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Note d'application

Analytical Scale 96-well Protein A Affinity Resin-Based Purification using Andrew+™ Automation Robot Supporting Upstream Bioprocessing

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Abstract

An automated Protein A affinity purification protocol for up to 96 culture media samples in a well plate format is described. The automation uses Cytiva™ antibody purification resin on Andrew+ Liquid Handling Robot. The purification consumes 120 µL HCCF to generate 100 µL of antibody in neutral pH buffer solution. For 96 samples, the purification protocol is comprised of a 55 minute liquid handling step and a 20 minute offline mixing during the Protein A binding step. The method was validated based on 96 samples each containing 173.5 µg of antibody. The purified amount as determined by LC-UV using Waters™ BioResolve™ Protein A Affinity Column was 167.7±14.2 µg, or recovery of 96.8±8.2%. Reproducibility of the 96-sample preparation was also excellent at ±8.5%. The Andrew+ automation protocol has been published in OneLab™ library and is available for download under the name "ProA-resin purification".

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Benefits

- Rapid and automated Protein A purification using Andrew+ Robot

- Automation protocol readily available for download in OneLab library

- Dynamic protocol to cover any number of samples from 1 to 96 in a well plate format

- Robust protocol with excellent recovery and reproducibility

- Excellent chromatogram characteristics of Waters BioResolve Protein A Affinity Column for the quantification of monoclonal antibodies

Introduction

Affinity purification of recombinant monoclonal antibody (mAb) refers to the use of a binding agent to extract an antibody from a complex matrix such as harvested cell culture fluid (HCCF). It is a reversable process where the binding occurs under physiological conditions and the antibody is released by a releasing reagent, typically under acidic conditions. The pH of the final solution is neutralized to maintain the antibody's stability for long-term storage. In recombinant mAb production and analysis, Protein A (ProA) ligand, immobilized on a solid surface, is the most frequently used affinity agent.

In biotherapeutic process optimization, monitoring process- and product-related quality attributes are routinely performed in a typical BioPharm setting. The Ambr250® multi-parallel bioreactor system is a widely used platform for upstream bioprocess development. In a typical fed-batch experiment, a design of experiment (DoE) setup with sampling up to 14 days can generate a large number of samples. To enable time-course analysis of multiple product attributes efficiently and consistently, automated purification becomes a critical step of the overall workflow.

This application note describes automated purification using Andrew+ Robot and ProA ligand immobilized on resin for up to 96 HCCF samples in a 96-well plate format.[1] It is a dynamic protocol, meaning when fewer than 96 samples are needed, the user simply enters the number of samples at the start of a run. The protocol will automatically adjust and prepare the specified number of samples. The recovery and repeatability of the automated purification was evaluated by LC-UV analysis using Waters BioResolve Protein A Affinity Column where ProA affinity ligand was immobilized on chromatographic media.

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Experimental

HCCF Sample Generation

A commercially available NISTCHO cell line (RM8675 ext. NIST.gov) expressing mAb was cultured for 14 days to generate HCCF samples. Day 14 samples from multiple reactors were pulled, centrifuged, and filtered through 0.2 µm syringe filter. Samples were stored in a -80 °C freezer.

Sample Preparation for Recovery and Reproducibility

The HCCF sample was transferred into a 96-well plate and mAb was purified using Andrew+ Robot. The purified mAb and unpurified mAb in HCCF were subject to LC-UV analysis with NISTmAb (RM8671 ext. NIST.gov) as the calibration standard. All samples were injected in triplicate.

LC Method Conditions

System:	ACQUITY™ Premier BSM LC-UV System	
LC conditions:	Waters BioResolve Protein A Affinity Column, MaxPeak™ Premier, 2.1 mm × 20 mm (p/n: 186011369)	
	Mobile phase:	A: 1xDPBS B: 0.1%Formic acid
	Column temp.	25 °C
	Sample temp.	8 °C
	Injection volume	2 µL
	UV Wavelength	280 nm
	Sampling rate	80 /s
	Run time (gradient):	3 minutes
LC-software:	waters_connect Software	

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Gradient Table

Time (min)	Flow Rate (mL/min)	Composition A (%)	Composition B (%)	Curve
0.00	0.75	100	0	initial
0.50	0.75	100	0	6
0.51	0.75	0	100	6
1.00	0.75	0	100	6
1.01	0.75	100	0	6
1.20	0.75	100	0	6
1.21	0.75	0	100	6
1.40	0.75	0	100	6
1.41	0.75	100	0	6
1.60	0.75	100	0	6
1.61	0.75	0	100	6
1.80	0.75	0	100	6
1.81	0.75	100	0	6
3.00	0.75	100	0	6

The gradient wash cycle is added to minimize potential carryover.

Results and Discussion

I. Description of automated ProA affinity purification based on 96-well plate

A OneLab protocol for automated ProA affinity purification was developed for up to 96 samples in a 96-well plate format. For an Ambr250 System with 8 bioreactors and daily HCCF sampling over 14 days, one possible 96-sample layout is displayed in Figure 1.

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Figure 1. Layout of 96 samples from the Ambr250 incubation of 8 bioreactors and HCCF sampling for 14 days (samples from days 0–6 are analyzed every other day).

The automated ProA affinity purification protocol used Cytiva MabSelect™ Antibody Purification Chromatography Resin. A general description for the OneLab protocol is summarized in Table 1 and the Andrew+ Robot deck layout is shown in Figure 2A. Briefly, Protein A resin was added to a filter plate and washed with water and phosphate buffer solution using an on-board Extraction+ vacuum filtration unit. After the bottom of the filter plate was covered with a film, HCCF solution was added. The top of the filter plate was then covered with a film and the plate was transferred to an offline Eppendorf® shaker and mixed for 20 minutes. After the mixing, both top and bottom films were removed, and the plate was returned to the Andrew+ Robot. The media solution was removed via vacuum filtration; the resulting resin with bound protein was washed with phosphate buffer and water. Finally, the mAb bound to ProA resin was released following three incubations with 100 mM glycine elution buffer at pH 3.0. The eluted mAb solution was neutralized with Tris buffer solution to pH 7.2 for protein stability and long-term storage.

The total recovered volume was 100 μL, approximately 20% enrichment of mAb concentration in media solution. The total time for the purification is 75 minutes for a plate of 96 samples. Excluding 20 minutes offline mixing using Eppendorf shaker, the total unattended liquid handling time was 55 minutes. The protocol is written as a dynamic protocol, allowing it to purify any number of samples from 1 to 96 samples in a well plate. Figure 2B shows the dialog box "Select the number of samples" that appears upon execution of the protocol where the number of samples to be prepared is entered. It is recommended to enter samples as multiples of eight to enable the use of 8-channel pipette for maximized speed. A detailed part list for Andrew+ Robot Dominoes and consumables are summarized in the Appendix.

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	Description of Automated ProA Purification
1	Load ProA resin to filter plate, wash the resin with water and phosphate buffer
2	Add 120 µL HCCF solution to the filter plate
3	Shake the plate at 1250 RPM and 12 °C for 20 minutes using Eppendorf shaker
4	Wash resin-bound antibody with phosphate buffer and water
5	Add Tris neutrilization buffer to receiving plate
6	Release antibody with 30 µL 0.1 M glycine three times. Incubate for 5 minutes each time.

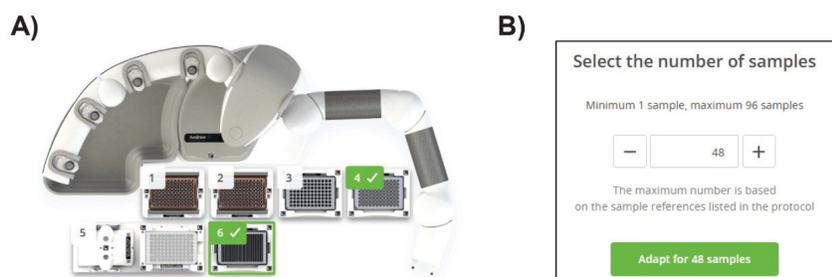*Table 1. An overview of ProA purification automation steps using Andrew+ Robot.*

Figure 2. (A) Placement of Andrew+ Dominos for the ProA affinity purification of 96 samples. Position 1&2, 300 µL pipette tips; 3, QuanRecovery™ plate; 4, HCCF sample; 5, Extraction+ with filter plate; and 6, reagent trough. Users should follow OneLab screen instruction for the final placement. (B) Dialog box for entering number of samples upon execution of the protocol.

II. Sample recovery and reproducibility

The sample recovery was determined by LC-UV using Waters BioResolve Protein A Affinity Column. The samples used were 120 µL of pooled HCCF sample containing 1.44 mg/mL of mAb; the amount of antibody in the sample is 173 µg. For reference, the binding capacity for 60 µL of 25% resin slurry used in the present protocol is calculated to be 450 µg based on 30 mg/mL dynamic binding capacity from Cytiva's product brochure.[2]

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Figure 3 shows chromatograms and calibration curve of the NISTmAb standard solutions. At the prepared

standard concentration range of 0.05–5 mg/mL, there is excellent linearity R2 = 0.998 and %deviation <10%. In

general, the column produced excellent peak characteristics, 5x narrower peak width than other columns

commonly used for titer determination.[3] The sharp peak produced by the column resulted in excellent

reproducibility for the triplicate injections used in sample analysis. Figure 4 shows %RSD of peak area from

triplicate injections of the 96 samples analyzed. The observed %RSD for all samples is <1%.
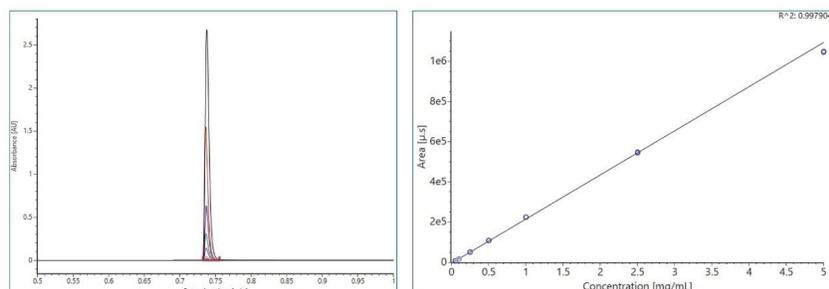

Figure 3. Chromatogram and calibration curve of NISTmAb standard

solution with concentration ranging from 0.05 mg/mL to 5 mg/mL

collected with LC-UV using Waters BioResolve Protein A Affinity Column.

%RSD	1	2	3	4	5	6	7	8	9	10	11	12
A	0.42%	0.07%	0.36%	0.10%	0.37%	0.18%	0.21%	0.10%	0.56%	0.11%	0.27%	0.28%
B	0.36%	0.08%	0.53%	0.06%	0.06%	0.31%	0.08%	0.57%	0.05%	0.09%	0.25%	0.04%
C	0.21%	0.05%	0.25%	0.02%	0.31%	0.31%	0.11%	0.50%	0.12%	0.04%	0.23%	0.07%
D	0.25%	0.04%	0.32%	0.11%	0.56%	0.20%	0.08%	0.37%	0.06%	0.06%	0.29%	0.03%
E	0.38%	0.09%	0.40%	0.06%	0.04%	0.30%	0.02%	0.20%	0.26%	0.07%	0.49%	0.26%
F	0.43%	0.11%	0.16%	0.23%	0.09%	0.46%	0.21%	0.07%	0.42%	0.27%	0.02%	0.41%
G	0.07%	0.34%	0.14%	0.05%	0.36%	0.04%	0.12%	0.15%	0.05%	0.17%	0.26%	0.10%
H	0.30%	0.12%	0.41%	0.28%	0.48%	0.01%	0.07%	0.46%	0.13%	0.04%	0.38%	0.08%

Figure 4. Peak area %RSD of triplicate injections for each of 96 samples

analyzed by LC-UV using Waters Protein A Column. The observed %RSD

for all samples is <1%.

The mAb concentration was determined for the purified samples as well as the unpurified HCCF for the

estimation of recovery of the automated purification. The determined concentration of mAb in purified sample is

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1.68±0.14 µg/µL or the amount of 168±14 µg in 100 µL final volume. The reproducibility is excellent with 8.5% RSD for 96 samples. Compared to the amount of mAb in unpurified sample 173.4 µg, the overall recovery for 96 samples was calculated to be 96.8±8.2%. Figure 5A shows the average mAb recovery obtained per well in the 96-well plate format, and Figure 5B shows the histogram of the %recovery distribution. The data showed that the reproducibility of the %recovery for 70 out of 96 samples (73%) were within ±10%, 21 out of 96 samples (22%) were within ±15%, and only 5 out of 96 samples (5%) were within ±20%. Overall, the automated purification using Andrew+ Robot is robust and highly reproducible.

A)

%RSD	1	2	3	4	5	6	7	8	9	10	11	12
A	109%	84%	86%	100%	112%	92%	92%	93%	91%	96%	87%	91%
B	98%	86%	90%	95%	94%	97%	83%	94%	85%	94%	98%	93%
C	100%	110%	95%	90%	91%	112%	96%	109%	93%	93%	88%	95%
D	100%	113%	109%	88%	108%	113%	117%	92%	103%	107%	105%	90%
E	102%	104%	92%	107%	108%	93%	92%	89%	88%	95%	91%	94%
F	104%	109%	92%	90%	109%	111%	106%	89%	95%	96%	102%	94%
G	93%	89%	106%	87%	107%	95%	88%	87%	92%	96%	84%	105%
H	97%	95%	100%	110%	94%	88%	82%	93%	93%	104%	99%	95%

B)

Figure 5. (A) Average mAb recovery per well in 96-well plate following automated ProA purification protocol, n=3. Wells are color-coded according to their value, with dark green values falling within 10% of 100% mAb recovery relative to the unpurified sample (full recovery); light green values falling within 15% of full mAb recovery; and pale yellow falling within 20% of full mAb recovery. (B) Distribution of average mAb recovery in the 96-well plate, following the same color scheme as (A). The cumulative count contributed by each range is represented as a percentage by the orange overlay line.

Conclusion

An automated ProA affinity purification protocol for 96 samples using Andrew+ Robot has been streamlined and uploaded to OneLab library. The protocol can be downloaded free of charge. The rapid purification produced a sufficient amount of antibody for multiple critical quality attribute (CQA) analyses, including intact protein, reduced protein analysis, released glycan profiling, size exclusion chromatography (SEC) and charge variant

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analysis, among others. An example of sample distribution from HCCF and ProA purified sample for various analysis is summarized in Figure 6.

Additional highlights of the automation protocol include:

- The total run time is 75 minutes. Excluding the offline plate shaking time of 20 minutes, the total unattended liquid handling time is 55 minutes.

- Minimal sample consumption (120 μL HCCF per sample).

- There is an excellent recovery of 96.8±8.2% for 96 samples preparation.

- The recovery is highly reproducible with RSD of 8.5% for 96 samples.
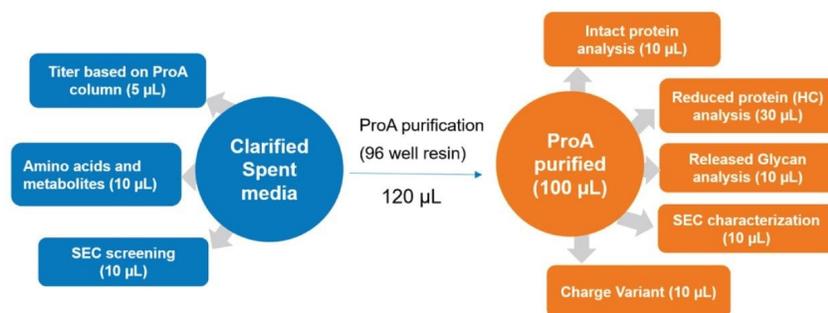
Figure 6. HCCP sample distribution for various process and product attrbutes measurement.

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Appendix

Table 2 is a summary list of Dominos and consumables used in ProA affinity purification using Andrew+ Robot. Table 3 is a summary of reagents used and their placement in reagent trough (Figure 7).

Equipment and Andrew+ Domino	Quantity	Description	Part Number
Extraction+ vacuum manifold	1	Applies vacuum to filter plate	176005201
Plate Manifold Spacers	2	One 15mm and one 17mm spacer to raise Quan Recovery plate to the filter plate	186010527
Tip Insertion System Domino	2	At least 136 tips (300 µL) distributed between two dominos arrayed in columns	186009612
Deepwell Microplate Domino	1	Holds the 12-channel reservoir	186009597
Storage plate Domino	1	Holds the QuanRecovery plate	186009596
Microplate Domino	1	Holds the 96-round well collection plate	186009600
Microplate Gripper	1	Moves the QuanRecovery plate	186009776
Pipette 8 ch-300 µL	1	Pipettor which pipettes into one 8-well column at a time	186009607
Eppendorf Thermomixer C	1	ProA resin mixing	Eppendorf 5382000023
Eppendorf SmartBlock for Microplates and Deepwell Plates	1	Holds filter plate	Eppendorf 5363000039
Consumables			
Axygen 12-channel trough	1	Holds the five reagents listed in table below	Corning RES-MW12-HP
AcroPrep Advanced 350 µL 96-well filter plate 10K membrane	1	For ProA affinity capturing	Pall or Cytiva 8019
Waters QuanRecovery 700 µL 96-well plate	1	Receives samples for released mAb from ProA resin	186009184
300 µL pipette tips	2 boxes	For use by 8-channel pipettor	700013297 (refill)
Waters 350 µL 96-round well collection plate	1	Holds 96 HCCF samples containing IgG mAb to be purified	186002643

Table 2. List of equipment, Dominos, and consumables.

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Column of 12 channel plate

Figure 7. Reagent placement in 12-channel plate.
The amount of each reagent is summarized in
Table 3.

Reagents for 96 Samples	Well Location	V (ML) for 96 Samples	Description
Water	A1-A2 (blue)	2 × 16 mL	Resin washing
Binding /wash buffer (1 x PBS)	A4-A7 (yellow)	4 × 16 mL	Resin conditioning (Sigma-Aldrich DPBS p/n D1408)
ProA Resin	A8 (light blue)	7 mL	mAb affinity capture (Cytiva, p/n 17519901)
Neutralization buffer	A10 (orange)	2 mL	1M Tris buffer, pH 7.5 (Invitrogen, p/n 15567-027)
Elution Buffer	A12 (brown)	10 mL	100 mM glycine, pH 3.0 (Sigma-Aldrich, p/n G7126)

Table 3. Reagent placement in the 12-channel reagent trough.

Instruction for reagent preparation

1. Binding/wash buffer: 1xDPBS, perform 1:10 dilution of 10xPBS (Ex. Sigma-Aldrich) using H_2O

2. ProA resin: The resin comes as 50% resin in 20% ethanol; following Cytiva's instruction, centrifuge at 1000 g for 3 minutes, then replace supernatant with 400 mM NaCl in 20% ethanol to 50% resin level. Further dilute with 1xDPBS to 25% resin as the working solution. Thoroughly mix prior to transfer to reagent trough. After each use, mark the liquid level on the tube. In next use, if liquid level is reduced due to evaporation, fill to mark with 20% ethanol, 0.4 M NaCl solution.

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3. Elution buffer: 100 mM glycine, pH adjusted to 3.0 using 5 N HCl.

4. Neutralization buffer: 1 M Tris-HCl, pH 7.5.

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