The unbound fraction was cleared by centrifugation (1000 g for 1 min). Resin was washed 3 times with 0.5 mL Wash Buffer by centrifugation (1000 g for 1 min). Protein was eluted in 0.5 mL Elution Buffer by centrifugation (1000 g for 1 min). The elution steps were repeated two more times, yielding a total of 1.5 mL eluted sample.

Affinity purification by batch/slurry method.
*E. coli* lysate (0.5 mL) was incubated with 100 µL Ni-NTA HIS•Bind® Resin in a 1.5 mL centrifuge tube for 60 min at room temperature with end-over-end mixing. The unbound fraction was cleared by centrifugation (1000 g for 2 min). Resin was washed 3 times with 0.5 mL Wash Buffer by centrifugation (1000 g for 2 min). Protein was eluted in 0.5 mL Elution Buffer by centrifugation (1000 g for 2 min). The elution steps were repeated two more times for a total of 1.5 mL eluted sample.

Affinity purification using gravity-driven column.
His-CRP was purified using a 0.5-2 mL disposable plastic column, according to the user manual. Each packed column was prepared with 100 µL Ni-NTA HIS•Bind® Resin and allowed to settle for 30 min. The column was equilibrated by washing twice with 0.5 mL Binding Buffer. *E. coli* lysate (0.5 mL) was applied to the column and allowed to flow through. The column was washed 3 times with 0.5 mL Wash Buffer and protein was eluted in 1.5 mL (3 X 0.5 mL fractions) Elution Buffer.

Protein concentration and buffer exchange using the Amicon® Ultra 0.5 mL device.
Samples were processed according to the Amicon® Pro user manual. The Amicon® Ultra 0.5 mL device was inserted into a 1.5 mL microcentrifuge tube. 500 µL of the eluted fraction was added and centrifuged at 14,000 g for 15 min. This process was repeated twice more to concentrate the entire sample. In certain cases, the resulting sample was buffer-exchanged by centrifugation (14,000 g for 15 min) using 0.5 mL PBS. The process was repeated twice more to complete buffer exchange. The concentrated His-CRP sample (40 µL) was recovered from the Amicon® Ultra 0.5 mL device by reverse spin in a microcentrifuge (1000 g for 2 min).

Protein quantitation using the Direct Detect™ infrared (IR)-based spectrometer.
Assays were performed using Direct Detect™ Assay-free Sample Cards and Direct Detect™ Spectrometer. Each card contains four hydrophilic polytetrafluoroethylene (PTFE) membrane positions surrounded by a hydrophobic ring to retain analyzed sample within the device’s IR beam. All measurements were performed using 2 µL of sample per membrane position. A “buffer only” sample was also run and served as a reference blank. Sample concentration was determined in reference to a calibration method. The Direct Detect™ system requires generation of a standard curve only once, upon system installation and calibration. For all experiments, the system was calibrated using National Institute of Standards & Technology (NIST) -certified Bovine Serum Albumin (BSA) SRM927d in PBS. A series of ten concentration points from 0.125 mg/mL to 5 mg/mL was used to generate the instrument calibration curve.

Electrophoresis.
Samples (in 1X SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) loading buffer) were denatured at 70°C for 10 min and loaded onto 4-12% gradient NuPAGE® Bis-Tris (MES) Gels (Life Technologies) with 5 µL of protein molecular weight standards. Gels were run at 200 Volts for 40 min, stained/destained with SimplyBlue™ Safe Stain (Life Technologies) using the microwave method and imaged.
Results
Comparative advantages of an all-in-one protein preparation workflow

In a typical research and development-scale affinity purification workflow, sample neutralization, buffer exchange, and/or concentration are often required following the purification step. While these steps are achievable through numerous methodologies, including centrifugal platforms, no traditional device can perform all the requisite tasks in this workflow. We compared the relative performance of the Amicon® Pro purification system with a traditional 0.5 mL affinity-based spin column. Since these spin columns alone are not able to concentrate or buffer exchange samples, an Amicon® Ultra 0.5 mL device was included as part of the spin column workflow.

As shown in Table 1, the Amicon® Pro device offered a significant reduction in overall processing time (33%, a savings of 63 min) while also minimizing hands-on time by decreasing the number of steps from 31 to 15 (52% reduction). One of the key factors underlying time savings is the reduction in spin steps (3 to 1, in each case) required during the wash, elution, concentration, and buffer exchange phases.

More importantly, the Amicon® Pro device’s faster, easier process did not compromise sample yield or purity. Given the same starting parameters (resin, lysate, and binding time), the Amicon® Pro device yielded 18% more His-CRP than the 0.5 mL spin column and 53% more His-CRP than the batch slurry method (Figure 2A). One factor that contributed to this improved yield was that the Amicon® Pro device minimized sample loss during transfer between devices that typically occurs with affinity-based spin columns or slurry methods. This result also further substantiated the finding that binding of a target protein to a packed affinity resin is more efficient than in the slurry format. Both the Amicon® Pro device as well as the traditional affinity-based spin column, in which the affinity resin is sequestered and separated from the removed fraction, yielded more His-CRP than did the slurry method, in which there is loss of His-CRP (and resin) during aspiration of wash fractions. The gel image confirmed the purity of the Amicon® Pro elution fraction; even with fewer separate wash steps, no contaminating species contributed to the yield value (Figure 2B).

Table 1. Less time, fewer steps required by the Amicon® Pro workflow compared to traditional methods. The table outlines the differences between the two spin-based methodologies at the individual step and overall process levels following purification of a His-tagged protein from 0.5 mL E. coli lysate. The Total time values represent the average and standard deviation of three independent trials involving 4 devices each.

<table>
<thead>
<tr>
<th>Step</th>
<th>Traditional (0.5 mL affinity-based spin column + Amicon® Ultra 0.5 mL device)</th>
<th>Amicon® Pro purification system</th>
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<tr>
<td>Wash resin</td>
<td>2 x 1 min</td>
<td>2 x 1 min</td>
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<tr>
<td>Bind</td>
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<td>1 hour</td>
</tr>
<tr>
<td>Clear</td>
<td>1 min</td>
<td>1 min</td>
</tr>
<tr>
<td>Wash</td>
<td>3 x 1 min</td>
<td>1 x 1 min</td>
</tr>
<tr>
<td>Elute</td>
<td>3 x 1 min</td>
<td>1 x 15 min</td>
</tr>
<tr>
<td>Concentrate</td>
<td>3 x 15 min</td>
<td></td>
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<tr>
<td>Buffer exchange</td>
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<td>1 x 15 min</td>
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<tr>
<td><strong>Total time (average)</strong></td>
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<td><strong>127 +/- 2.5 min</strong></td>
</tr>
<tr>
<td><strong>Number of steps</strong></td>
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<td>15</td>
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</table>

Figure 2. Better yield, equal protein purity from Amicon® Pro purification compared to two traditional affinity purification schemes. In each case, 100 µL settled resin was used to purified His-CRP from 0.5 mL E coli lysate. (A) The graph shows the difference in total protein yield (His-CRP) from the three different bind–wash–elute methods. Bars represent the average of 12 (Amicon® Pro), 6 (0.5 mL spin column), or 4 (slurry method) independent tests. (B) A representative gel showing the various fractions derived during purification using the three methods being compared.
More efficient washing enabled by large volume reservoir

Much of the relative improvement in overall performance can be attributed to the Amicon® Pro device’s design. The large chamber of the upper exchange device has a 10 mL capacity. This larger volume provides two advantages over smaller spin-based purification devices. First, the wash and elution steps can each be achieved with a single one-minute spin. While the Amicon® Pro device was able to eliminate nearly all non-target proteins with a single 1.5 mL wash step, the smaller affinity-based spin columns and gravity column-based methods required three washes to effectively clear the resin of nonspecifically bound species (Figure 3). The large chamber capacity is even more advantageous in cases requiring more stringent washing, in which the amount of wash buffer used can easily be increased without increasing process time.

Elution without dilution

Similarly, for the elution phase, a single one-minute spin was sufficient to release >90% of bound protein (Figure 4). In fact, the single spin resulted in recovery of more recombinant protein than three elutions from the 0.5 mL spin column. Moreover, when the included Amicon® Ultra 0.5 mL filter was attached, the sample underwent simultaneous concentration during the elution step. The fraction’s final concentrated volume was, on average, 45 µL following a 15 minute spin. By combining these two steps, the Amicon® Pro device further eliminated the tedious requirement of determining protein content across the various eluted fractions before concentrating.

Figure 3. Efficient clearance of nonspecific protein with a one-spin wash. Ni–NTA agarose resin (100 µL packed volume) was used to purify His-CRP from 0.5 mL lysate using gravity-driven columns or centrifugal devices (n = 3). After unbound protein was cleared (Flowthrough), devices were washed three times (3 mL/wash for the gravity column and Amicon® Pro, 0.5 mL/wash for the competitor spin device). Each fraction was collected and total protein content was determined using the Direct Detect™ spectrometer.

Figure 4. Protein elution without sample dilution. 100 µL Ni–NTA agarose resin and 0.5 mL His-CRP lysate were added to Amicon® Pro or traditional devices (n = 3) and incubated for 1 hour. After clearance and washing, proteins were eluted from spin devices, either once in 1.5 mL Elution Buffer (Amicon® Pro) or 3 times with 500 µL Elution Buffer (Competitor spin column). While three elution steps were required for the smaller spin columns, a single spin in the Amicon® Pro device was sufficient to recover > 90% of affinity-captured protein in an average of 45 µL final volume*. For this sample, the included Amicon® Ultra 0.5 mL filter was attached, so that the sample underwent simultaneous concentration during the elution step. The fraction’s final concentrated volume was, on average, 45 µL following a 15 minute spin.
Excellent yield over wide range of sample volumes

The larger volume of the exchange chamber also allows for processing of a wider range of sample volumes. To assess the scalability of the Amicon® Pro purification process, all components (resin, lysate, buffers) in the system were linearly scaled for 0.5 – 9 mL lysate (25-900 µL resin, Figure 5). As demonstrated by the high R² value, the Amicon® Pro device showed linearity in protein yield for the range of reaction volumes tested (Figure 5A). Because of this excellent yield, even for larger sample volumes, the Amicon® Pro device facilitates purification from samples in which the concentration of target protein is low (illustrated in Figure 5B).

A.

B.

In this scalability assessment, 0.5 mL of His-CRP-containing lysate was diluted to varying degrees and subjected to purification using the Amicon® Pro device. For 100 µL resin, yield was roughly equivalent for up to 2.5 mL diluted sample volume. However, percent recovery declined significantly at sample volumes ≥ 5 mL, most likely due to reaction dilution and inadequate mixing conditions (lowered resin-target interaction). To address these challenges, binding reactions were carried out by end-over-end mixing and extended to 16 hours, and volume of packed resin was increased to 500 µL. When combined, these modifications resulted in percent recovery that was nearly equivalent to that of the standard reaction.

For larger scale reactions, two caveats must be considered. First, because the Amicon® Pro device’s ability to concentrate a sample is limited by the processing capacity of the Amicon® Ultra 0.5 mL centrifugal device, larger reactions (> 200 µL packed resin) are restricted to the Bind-Wash-Elute workflow (upper path in Figure 1). Second, to accommodate the volumes in the two largest reactions shown, it was necessary to increase the speed and duration of centrifugation to 2000 g for 3 min following bind, wash, and elution steps.

Functional benefits of the Amicon® Pro device’s unique buffer exchange design

As a final step in the purification process, buffer exchange is often required to put the protein in the proper context for downstream applications. Critical here is the ability to maintain high activity (relative to yield) of the purified fraction. Although it is gentle, dialysis is time-consuming, subject to sample loss, requires large volumes of buffer for exchange and necessitates an additional concentration step. Current centrifugal diafiltration methods require multiple rounds of buffering (with repeated sample concentration and dilution), which greatly increases the potential for protein aggregation and precipitation or loss of structural integrity. To maximize the buffering capacity of the Amicon® Pro device, the exchange tip was designed to closely match the contour of the Amicon® Ultra 0.5 mL filter, increasing active membrane surface area while reducing space between the plastic sidewalls (Figure 6) and yet metering in the new buffer with sufficient mixing to displace the original buffer in a single spin.

Figure 6. The uniquely designed interface between the exchange tube tip and the Amicon® Ultra device enables greater than 99% buffer exchange in a single spin. Buffer exchange, as shown in this diagram, was measured by the replacement of a low-molecular weight dye (yellow) with clear buffer (black arrows), while a high-molecular weight dye (blue) was retained inside the Amicon® Ultra device.
To assess diafiltration performance, equivalent eluted samples of GST–LPP (lambda protein phosphatase; a protein known to lose activity under standard 3-spin diafiltration conditions) were buffer-exchanged using the Amicon® Pro device (one 15-min spin using 1.5 mL) or an Amicon® Ultra 0.5 mL device (three 15-min spins using 0.5 mL buffer each). Following exchange, resulting fractions were assayed for phosphatase activity using paranitrophenyl phosphate (pNPP) substrate. Amicon® Pro-derived GST-LPP fractions retained higher activity than similar samples buffer-exchanged using a three-spin method (Figure 7). This finding suggested that the elimination of multiple concentration/dilution cycles had a positive impact on sample integrity, providing greater consistency of sample performance in downstream applications.

Figure 7. Gentler buffer exchange = greater sample activity. Current centrifugal diafiltration methods require multiple spin steps to achieve efficient buffer exchange. Repeated concentration/dilution cycles can have deleterious effects on protein structure, causing a reduction in total as well as specific activity. GST–LPP was buffer-exchanged using an Amicon® Pro device or an Amicon® Ultra 0.5 mL device (n = 4). Following buffer exchange, samples were brought to an equivalent volume (50 µL) and assayed for phosphatase activity using pNPP substrate. The Amicon® Pro–derived GST-LPP demonstrated greater total activity compared to that of the same protein buffer-exchanged using a 3-spin method.

Conclusions
Reliable affinity-based purification is a prerequisite for unlocking the biochemical functionality of proteins. While numerous methods exist to handle this task, the overall workflow is tedious and suffers from a lack of continuity, increasing the potential for sample loss due to transfer between devices. As outlined above, the Amicon® Pro device provides a single centrifugal platform for small-scale purification. In comparative analyses, purification of His-tagged protein using the Amicon® Pro device proved to be faster, easier, and garnered better yields than other traditional methods.

The key benefits include reduced spin numbers at every step, simultaneous concentration during elution (or buffer exchange) step through attachment of the Amicon® Ultra 0.5 mL filter and no sample transfer during the entire process. Recovery is improved by confining the sample and workflow to a single device. Moreover, from the protein activity perspective, highly effective single-spin diafiltration offers a more efficient approach to buffer exchange than other current centrifugal options. Lastly, although designed to encompass the total workflow for a certain range of sample sizes (up to 200 µL resin), the device’s modularity permits its use in larger scale purifications (up to 10 mL total volume; 1 mL resin), enrichment of dilute samples, and single-spin buffer exchange. In summary, the Amicon® Pro purification system offers a robust, rapid solution for small-scale protein purification.

References
### Ordering Information

To choose the appropriate Amicon® Pro device, determine the molecular weight cut-off (MWCO) of your protein of interest and your desired affinity purification scheme. For convenience and ease of use, the Amicon® Pro purification kits contain devices, reagents and buffers optimized for twelve reactions. These kits are ideal for affinity purification of tagged recombinant proteins, antibody purification and depletion.

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