#507- Rapid, Ultrafiltration-based Method for Purification of Monoclonal Antibodies from Hybridoma Supernatants

Darshan K. Koticha, Christopher A. Barbagallo and Peter J. Rapiejko
Millipore Corporation, Biosciences Division, 17 Cherry Hill Drive, Danvers, MA, USA 01923

Abstract

Monoclonal antibodies (mAB) continue to gain importance as therapeutic and diagnostic agents for many types of cancer. The process of screening hybridoma libraries for candidate mABs is both time consuming and labor intensive. Once a hybridoma cell line expressing a suitable mAB is established, a bench-scale purification methodology (e.g. 50-500 mL) must be developed to produce sufficient mAB for further characterization. A traditional method for purifying mABs involves clarification of the hybridoma supernatant by centrifugation, followed by an ammonium sulphate precipitation to concentrate the mABs. The precipitate is then recovered by centrifugation, resolubilized and desalted using dialysis. After these steps, the mAB is further purified using Protein A/G affinity chromatography. The purified antibody is desalted and exchanged into a biological buffer using dialysis. The entire process typically requires several days to complete and can be particularly onerous if multiple mABs are to be evaluated in parallel.

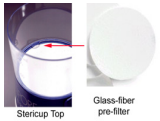
We describe a new and simplified method that minimizes the processing time to less than a day to obtain pure mAB. The method involves clarification of the hybridoma supernatant by microfiltration, followed by concentration using ultrafiltration (Saha K, et al, 1992). The mAB is further purified on Protein A/G beads. The purified mAB is desalted and buffer-exchanged using ultrafiltration. This approach was successfully used to purify an anti c-myc antibody secreted by the hybridoma clone 9E10 (Evan G, et al., 1985). The mAB purified by this new method performed comparably to the commercially purified mAB in downstream applications such as western blotting and ELISA.

The data demonstrate that the new protocol is robust and delivers mAB of a high purity and yield as compared to the traditionally purified mAB. The application of ultrafiltration to mAB purification will be of considerable value to any researcher interested in screening hybridoma libraries and accelerating the purification of mABs.

1 CLARIFICATION

Microfiltration is significantly faster and easier to use than centrifugation, for clarifying cell-containing supernatants.

Workflow for obtaining Clarified Supernatant	
Traditional	**Microfiltration**
Pipette cells into centrifuge bottles and balance weights	Pipette cells into Stericup device with AP-40 prefilter
Centrifuge to remove cells ~15 min	Apply vacuum for 1-2 min and collect supernatant in clean receiver
Collect supernatant in clean receiver	
Clean centrifuge tubes	
Total time = ~ 30 min	Total time = ~ 5 min

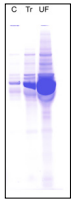


Hybridoma cells, clone 9E10 (ATCC, Manassas, VA) were grown at 37°C, 5% CO_2, in RPMI + 10% low Ig FBS (Invitrogen, Carlsbad, CA) to a final density of ~1.5 x 10^6 cells/ mL. The cells were either centrifuged at 600 x g or filtered using a Stericup device (Millipore, Billerica, MA). A glass fiber prefilter, AP40 (Millipore) was inserted into the tab in the Stericup device (red arrow), to prevent fouling and to increase speed of filtration.

2 CONCENTRATION

Ultrafiltration enables the concentration of mAB supernatant in less than 1 hr

Centricon Plus 70 device



Workflow for obtaining Concentrated Supernatant	
Traditional	**Ultrafiltration**
Weigh out ammonium sulfate	Aliquot clarified supernatant in Centricon Plus 70 device
Add ammonium sulfate to clarified supernatant with constant stirring (20-30 min). Store at 4°C	Centrifuge (20-30 min)
Centrifuge to recover pellet	Invert spin to collect sample (2 min)
Resuspend pellet	Load on protein-G column
Prepare dialysis membrane and check for integrity	
Collect resuspended precipitate in dialysis tubing/device	
Dialyze with three changes of PBS (18-24 hrs)	
Collect dialysate in centrifuge tube(s)	
Centrifuge (20-30 min)	
Load on protein-G column	
Total time required = 20 - 26 hrs	**Total time required = ~ 40 min**

C: Clarified supernatant
Tr: Supernatant precipitated with ammonium sulphate and dialyzed using 10K MWCO membrane (Spectrapor, Rancho Dominguez, CA)
UF: Supernatant concentrated by ultrafiltration on Centricon Plus 70 device (Millipore).
Samples were run on 4-12% NuPAGE® gels (Invitrogen), under reducing conditions, stained with Sypro® Orange dye (Invitrogen) and imaged on the Kodak 4000 MM gel scanner (Kodak, Rochester, NY). Protein bands are pseudo-colored blue.

3 PURIFICATION

mAB purification on Montage centrifugal columns with Prosep-G media is complete in less than 1 hr

Equilibrate the column
Add 20 mL Binding Buffer A
Spin 500 x g for 5 min

Load mAB concentrate
Spin 150 x g up to 20 min

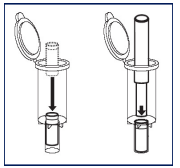
Wash the column
Add 10 mL Binding Buffer A
Spin 500 x g for 2 min

Repeat wash twice

Elute the purified mAB
Add 10 mL Elution Buffer to column
Add 1.3 mL Neutralization Buffer to collection tube
Spin 500 x g for 5 min

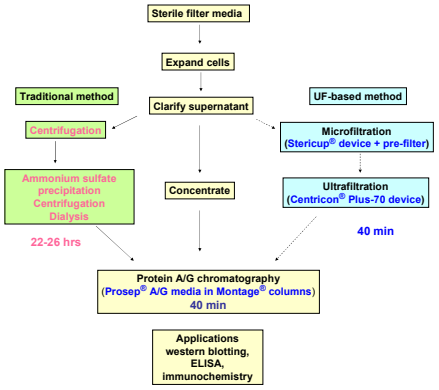
Regenerate the column
Add 10 mL Binding Buffer A to column
Spin 500 x g for 2 min
Store column at 4°C

Loading Protein-G plug in Montage column



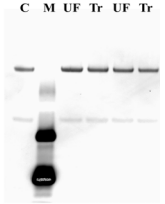
Total time required = 40 min

Process flow of mAB purification from hybridoma cells



4 PURITY

Montage columns with Prosep-G media deliver mAB of comparable purity to the commercially available mAB

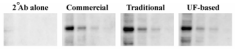


C: commercially available anti-cmyc antibody (Invitrogen)
M: Molecular weight marker
UF: mAB purified using UF-based method
Tr: mAB purified using traditional method

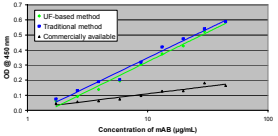
Samples (2.0 µg total protein loaded in each lane) were run on 4-12% NuPAGE gels, under reducing conditions, stained with Sypro Orange dye to assay for purity and imaged on the Kodak scanner.

5 ACTIVITY

UF-purified mAB has comparable activity to the traditionally purified mAB



Western blotting
Serial dilutions, 3-fold, of nuclear extracts of HEK 293 cells were run on 4-12% NuPAGE gels and transferred to Immobilon™-P membranes (Millipore). Blots were probed with the indicated primary antibodies and secondary anti-mouse alkaline phosphatase conjugate (Sigma, St. Louis, MO). The blots were developed with Immobilon™-AP reagent (Millipore) and imaged on the Kodak scanner



ELISA
HEK 293 cells were grown on 96-well plates (BD Biosciences, Franklin Lakes, NJ). After fixation and epitope-retrieval by heating for 10 min in a microwave, cells were permeabilized with 1% saponin (Sigma) in PBS + 2% normal donkey serum (Jackson Immunoresearch, West Grove, PA) and treated with serial dilutions of the indicated purified mABs. The cells were then washed and treated with goat anti-mouse HRP-conjugate antibody (Sigma). The reactions were developed using a SureBlue™ TMB HRP substrate (KPL, Gaithersburg, MD). The readings were measured on a Spectromax plate reader (Molecular Devices, Sunnyvale, CA).

6 SUMMARY

Purification method	Traditional	UF-based
Supernatant Starting volume	200 mL	200 mL
Supernatant Ending volume	30 mL	3.0 - 6.0 mL
Concentration factor	~7X	30 -70X
Time for concentration	18-24 hrs	30 - 40 min
Number of steps	10	3
Montage columns with Prosep-G media	~ 40 min	
Total time	~ 2 days	~ 2 hrs
Yield of purified mAB (mg)	1.6 +/- 0.2	2.06 +/- 0.2
Purity by SDS-PAGE	Comparable to commercially purified mAB	
Specific activity by ELISA-based assay		

CONCLUSIONS

- Our data show that the combination of microfiltration and ultrafiltration is a rapid method for mAB purification.
- mAB purified on Montage centrifugal columns with Protein G media, is comparable to the commercially available mAB in purity and activity.
- The UF-based mAB purification method, compared to the traditional method is:
  - faster (~2hrs compared to 2 days)
  - easier to use (fewer steps)
  - results in a higher recovery
  - and is comparable in cost

References:
1) Saha K., Case R., and Wong PK. "A simple method of concentrating monoclonal antibodies from culture supernatant by ultrafiltration". *J Immunol Methods.* 1992 Jul 6;151(1-2):307-8
2) Evan GI, Lewis GK, Ramsay G and Bishop JM. "Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product". *Mol Cell Biol.* 1985 Dec;5(12):3610-6.