MILLIPORE

Monoclonal Antibody Isolation

94-0326

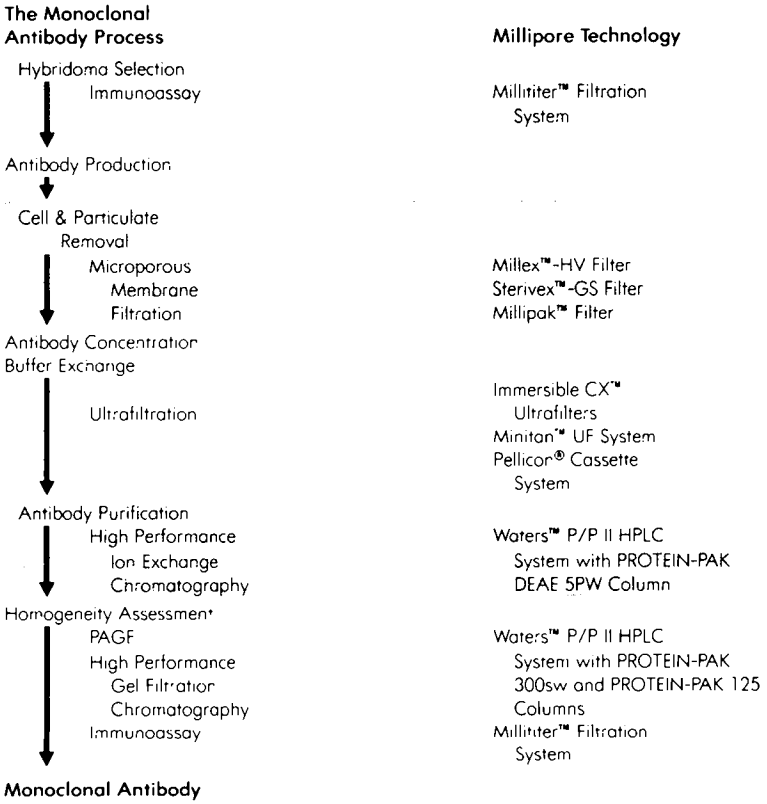
Technical Bulletin

Monoclonal antibodies, immunoglobulin proteins that are specific to a single antigen, represent a major research breakthrough. Many applications for monoclonal antibody technology have already been realized in biological research and diagnostic testing. Future expansion into medical therapy, veterinary science, and industrial purification is on the horizon. The potential for production of monoclonal antibodies in a uniform state and in unlimited quantities demands that the ability to harvest, isolate, and purify these proteins be well understood and documented.

Continuous biosynthesis of monoclonal antibodies is accomplished through the fusion of lymphocytes (B-cells) with myeloma cells to produce immortal hybridoma cells. The steps involved in the antibody production process are highlighted in Figure 1. The Millipore Corporation combines the membrane technology of the Millipore Products Division with the HPLC technology of the Waters Chromatography Division to provide an optimized scheme to facilitate the selection of individual hybridoma cells and the isolation and purification of monoclonal antibodies with high purity and yield. This scheme utilizes membrane-containing microtitration plates for hybridoma screening and antibody titer assays, microfiltration devices for cell and particulate removal, ultrafiltration devices for antibody concentration and buffer exchange, and high performance ion exchange chromatography for rapid isolation of high purity antibodies.

An entire line of Millipore and Waters products are available for each step in the monoclonal antibody process. Applications details are found in the appropriate sections of this bulletin.

Figure 1 **Millipore and Waters products for monoclonal antibody production**



Hybridoma selection

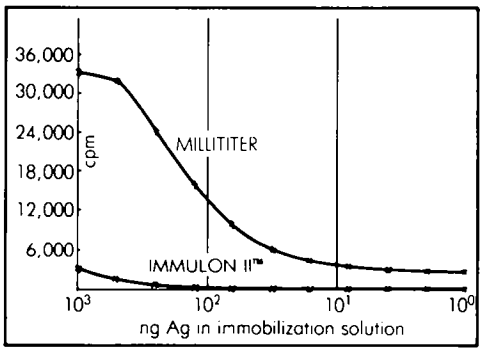
The hybridomas obtained by the fusion process must first undergo a screening procedure to identify the cells which produce the desired antibody. This involves testing the supernatant fluid from each cell line for specific immunological activity. This multi-step procedure is the most labor-intensive portion of the monoclonal antibody production process and is difficult to automate due to the complexity of assay protocols. Both radioimmunoassay (RIA) and enzyme immune assay (EIA) procedures have been developed for hybridoma screening.[1]

The time required to complete a screening assay can be reduced through the use of the Millititer™ Filtration System which combines a standard 96-well plate with membrane technology. This system utilizes vacuum filtration to eliminate tedious centrifugation or aspiration steps and permits simultaneous processing of all 96 wells. Additionally, the microporous membrane forming the base of the 96-well plate provides one hundred times more surface area than a standard well and has been shown to bind as much as ten times more IgG in a half-sandwich assay (Figure 2). Three types of microporous membranes bound to 96-well plates are available to meet the requirements of specific screening assays (Table 1).

[1]M. Stya, R. Wahl, and W. H. Beierwaltes, *Journal of Immunological Methods*, 75, 75-82 (1984)

A typical dilution curve obtained for the Millititer plates. Not only was more mouse IgG immobilized compared to the standard plate, but this antigen remained available for interaction and detection by the radiolabeled antibody.

Figure 2 Millititer plate dilution curve



Immulon II™ is a trademark of Dynatech Laboratories, Inc.

A variety of membrane types and porosities allow you to optimize the use of Millititer plates for many specific applications.

Table 1 Membrane selection for Millititer™ plates

Membrane type	Pore size	Recommended applications
Type HATF Nitrocellulose	0.45 μm	Solid-phase immunoassays (RIA, ELISA)
Type SVLP Hydrophilic DURAPORE®	5.0 μm	Immunoassays for cell surface antigens and large particulate-type antigens
Type GVWP Hydrophilic DURAPORE®	0.22 μm	Studies involving bacteria, fungi and red blood cells or requiring low protein binding membrane

Isolation of monoclonal antibodies

The hybridomas which have been selected and cloned are now suitable for larger scale production. The remaining steps in the production process focus on the isolation, purification and analysis of the monoclonal antibody products. These steps utilize membrane and chromatography technologies to clarify the antibody-containing solution, to concentrate the products, to exchange the buffer if necessary and to isolate the desired immunologically active product.

Membrane Filtration

Both ascites and tissue culture supernatants require initial processing prior to the chromatographic isolation of the final products. Membrane filtration devices are uniquely suited to the removal of cells, cell debris and other particulates. Additionally, ultrafiltration membranes having molecular weight cutoffs can be used to reduce sample complexity and to concentrate the desired protein. This process is often used to reduce the volume of a cell culture supernatant solution. Finally, buffer exchange can be accomplished by membrane filtration to facilitate sample application to a chromatographic column.

Cell and Particulate Removal

Microporous membrane filtration systems may be utilized for processing culture media, ascites, buffers and aqueous-based solutions. The selection of the device most appropriate for a specific application is dependent on the volume of fluid to be processed. The Millex™, Sterivex™ or Millipak™ products are all available with low protein binding membranes ideal for removing particulates and microorganisms from antibody solutions. Table 2 summarizes the proper microfiltration system for various application needs.

Antibody concentration and buffer exchange

To concentrate or desalt antibody samples smaller than 50 milliliters, Immersible CX ultrafilters provide optimum results. Sample volumes of 50 milliliters containing 1-10 mg/ml protein can be reduced in volume to as low as 1-2 milliliters. Immersible CX ultrafilters are available with the same polysulfone membranes used in the larger ultrafiltration systems. For example, solutions of IgE used in antigen binding assays have been concentrated using the Immersible CX devices[2]. Immersible CX ultrafilters are easy to use. Simply immerse in the solution and draw a vacuum.

If the isolation process begins with 0.2-2 liters of fluid, the Minitan™ Ultrafiltration System is best for desalting and concentrating samples to 25-30 milliliter final volumes. The system may be equipped with either microporous, low protein binding DURAPORE® membrane or highly retentive polysulfone ultrafiltration membrane plates. Using a PTHK type membrane (100,000 MW cutoff), 0.5 liter of a conditioned media containing 10 percent fetal calf serum was concentrated to 30 milliliters with 98 percent recovery of total protein. This process required one hour to complete.

The Pellicon® Cassette System is a tangential flow ultrafiltration system capable of concentrating 2-200 liters of fluid to final volumes of 0.2-2 liters. The system is ideal for concentrating and desalting high volume protein solutions. For example, this ultrafiltration system was used to process 60 liters of cell culture fluid containing monoclonal antibodies. Using a PTHK type polysulfone membrane having a 100,000 Dalton nominal molecular weight limit (NMWL), the culture fluid was concentrated in just nine hours to a final volume of four liters. In fact, this 100K NMWL membrane is tested in manufacture specifically with immunoglobulin G for quality control purposes and is guaranteed to retain at least 95 percent of the applied immunoglobulin. Table 2 summarizes the ultrafiltration systems applicable to a range of sample volumes.

[2]D. MacGlashan and L. M. Lichtenstein, *Journal of Immunology*, 130 (5), 2330-2336 (1983)

Millipore offers a variety of microfiltration and ultrafiltration systems that are selected based on initial sample volumes and solute concentrations.

Table 2 Filtration selection guide

Volume	Cell & particulate removal microfiltration	Antibody concentration ultrafiltration	Buffer exchange ultrafiltration
1-10 ml	Millex-HV	Immersible CX-30	Immersible CX-30
10-50 ml	Sterivex	Immersible CX-30 (x2)	Minitan System (1 plate)
50-200 ml	Sterivex	Minitan System (2 plates)	Minitan System (2-4 plates)
200-2000 ml	Millipak-20	Minitan System (2-4 plates)	Minitan System (6-8 plates)
2-10 L	Millipak-60	Pellicon Cassette System	Pellicon Cassette System

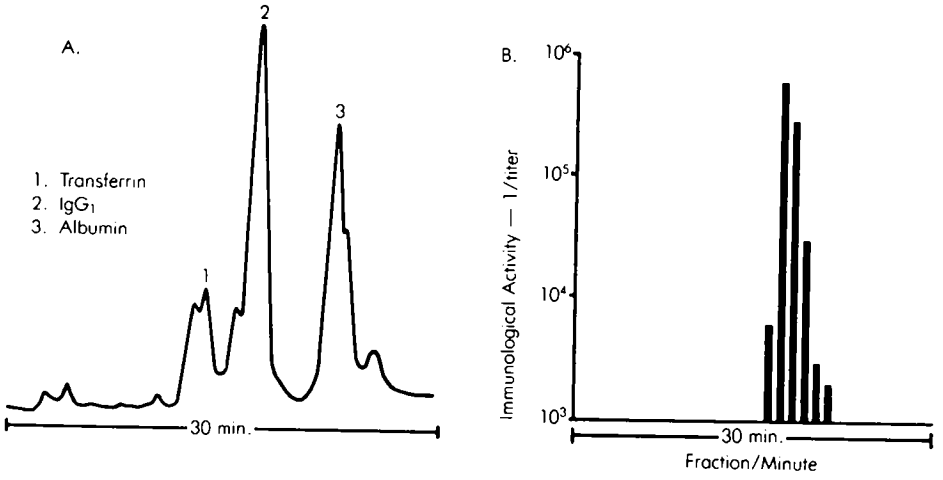
Antibody purification by high performance ion exchange chromatography

The specific immunogloblins have traditionally been isolated and purified by a combination of ammonium sulfate precipitation, affinity chromatography, i.e. Protein A or anti-Ig, and/or anion exchange chromatography. Waters HPLC systems for the high performance ion exchange isolation of proteins permit direct purification of immunoglobulins from ascites or cell culture supernatant solutions. In many cases, this high resolution technique eliminates the need for an initial affinity purification step.

The goal of the chromatographic separation is to separate the monoclonal antibody from the primary constituents of ascites and cell culture supernatants—albumin, transferrin and host immunoglobulins. The HPLC isolation uses a high efficiency anion exchange column, the PROTEIN-PAK DEAE 5PW, and a gradient elution scheme incorporating simultaneous ionic strength and pH changes. Figure 3 illustrates the separation achieved for one milliliter of a 1/20 diluted ascitic fluid sample after filtration through a Millex-HV Filter Unit. One-minute fractions were collected and assayed for immunological activity by solid-phase RIA. All injected activity was recovered with fractions 17 and 18 exhibiting the highest titer. This particular antibody was isotyped IgG_1.

A high performance ion exchange isolation of IgG from mouse ascites shows excellent resolution of IgG, transferrin and albumin (A), with high recovery of active monoclonal antibody as determined by solid-phase RIA (B). Greater than 95% of immunological activity is contained in fractions 17 and 18. Chromatographic conditions: Column: PROTEIN-PAK DEAE 5PW, 7.5 mm ID × 7.5 cm; Buffer A: 20mM TRIS, pH 8.5; Buffer B: 20mM TRIS, pH 7.0 + 0.3M NaCl; Gradient: 0 to 100% B, linear, 30 min.; Flow rate: 1.0 ml/min; Detection: 280 nm, 0.2 AUFS.

Figure 3 **Antibody isolation from mouse ascites**

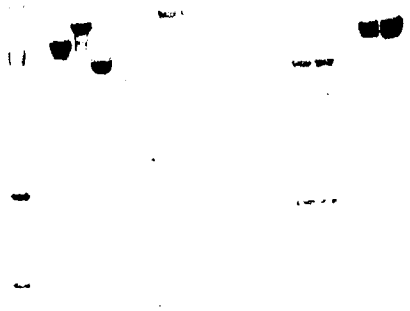


Homogeneity assessment

In addition to monitoring the activity of the isolated proteins by radioimmunoassay, fractions should also be analyzed for homogeneity. A traditional method of assaying protein samples is polyacrylamide gel electrophoresis. Using a 16 percent polyacrylamide gel run under denaturing conditions in SDS, proteins are separated on the basis of size and IgG will be denatured into its heavy and light chain subunits. Figure 4 illustrates the results obtained for the analysis of the fractions from the chromatographic isolation. Fractions 17 and 18 are shown to be free of transferrin or albumin contamination. Some minor IgG carryover is observed in the early albumin fractions. It can be concluded that the procedure successfully isolates immunologically active and homogeneous monoclonal antibody.

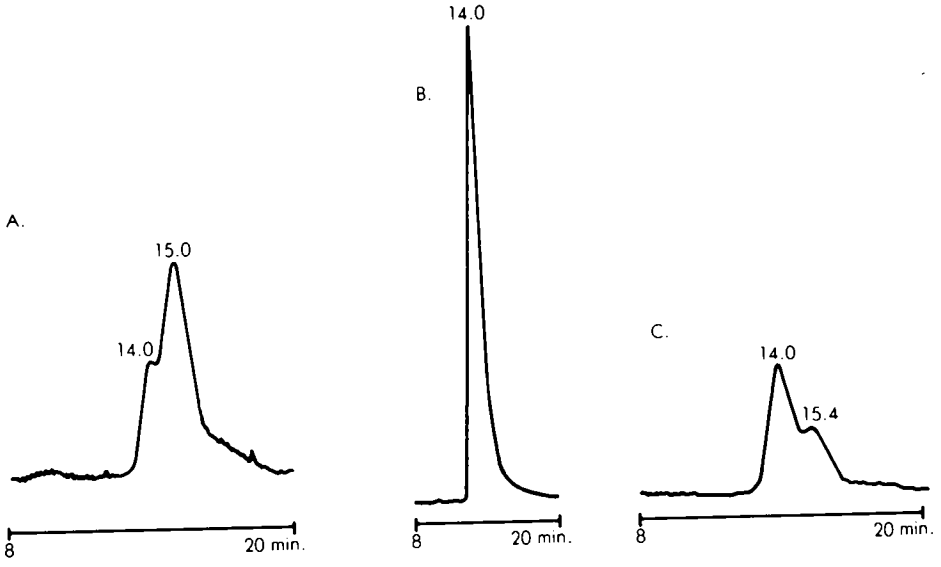
An alternative method for rapidly screening protein fractions is gel filtration HPLC. Figure 5 illustrates the analysis of selected fractions from the isolation scheme of Figure 3 using the PROTEIN-PAK 300sw and PROTEIN-PAK 125 gel filtration columns. Isocratic elution conditions in 0.1M phosphate buffer provide IgG elution at 14.0 minutes, transferrin at 15.0 minutes, and albumin at 15.4 minutes. Detection at 214 nanometers maximizes sensitivity to allow for direct analysis of small fraction aliquots. As can be seen, trace contamination of active fractions with albumin or transferrin can be readily detected. Additionally, fractions that contain IgG can be identified within 15 minutes of their collection.

Figure 4 **Analysis for homogeneity**



Polyacrylamide gel electrophoresis profiles of the fractions from the IgG isolation shown in Figure 3. Lane 2: molecular weight markers; Lanes 4-6: BSA, human transferrin and mouse IgG standards respectively; Lanes 8-20: chromatographic fractions 9-21.

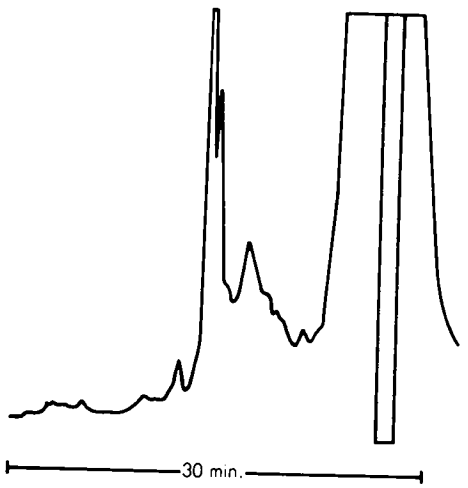
Figure 5 **Rapid screening of fractions for IgG**



High performance gel filtration analyses of active fractions show a trace of IgG with transferrin in fraction 15 (A), homogeneous IgG in fraction 17 (B), and a trace of IgG with albumin in fraction 19 (C). Chromatographic conditions: Columns: PROTEIN-PAK 300sw and PROTEIN-PAK 125; Elution buffer: 0.1M sodium phosphate, pH 6.8; Flow rate: 1.0 ml/min.; Detection: 214 nm, 0.05 AUFS.

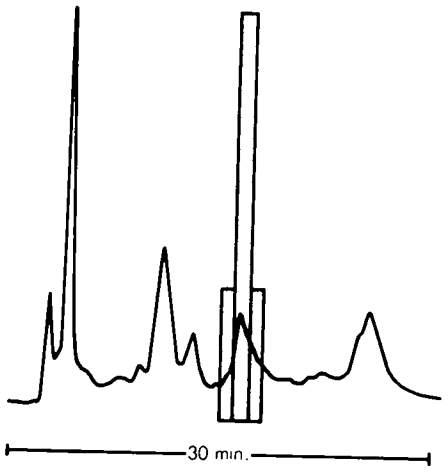
High performance ion exchange isolation of IgM from mouse ascites. Shaded area indicates immunological activity as determined by ELISA. Chromatographic conditions as in Figure 3.

Figure 6 **Isolation of IgM**



High performance ion exchange isolation of IgG, from Iscove serum-free media. Shaded area indicates immunological activity as determined by immunoperoxidase assay. Chromatographic conditions as in Figure 3.

Figure 7 **Antibody isolation from tissue culture fluid**



Additional applications of HPLC isolation of antibodies

Isolation of IgM antibodies

A second class of immunoglobulins that can be isolated by anion exchange HPLC is represented by IgM compounds. These antibodies are the first to be produced by an organism after introduction of an immunogen. These immunoglobulins have an average molecular weight of 950K Daltons and illustrate the potential for the isolation procedure to accommodate large proteins. Figure 6 illustrates the isolation of IgM from ascites. Recovery of IgM immunoactivity was documented by ELISA. Fraction 27 contained the bulk of the activity and was found to contain only a trace of albumin by gel filtration HPLC.

Isolation of monoclonal antibodies from culture fluid

As the applications for monoclonal antibodies have increased, the need for scaling-up production procedures has fostered advances in tissue culture techniques which are more easily adaptable to commercial production. Fortunately, the same procedures utilized for isolation of monoclonal antibodies from ascites can also be applied to tissue culture supernatants. Figure 7 illustrates the isolation of an IgG monoclonal antibody from an Iscove serum free media. An immunoperoxidose assay indicated that fraction 17 contains the highest immunological activity titer. Use of the Iscove media reduces the albumin content of the tissue culture supernatant solution as well as the amount of polyclonal antibodies introduced through the use of serum-containing culture media.

The choice of column for ion exchange isolation will be determined by the quantity of antibody desired. For isolations of less than 20 milligrams of antibody per hour, the PROTEIN-PAK DEAE 5PW, 7.5 mm ID × 7.5 cm is chosen. For an increased load of up to 500 milligrams of antibody per hour, the 21.5 mm × 15 cm column is employed. Table 3 summarizes the products available for the HPLC isolation and analysis of monoclonal antibodies.

Summary

The production of monoclonal antibodies requires a variety of procedures to screen hybridomas, cultivate clones and isolate pure immunoglobulins.

The Millipore Products Division and Waters Chromatography Division of the Millipore Corporation combine to offer the application expertise and optimized products for use in each step of the antibody production process. Sample processing protocols have been presented which will facilitate the rapid production of homogeneous monoclonal antibodies for subsequent use in diagnostic procedures, production purifications or other areas of basic research.

Source: M. J. Gemski and M. P. Strickler
Waters Life Science Applications Laboratory, Rockville, MD.
M. K. Gentry and B. P. Doctor
Walter Reed Army Institute of Research, Washington, DC.

Waters high performance ion exchange and gel filtration columns provide highly predictable and reproducible results when used with the Waters Protein/Peptide Isolation System II.

Table 3 High performance column selection guide

Column	Packing type	Application
PROTEIN-PAK DEAE 5PW	1000Å pore size, diethylaminoethyl-bonded hydrophilic polymer	Ion exchange isolation of monoclonal antibodies from ascites, tissue culture supernatant, or serum free media. Suitable for IgG, IgA and IgM isolations.
7.5 mm ID × 7.5 cm		≤ 20 mg antibody per hour
21.5 mm ID × 15 cm		≤ 500 mg antibody per hour
PROTEIN-PAK 300sw 7.8 mm ID × 30 cm	300Å pore size, polymerized diol-bonded silica	Gel filtration analysis of fractions from the anion exchange isolation of monoclonal antibodies
PROTEIN-PAK 125 7.8 mm × 30 cm	125Å pore size, polymerized diol-bonded silica	

Product and ordering information

Should you require additional product information, please call Millipore Corporations' respective Divisions and request the literature codes noted below.

Waters Chromatography Division,
34 Maple St., Milford, MA 01757
(617) 478-2000

Customer Sales (ordering information only) Ext. 2720

Customer Service and Applications Ext. 2630

	Literature No.	Catalog No.
Protein/Peptide Isolation System II	L22	97888 (110V/50Hz)
510 HPLC Solvent Delivery Systems (2)		97889 (120V/50Hz)
680 Automated Gradient Controller		
441 UV Absorbance Detector		
U6K Injector		
PROTEIN-PAK DEAE 5PW	L22	
7.5 mm × 7.5 cm		88044
21.5 mm × 15 cm		10640
PROTEIN-PAK 300sw, 7.8 mm × 30 cm	L22	80013
PROTEIN-PAK 125, 7.8 mm × 30 cm		84601

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Ashby Rd., Bedford, MA 01730
Technical Service Group
(800) 225-3384

Millipore membrane products

Millititer™ Vacuum Filtration Holder	PB086	XX28 096 00
Millititer HA Plate, 0.45 μm, sterile, 10/pk		STHA 096 10
Millititer SV Plate, 5.0 μm, sterile, 10/pk		STSV 096 10
Millititer GV Plate, 0.22 μm, sterile, 10/pk		STGV 096 10
Complete, Assembled Pellicon® Cassette System, 115V	AB822	XX42 00K 60
Complete, Assembled Pellicon® Cassette System, 230V		XX42 00K 50
Type PTHK Filter Cassette, 100,000 NMWL, 5.0 ft²		PTHK 000 05
Complete, Assembled Minitan™ UF System, 115V	AB822	XX42 MT0 60
Complete, Assembled Minitan™ UF System, 230V		XX42 MT0 50
Type PTHK Minitan Plates, 100,000 NMWL, 4/pk		PTHK OMP 04
Immersible™-CS Ultrafilters, 10,000 NMWL polysulfone, 25/pk	AB822	PTGC 11K 00
Immersible-CS Ultrafilters, 30,000 NMWL polysulfone, 25/pk		PTTK 11K 00
Immersible-CX Agitator, 120V		XX42 AG1 15
Immersible-CX Agitator, 230V		XX42 AG2 30
Millex® -HV Filter Unit, 0.45 μm, 50/pk	AB823	SLHV 025 NS
Millex-HV Filter Unit, 0.45 μm, 250/pk		SLHV 025 NB
Sterivex™-GS Filter Unit with filling bell, 0.22 μm, sterile, 10/pk		SVGS B10 10
Sterivex-GS Filter Unit without bell, 0.22 μm, sterile, 15/pk		SVGS 010 15
Millipak™-20 Filter Unit, 0.22 μm, sterile, 2/pk		MPGL 02S H2
Millipak-40 Filter Unit, 0.22 μm, sterile, 2/pk		MPGL 04S H2
Millipak-60 Filter Unit, 0.22 μm, sterile, 2/pk		MPGL 06S H2

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