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Application Note

Fast and Efficient Elution of Proteins from Polyacrylamide Gels Using Nanosep® Centrifugal Devices

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Polyacrylamide Gel Electrophoresis

Polyacrylamide Gel Electrophoresis (PAGE) is both an analytical and preparative tool widely used in protein analysis. Proteins are separated by PAGE in an electric field according to their size, shape and charge both in their native configuration (native PAGE) or in the presence of denaturing agents such as the ionic detergent sodium dodecyl sulfate (SDS-PAGE). A third type of electrophoretic technique, isoelectric focusing (IEF), separates proteins according to differences in their isoelectric points.

One of the most powerful applications of PAGE combines SDS-PAGE and IEF to separate proteins from complex preparations in two dimensions, first by their isoelectric points and then by their molecular weight in the presence of SDS, a technique generally known as high-resolution two-dimensional PAGE (2D-PAGE).

Advances in 2D-PAGE have revolutionized proteomics and its applications in biomedicine, biotechnology or pharmaceuticals. Proteins in complex sample preparations are first separated by 2D-PAGE, stained with fluorescent dyes, and then scanned, allowing for detailed measurements of expression levels and protein mapping. In this way, specific proteins may be identified and further characterized. This stage of protein characterization often requires extracting the proteins of interest from the polyacrylamide gels for further analyses such as mass spectrometry, HPLC, amino acid sequencing or composition analyses, and post transcriptional modification analyses.

Proteins separated on polyacrylamide gels can be transferred onto immobilized membranes (blotting). However, in many instances, a gentle method to elute the proteins directly into a liquid phase is required to avoid chemical modification or denaturation of the eluted proteins. Common approaches include elution by diffusion and electroelution[1, 5]. In the diffusion method, the protein band or spot of interest is sliced from the gel and minced into small gel pieces to increase the diffusion surface. These gel pieces are then mixed in an SDS solution with gentle agitation until the protein fraction diffuses out of the gel[1]. This method, although relatively simple and inexpensive, is not as efficient as the electroelution. The polyacrylamide matrix tends to expand in solution trapping a considerable amount of protein, which reduces the final protein recovery; therefore, it is not the method of choice when working with the microquantities of protein required in most proteomic applications.

Electroelution is more efficient than direct diffusion but requires specific electroelution devices and the eluted protein often needs further concentration and desalting[2]. As in the diffusion method, proteins are first separated by gel electrophoresis and the protein band of interest is excised from the gel. Elution is accomplished in an electric field that moves the protein out of the gel slice into a gel zone made of agarose[5], polyacrylamide[2] or polypropylene[1], depending on the specific electroelution device used. The eluted fraction is finally collected in reservoirs. The eluted protein is diluted significantly during the process and often requires further concentration and desalting. Although electroelution is generally more efficient than direct diffusion, some proteins are more resistant to electrotransfer than others and recovery yields vary[1]. Also, elution times can be long for some protein preparations, which might lead to protein denaturation.

Use of Ultrafiltration for High-Yield Protein Purification

Ultrafiltration is a technique based on membrane separation that has been applied to the separation of dissolved small molecules[1]. When a force, such as a centrifugal force, is applied, molecules smaller than the pore size of the membrane will pass through it while larger molecules will be retained. Nanosep Centrifugal Devices have been designed for application of this technique in manipulation of both protein and DNA. They are equipped with specially prepared membranes having different Molecular Weight Cutoffs (MWCO). Protein solutions can be efficiently concentrated by means of Nanosep centrifugal devices. The choice of the correct MWCO (see Table 1) also allows for fast and effective salt removal, buffer exchange and protein fractionation.

Table 1

Selection guide based on Molecular Weight Cutoff

Solute	Molecular Weight Cutoff					
	3K	10K	30K	100K	300K	0.2 µm
Vitamin B12 (1.3 kD)	5-10%	0-4%	11-79%	11-79%	11-79%	11-79%
Bacitracin (1.4 kD)	0-4%	0-4%	11-79%	11-79%	11-79%	11-79%
Cytochrome C (12.5 kD)	90-100%	80-89%	0-4%	11-79%	11-79%	11-79%
Myoglobin (17.8 kD)	90-100%	90-100%	90-100%	0-4%	11-79%	11-79%
α-Chymotrypsinogen (24.5 kD)	90-100%	90-100%	80-89%	0-4%	11-79%	11-79%
Albumin (67 kD)	90-100%	90-100%	90-100%	5-10%	0-4%	11-79%
Gamma Globulin (160 kD)	11-79%	90-100%	90-100%	11-79%	0-4%	11-79%
Thyroglobulin (669 kD)	11-79%	11-79%	90-100%	90-100%	80-89%	0-4%
IgM (960 kD)	11-79%	11-79%	11-79%	90-100%	80-89%	0-4%
Yeast	11-79%	11-79%	11-79%	11-79%	90-100%	90-100%

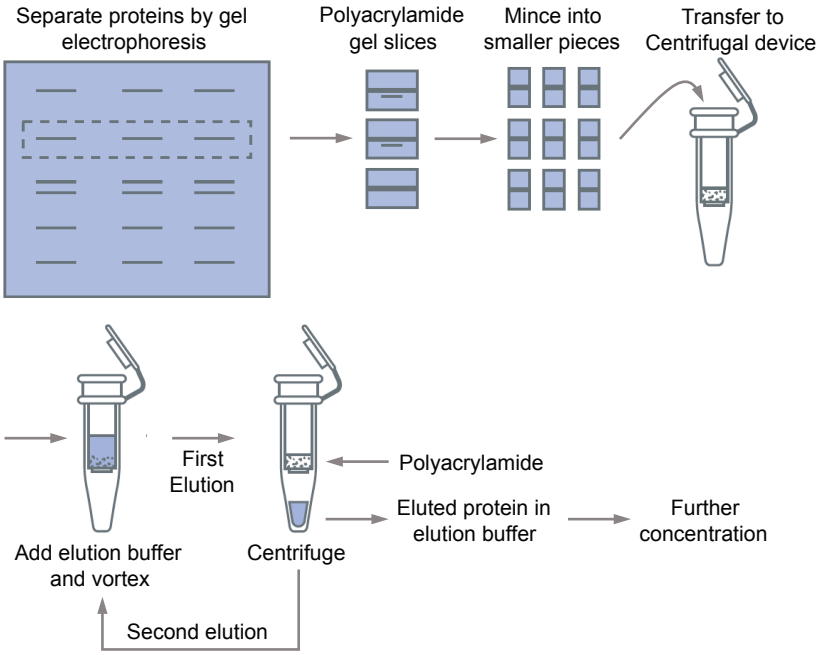
90-100%
80-89%
11-79%
5-10%
0-4%

0.1% solute concentrated to 35-50 µL. Recoveries shown as percent of starting material.

Nanosep centrifugal devices provide a fast and inexpensive alternative to commonly used methods for the elution and concentration of proteins that have been separated on polyacrylamide gels. The results of our study show that, due to its gentle nature, this elution method is especially effective for eluting biologically-active molecules and preparations of protein complexes. Figure 1 shows a step-by-step diagram of the general procedure. Proteins are first separated by gel electrophoresis and the protein or proteins of interest are identified by running a duplicate gel and staining it. Alternatively, the gel containing the protein of interest can be stained, identified, and later destained. Once identified, the protein band of interest is excised with a clean razor blade and minced into smaller pieces to maximize the gel surface area during protein elution. The gel slices are then placed into a Nanosep centrifugal device equipped with a membrane with the appropriate MWCO. The elution buffer is added directly to the tube and mixed with the gel slices by vortexing. The centrifugal device is then centrifuged following manufacturer's instructions (in general, 14,000 x g for 2 to 20 minutes depending on the sample) and the filtrate containing the eluted protein is collected. In order to increase protein recovery, the polyacrylamide retained in the upper chamber can be rinsed with elution buffer and centrifuged again to elute additional protein from the gel. The eluted protein sample can be further concentrated using a new Nanosep centrifugal device equipped with the appropriate MWCO-membrane.

Figure 1

Protocol for the elution of proteins from polyacrylamide gels by ultrafiltration with Nanosep Centrifugal Devices

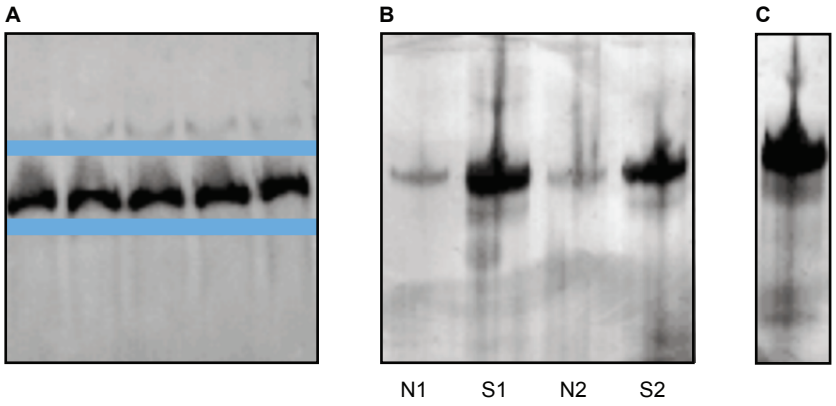


Parameters Affecting Protein Elution

Protein recovery was studied using nondenaturing (native elution) or denaturing (SDS elution) conditions. In order to quantify protein recovery using a standard protein assay, bovine serum albumin (BSA) was heavily loaded (6 µg per lane) in all the lanes of a polyacrylamide gel (4-15% Mini ReadyGel◆ precast gel, Bio-Rad, Hercules, CA, USA). Protein samples were boiled for 5 minutes in sample buffer[3] before they were subjected to PAGE using a Mini-PROTEAN◆ II electrophoresis system (Bio-Rad, Hercules, CA, USA) at 200 V. A duplicate gel stained with Coomassie Blue R-250 was used as a reference to locate the position of the protein band in the gel. A 5 mm strip, spanning five gel lanes, was excised from the gel at the appropriate position (approximately 130 mg of polyacrylamide) (Figure 2A), minced, and transferred to the upper chamber of a Nanosep centrifugal device (Pall Laboratory equipped with a 300K MWCO Omega™ membrane. The protein trapped in the gel pieces was eluted in the presence (SDS elution) or absence (native elution) of SDS. Four hundred microliters of an SDS elution buffer (0.25 M Tris-HCl buffer, pH 6.8; 0.1% (w/v) SDS) or native elution buffer (0.25 M Tris-HCl buffer, pH 6.8) were added to the chamber and mixed with the gel slices by vortexing. After a 20 minute centrifugation, 300-350 µL of filtrate was recovered and stored for protein analysis (Figure 1, first elution). The elution was repeated a second time (Figure 1, second elution) by adding fresh elution buffer to the gel slices and centrifuging the samples for 20 minutes. A filtrate of approximately 250-300 µL was recovered. Each protein eluant was concentrated 3-fold using a new Nanosep centrifugal device equipped with a 10K-MWCO Omega membrane. The concentrated protein eluants were assayed for protein content using the Pierce◆ Bicinchoninic Acid (BCA) Protein Assay Reagent[4], with bovine serum albumin as protein standard, following the standard protocol described by the manufacturer (Pierce Biotechnology, Inc.). Samples of concentrated protein eluants were also subjected to SDS-PAGE using a PhastSystem◆ and PhastGel◆ (12.5% polyacrylamide, GE Healthcare Bio-Sciences AB). Proteins in gels were silver stained following manufacturer's recommendations.

Figure 2

Effect of Bovine Serum Albumin (BSA) from polyacrylamide in the presence or absence of SDS



BSA was subjected to SDS-PAGE and detected by Coomassie Blue R-250 staining (panel A). The protein in five lanes (30 µg protein) was eluted using a 300K Nanosep Centrifugal Device in the presence or absence of SDS. Panel B shows the protein eluted in two consecutive runs (1 and 2), with (S) or without (N) SDS, after SDS-PAGE and silver straining. A strip spanning the six lanes of the gel (8 µg protein) was used in the ultrafiltration procedure in the presence of SDS. Panel C shows the protein eluted after SDS-PAGE and silver straining.

Table 2 shows protein recoveries following the first and second elution using native or SDS elution buffers. Addition of 0.1% (w/v) SDS to the elution buffer clearly increased the efficiency of the elution, with five times more protein being eluted in the presence of the detergent (50% of protein was eluted with SDS and 10% without SDS). In both cases, the amount of protein eluted in a second elution decreased to half (25% with SDS and 5% without SDS). Combining the results of both elution runs, 75% of the protein was eluted in the presence of SDS, while only 15% was eluted without SDS in the elution buffer. Figure 2B shows the protein eluants obtained in the two runs of elution with native and SDS buffer.

Table 2

Effect of Elution Buffer on Protein Recovery During Elution Procedure

	Elution Run	SDS Buffer	Native Buffer
Experiment 1: Large Gel Slice	Elution 1	50%	10%
	Elution 2	25%	5%
	Total Recovery*	75%	15%
Experiment 2: Small Gel Slice	Elution 1 + 2	98-100%	ND**

**Total recoveries were increase by 10-15% when half the amount of polyacrylamide gel was loaded in the Nanosep Centrifugal Devices.*
***Not Determined*

This elution test was, however, performed using suboptimal conditions, i.e., overloading the centrifugal units with polyacrylamide gel slices. The polyacrylamide gel slices acted as a physical barrier to the flow of elution buffer, decreasing the flow rate and, thus, the overall efficiency. When the same protocol was used employing two centrifugal units, each containing half of the gel slices, the protein recovery was increased 10-15%, using either native or SDS elution protocols. Reducing the amount of polyacrylamide loaded into the centrifugal devices also considerably reduced the centrifugation time, which could be significant for biologically-active samples.

Recovery of Dilute Protein Samples

In a second experiment, a considerably more dilute BSA sample (1.4 µg protein per lane) was subjected to SDS-PAGE using a PhastSystem (Pharmacia) and 12.5% polyacrylamide PhastGel (Pharmacia). The distance migrated by the protein was determined using a duplicate, silver stained gel. Then, a 3-mm strip of the gel containing the protein bands in six lanes was excised. Protein elution was performed using SDS elution buffer, as described above, and the protein eluants of two consecutive elutions were combined and further concentrated. For each elution, only a 2-minute centrifugation was required to obtain the volume of SDS buffer that had been loaded. The concentrated protein eluant was subjected to SDS-PAGE in a 12.5% PhastGel (Figure 2C).

The polyacrylamide gel used in this experiment was smaller (4.3 x 5 mm) than the one used in the first experiment described above, therefore considerably less protein could be loaded onto the gel. As a result, amounts of eluted protein were below the sensitivity of standard protein assays. Protein concentrations were determined densitometrically using 1D Image Analysis Software (Kodak Digital Science◆). Under the conditions used in this experiment, 98 to 100% of the protein was eluted (Table 2, Experiment 2). The high protein recovery was due, primarily, to the reduced size of the polyacrylamide gel slices loaded into the centrifugal unit during this experiment, as compared to the first experiment performed, described above. Thus, the amount of polyacrylamide gel used in the procedure seems to be the most critical factor for efficient recovery, and, therefore, the number of centrifugal units used in each elution experiment should be determined empirically.

Conclusion

Nanosep centrifugal devices allowed for rapid and efficient elution of proteins from polyacrylamide gels. The amount of starting polyacrylamide gel material was the limiting factor for high protein recoveries. Reducing the amount of polyacrylamide not only increased the yields of eluted protein but also reduced centrifugation times significantly, a factor of consideration when eluting biologically-active proteins.

We have successfully applied this method to elute enzymatically-active protein mixtures in their native state under both nondenaturing and denaturing conditions, with no significant loss of activity (data not shown). The amount of SDS in the elution buffer can be empirically determined to increase recovery while preserving biological activity. Ultrafiltration is, therefore, a fast and gentle method, ideal for the elution of biologically-active proteins.

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