Preparing Samples for Protein Sequencing

A Newcomer's Guide

PERKIN ELMER

Next Page

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Previous Page | Next Page

Table of Contents

Click a topic to go to it.

Previous Page Next Page

Previous Page

1 Introduction

About This Guide

Preparing Samples for Protein Sequencing: A Newcomer's Guide focuses on the use of SDS-PAGE—a practical, low cost method of sample preparation. Although the techniques used with this and other methods will vary from lab to lab, the basic guidelines discussed in this booklet are applicable to many situations.

Other topics covered in this guide include:

- The most significant uses of protein sequencing information
- An overview of Edman degradation
- Common pitfalls in sample preparation and how to avoid them
- Sample preparation by electrophoresis and in solution
- Measuring the amount of protein in your sample
- Avoiding N-terminal blockage, and deblocking samples that won't sequence
- Internal and C-terminal sequencing

Numerous references to more comprehensive literature on the topics discussed are listed in the *References* chapter at the end of this guide. In addition, we have included a glossary of terms, a summary of do's and don'ts for sample preparation (Appendix A), and a section designed to help you with microgram / picomole conversions (Appendix B).

Protein Sequencing Overview

Protein sequencing provides information about the amino acids that make up a protein. During the sequencing process, amino acids are sequentially removed from the N-terminal end of the protein strand, and identified in the order they occur in the protein.

Many naturally occurring proteins, however, cannot be directly sequenced from the N-terminus due to chemical blockage. In such cases, internal or C-terminal sequencing can be performed. Internal sequencing is the sequencing of peptide fragments resulting from the enzymatic or chemical digestion of a specific protein. C-terminal sequencing is the sequential cleavage and identification of amino acids starting from the C-terminal end of a protein.

Advances in automated protein sequencing technology now make it possible for scientists to sequence very low picomole amounts of protein—as little as 1 to 5 pmol. Many sequencing projects, however, are often limited by an inability to obtain sufficient amounts of adequately purified protein. The use of extremely pure starting protein is critical for successful sequencing.

Sample Preparation

Many methods for sample preparation have been developed over the years. No particular method works best; each has distinct advantages and disadvantages. The best method for preparing a particular sample can only be determined empirically and with reference to your own criteria regarding yield, purity, cost and speed. The methods most commonly used to prepare samples for protein sequencing are:

- Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)
- High Performance Liquid Chromatography (HPLC)
- Capillary Electrophoresis (CE)

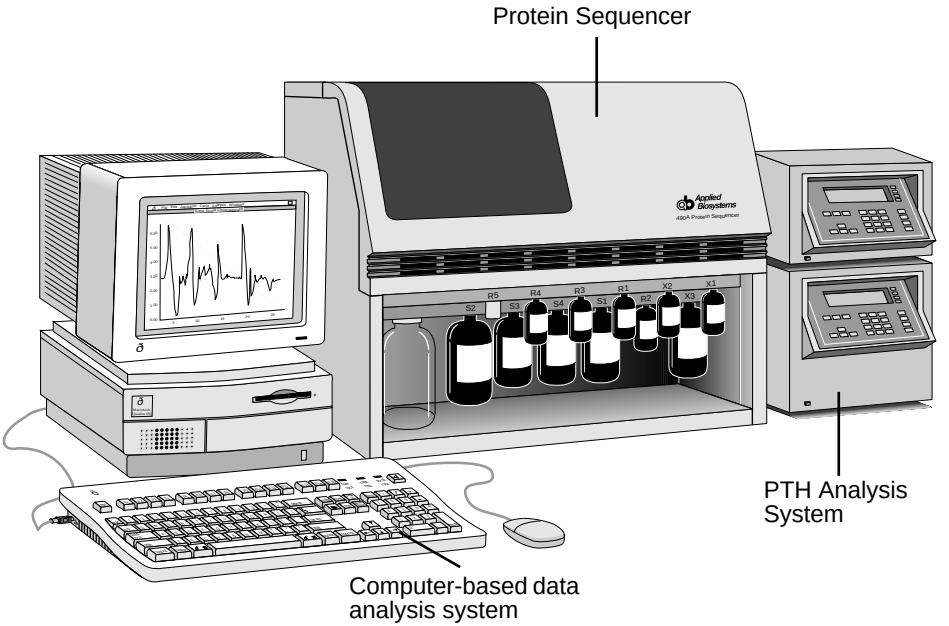


Figure 1. The Automated Protein Sequencer

Automated protein sequencers allow researchers to set up and control sequencing runs, and collect the resulting data via specialized software. These sequencers automatically remove and analyze amino acid residues from protein and peptide chains of various types and lengths.

Factors to consider when selecting your method for sample preparation are:

- The amount of protein required
- Time constraints
- The level of purity required

We hope you find this booklet a valuable reference—one that provides a foundation for your own exploration and discoveries in preparing samples for protein sequencing.

2 Using Protein Sequence Information

Information obtained from protein sequencing has many valuable uses including:

- The identification of new proteins
- Probe design for molecular cloning
- The manufacture of synthetic peptides for use as immunogens

Identification of New Proteins

Unknown bands on a gel can be identified using protein sequencing information. Once an N-terminal sequence is known, a computer-assisted database search can be carried out. Database searches are essential to:

1. Determine if the sequence represents a newly-identified protein.
2. Determine the relationship of the new protein to its homologues.
3. Identify the protein if it is not novel.

Seemingly unrelated proteins sometimes share significant homology in particular regions. These relationships provide insight into the possible function of the protein.

Probe Design for Molecular Cloning

Designing probes for molecular clone generation is one of the primary uses of protein sequence information. Once protein sequence information has been obtained, regions of minimal codon redundancy are selected for the design of probes such as PCR primers and oligonucleotides. These probes are used to screen cDNA or genomic libraries.

Recombinant proteins are sequenced to:

- Verify the authenticity of putative positive clones
- Monitor the genetic stability of the clone, and the proper processing of recombinant protein products

Manufacture of Synthetic Peptides for Use as Immunogens

Synthetic peptides have become an indispensable tool in cell biology, genetics, molecular biology, immunology and a variety of other life sciences. Antibodies raised against synthetic peptides can be used to identify and purify newly discovered proteins. Furthermore, synthetic peptide analogs can reveal important structural characteristics and suggest the functional properties of proteins. Protein sequencing information from a novel protein often provides the basis for the structure of synthetic peptides.

Antibodies to synthetic peptides are highly specific to the peptide sequence, and can differentiate between sequences containing a single amino acid substitution. Antibodies raised to a synthetic peptide whose composition is based on the predicted sequence of a specific region of a gene will recognize and bind only to proteins containing that sequence. Therefore, anti-peptide antibodies can be used to confirm that the gene and predicted protein sequences are both correct. The antibodies can then be used to isolate and purify the gene product in larger quantities for further study.

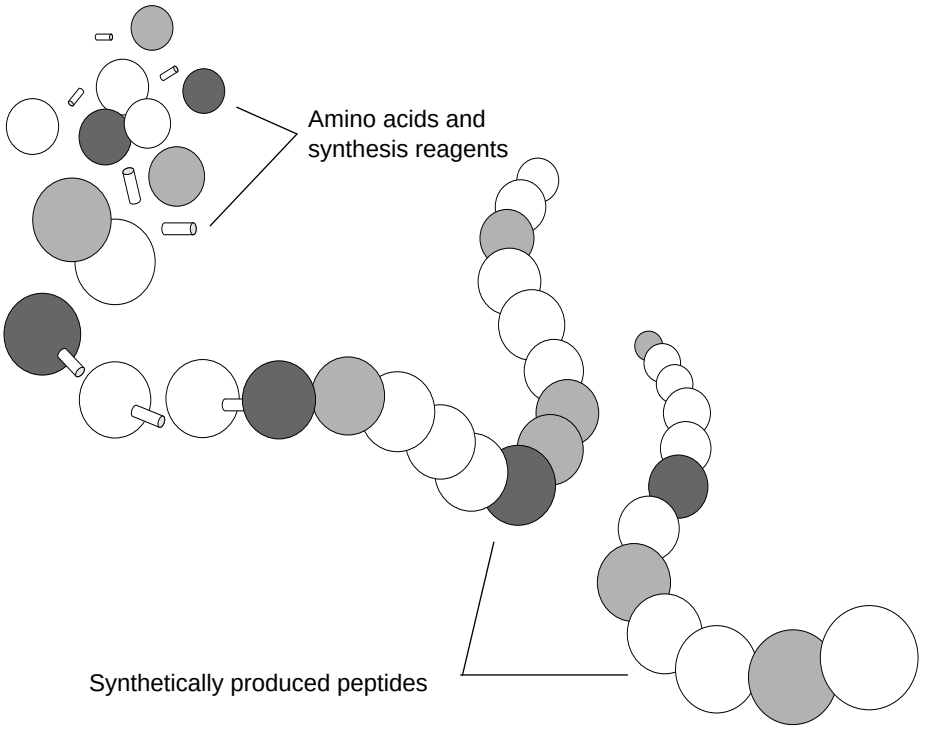


Figure 2. Manufacture of Synthetic Peptides

The antisera produced by whole protein immunizations will contain a mixture of antibodies, each specific to a different region of the protein. The antisera generated from synthetic peptide immunizations, however, will contain only one antibody specific to only one region of the protein—the region from which the sequence originated.

In vaccine development, synthetic peptides of 10 or more residues are typically synthesized and coupled to carrier proteins. Another approach using multiple antigenic peptides (MAPs) can also be employed. The MAP immunogen is composed of multiple copies of a single antigen attached to a small, non-immunogenic, poly-lysine core. As a result, the total weight of

the final product can consist of up to 95% synthesized peptide antigen. The MAP approach eliminates the need for antigen conjugation to a carrier protein; produces antibodies specific to the peptide antigen alone, not to some unknown site on the carrier; and results in the production of sera containing higher titers of specific, usable antibodies.

The selection of appropriate protein sequences to synthesize peptide antigens from is based on several methods of antigenicity prediction.[1,2] Hydrophilic sequences are often selected because they are more likely to represent amino acids on the surface of the intact protein, and be available for antibody binding. Various computer programs are available to help with the sequence selection process.

Peptides can be synthesized quickly and in large quantities. The Synergy™ Peptide Synthesizer can synthesize peptide antigens overnight. Designed by Perkin-Elmer for life science researchers, Synergy is easy-to-use, and is a cost-effective way to quickly produce synthetic peptides in-house. FastMoc chemistry has been optimized for this system; MAP synthesis columns are also available for use with Synergy. Contact Perkin-Elmer for more information on synthetic peptides or the Synergy system. In addition, the *References* chapter of this book lists three publications (reference numbers [3], [4] and [5]) which focus on the production and use of antibodies raised against synthetic peptides.

3 Edman Degradation

An Overview of Protein Sequencing Chemistry

The term *Edman degradation*, often used interchangeably with *Edman chemistry*, refers to the rich tradition of determining the sequence of amino acids in a protein by sequential chemical degradation from the N-terminus (or amino terminus) of the protein. Currently, all N-terminal sequencing performed on commercially available instruments is based on Edman degradation.

Edman degradation was developed during the 1950s by Pehr Edman. The key reagent used in this process is phenyl isothiocyanate (PITC), commonly referred to as the *Edman reagent*. Although different acids, bases, solvents, and detection methods have been used for protein sequencing over the years, PITC remains an essential ingredient of the chemistry required to perform Edman degradation.

In preparation for Edman degradation, the purified protein must be immobilized on a support. Typically the sample is either adsorbed to a chemically modified glass fiber disc, or electroblotted from a gel onto a porous polyvinylidene fluoride (PVDF) membrane such as the Perkin-Elmer ProBlott® PVDF membrane. Then Edman degradation is performed.

The Edman chemistry cycle consists of three stages.

1. Coupling

   The N-terminus of the protein couples with PITC under basic conditions to form a phenylthiocarbamyl (PTC)-polypeptide.

2. Cleavage

   The peptide bond of the N-terminal PTC-residue undergoes acid cleavage from the polypeptide chain. This results in the release of an unstable anilinothiazolinone (ATZ) derivative of the amino acid.

3. Conversion

   The unstable ATZ-amino acid is converted into the corresponding phenylthiohydantoin (PTH) derivative. The PTH-amino acid is stable.

Figure 4 on page 13 and *Figure 5 on page 14* illustrate the protein sequencing process from two different perspectives. As shown in *Figure 4*, the sample remains bound to the support during the coupling and cleavage steps which occur in a temperature controlled reaction chamber. After cleavage, the ATZ-amino acid is extracted from the support and transferred to a conversion flask. There the unstable ATZ-amino acid is converted into a stable PTH-amino acid.

At the end of each cycle of Edman degradation, the PTH-amino acid is separated from reaction by-products and identified, typically by HPLC chromatography and UV absorbance respectively. Although not part of the Edman degradation cycle per se, PTH-amino acid analysis, like Edman degradation, is an essential step in the protein sequencing process.

All 20 PTH-amino acids are resolved by gradient elution from a reverse-phase HPLC support (see *Figure 3 on page 10*). All commercially available automated protein sequencing systems perform automated PTH-amino acid analysis, where the PTH-derivatives are automatically injected onto an HPLC system.

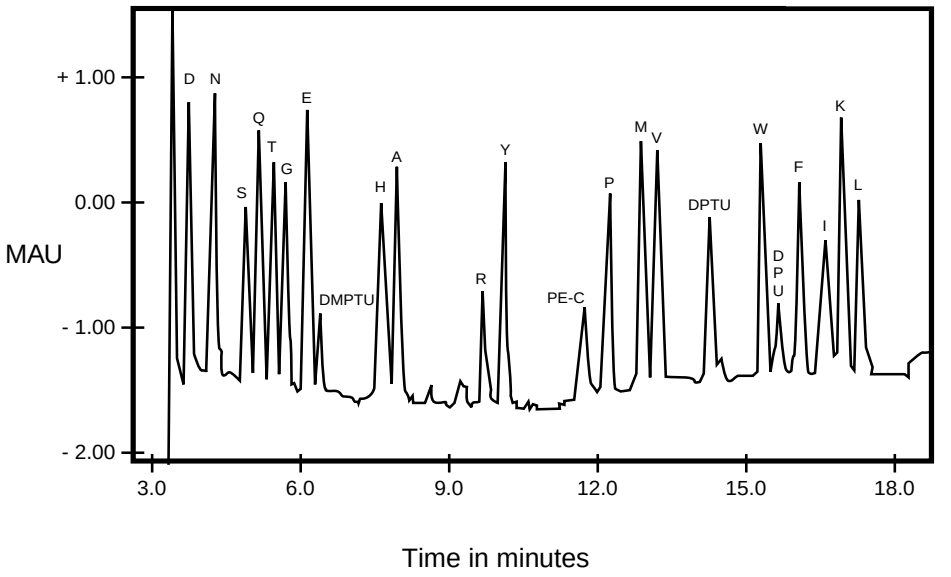


Figure 3. Typical Separation of PTH-Amino Acid Standards

Protein Sequencing is Not 100% Efficient

The goal of sequencing an unknown sample is to unambiguously identify as many amino acids as possible using the least amount of sample. The length of a sequence that can be successfully identified is limited by the chemical efficiency of Edman degradation as well as the purity, amount and molecular weight of the sample to be sequenced. Modern automated protein sequencers can routinely sequence low picomole levels of extremely pure samples. A sample that sequences well can provide accurate sequence information well below the 1 pmol level.

The efficiency of Edman degradation chemistry performed by automated protein sequencers is typically at or above 95%. Because the chemical efficiency is less than 100%, the amount of sequenceable sample decreases

slightly with each successive degradation cycle. With the exception of the initial coupling, the reaction of PITC with the amino-terminus or termini proceeds nearly quantitatively. The particular amino acid being reacted, or the local structure of the peptide chain, has little effect on the efficiency of the coupling reaction.

The cleavage reaction, however, requires a balance between complete cleavage of the ATZ-amino acid from the peptide and unwanted acid cleavage at other sites along the peptide chain. Because of this balance, cleavage efficiency is affected by the amino acid derivative being cleaved as well as the next amino acid in the chain. Incomplete cleavage of the ATZ-amino acid is referred to as *lag*. When lag occurs, the remaining uncleaved portion of the current N-terminal amino acid will appear in the chromatogram for the next cycle along with the next amino acid. Lag increases with each cycle in a sequencing run, and depending on the particular amino acids in the sequence, may be the primary reason a sample stops producing useful sequence data.

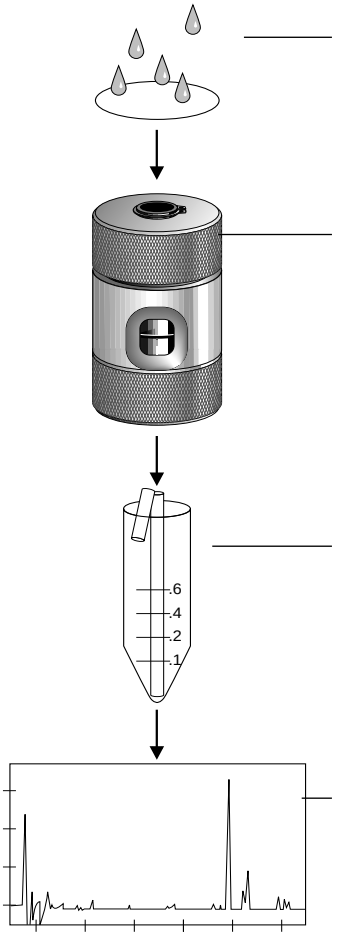
The repetitive exposure of the sample to strong acid during the cleavage reaction can also cause cleavage between amino acids elsewhere in the peptide chain. Each time *non-specific cleavage* of the peptide chain occurs, a new N-terminus is generated which can react with PITC. This will cause an increase in the *amino acid background*—the presence of other PTH-amino acids in the chromatogram which do not reflect the true N-terminal sequence.

At the start of a sequencing run, the amino acid background from non-specific cleavage is low. This background increases with each sequencing cycle. Luckily, non-specific cleavage is sequence specific, so cleavage will occur only between certain combinations of amino acids. This usually keeps the rate of increase of amino acid background quite low. However, when sequencing proteins with labile amino acid sequences and very large proteins, amino acid background will increase much more rapidly. In practical terms, while 50 pmol of a 100 to 200 amino acid protein may provide 40 to 50 cycles of interpretable sequence, the same amount of a 2,000 amino acid protein will probably provide only 10 to 15 cycles of sequence.

Although the amino acid background from non-specific cleavage at the start of a sequencing run is very low, amino acid background can also result from free amino acid contamination. In addition, the presence of other small molecules with strong UV absorbance or a PITC-reactive site will give rise to a number of chromatography peaks which may co-elute with PTH-amino acids. This background contamination can interfere with the identification of the first one or two amino acids in the sequence if the sample amount is small, but will typically wash away rapidly. Careful sample preparation will minimize this initial background problem.

In addition to the problems caused by decreasing signal and increasing background noise, not all of the protein delivered to the sequencer can be sequenced. For reasons not well understood, some of the N-termini seem to be unavailable for coupling to PITC in the first cycle. Since the amount of protein available varies from sample to sample, the initial yield of the first cycle of Edman degradation usually ranges from 30% to 70%. The yield can be even less, however, in cases where the protein is highly aggregated.

For all of these reasons, the average amount of a 30,000 dalton (Da) protein required by core facilities or service labs to obtain the first 15 residues of sequence is 84 pmol, or approximately 2.5 μg.[6] Many labs, however, can work well below this level.



Protein is adsorbed to a solid support such as a ProBlott PVDF membrane or glass fiber filter.

The support is placed in a reaction cartridge which is then placed into the protein sequencer.

Coupling & Cleavage Site

Steps 1 & 2 of the Edman degradation cycle take place in the reaction cartridge. The N-terminal of the protein couples with PITC to form a PTC polypeptide. The PTC N-terminal residue undergoes acid cleavage, resulting in the release of an unstable ATZ-amino acid. The ATZ-amino acid is then washed into a conversion flask.

Conversion Site

Step 3 of the Edman degradation cycle takes place in the conversion flask. Here the unstable ATZ-amino acid is converted into a stable PTH-amino acid. The PTH-amino acid is then automatically injected onto an HPLC.

Analysis

Using High-Performance Liquid Chromatography, each amino acid is identified by the elution time of the peak it produces on a chromatogram.

Figure 4. The Protein Sequencing Process

Figures 4 and 5 present two views of the Edman degradation chemistry cycle. Figure 4 illustrates where each part of the process takes place in the protein sequencer. Figure 5 conceptually illustrates how amino acids are sequentially cleaved from the protein strand one at a time through Edman degradation. The amino acids are then identified in the order that they occur in the protein through High-Performance Liquid Chromatography.

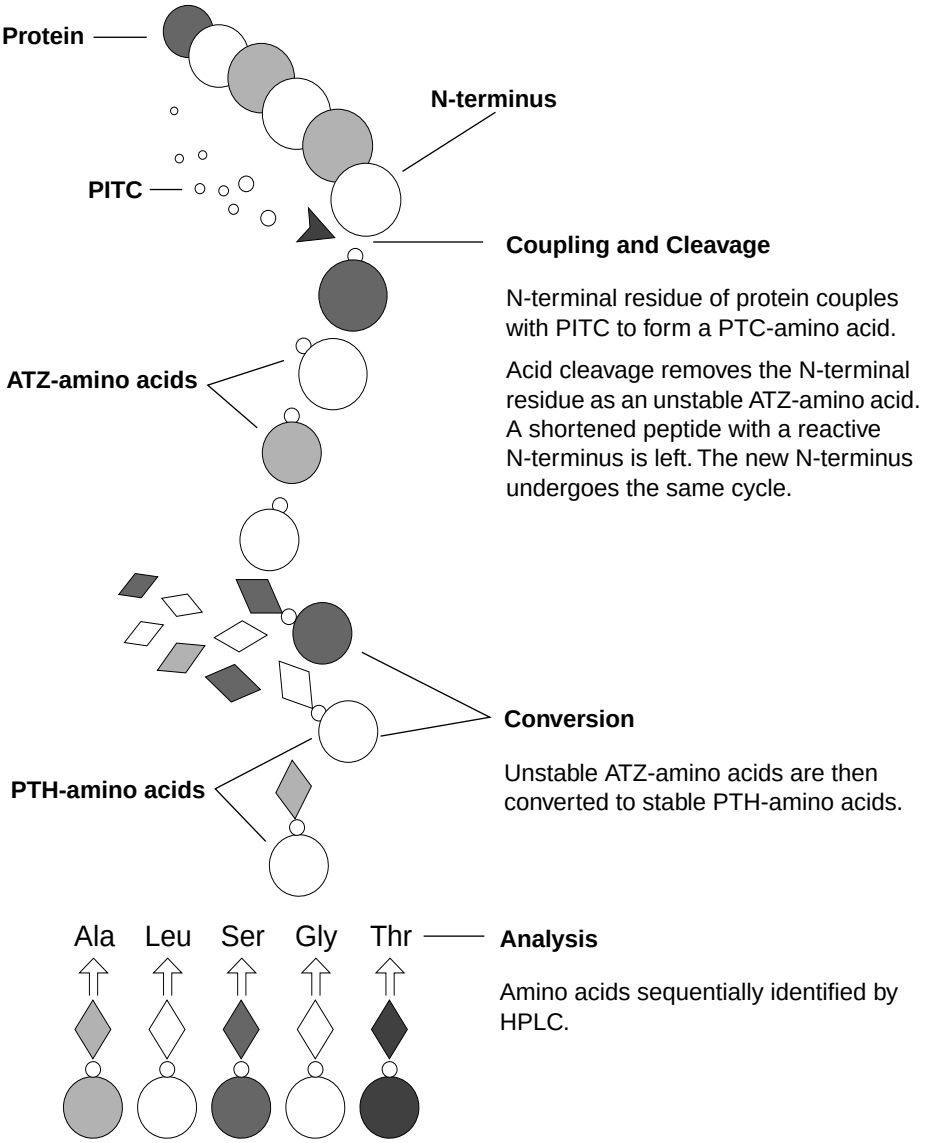


Figure 5. Edman Degradation and Amino Acid Identification

4 Sample Purity: Pitfalls to Avoid

Sample purity is one of the most critical factors for successful protein sequencing. Samples should:

- Contain one protein component only
- Be free of reagents which interfere with Edman degradation and the sequencing process

Two basic approaches that can be used to purify samples for protein sequencing are:

1. SDS-PAGE and electroblotting
2. ProSorb sample preparation cartridges

SDS-PAGE and electroblotting should be used if the protein of interest is in a complex mixture of proteins. For proteins isolated in solution which are free of contaminating proteins but contain salts, buffers and/or detergents, use ProSorb® cartridges.

Samples Containing More Than One Protein Component

Samples prepared by HPLC or SDS-PAGE which appear to be homogenous can yield ambiguous sequencing results. For instance, samples containing more than one protein component will produce more than one sequencing signal. If several proteins are present in nearly equivalent amounts, the sequence of any particular component is almost impossible to follow with confidence. If the molar ratio of major to minor components is high enough, the primary, secondary and even tertiary signals may be distinguished.[7]

Samples which have undergone incomplete processing, or partial enzymatic or chemical digestion can also produce more than one sequencing signal. Proteolytic fragments that differ only slightly can be difficult to separate by HPLC or SDS-PAGE because they are so similar in size and hydrophobic character.

HPLC fractions from chemical or enzymatic digests of proteins should be screened before committing them to sequence analysis. An HPLC column fraction can be screened for purity by capillary electrophoresis (requires less than 1 pmol[8]), or by mass spectrometry (also requires well under 1 pmol of sample).

Reagents That Interfere

Unexpectedly low sequencing signals, or unexpectedly high background noise can result if samples contain substances which:

- Interfere with the conditions required for efficient Edman degradation
- React with PITC
- React with the N-terminus of the protein

Tris, glycine, guanidine, glycerol, sucrose, ethanolamine, SDS, Triton X-100, Tween®, ammonium sulfate and other ammonium salts are among the reagents which can interfere with Edman chemistry. For example, a 2 µg sample of a 20,000 Da protein in 1 mL of 10 mM Tris contains a 100,000-fold molar excess of Tris to protein.

All reagents which interfere with protein sequencing should either be removed from samples prior to sequencing or not used at all. When purifying protein by electrophoresis and electroblotting, the protein sample is bound to a PVDF-type membrane. Contaminating reagents can easily be washed away with water and 50% methanol. When purifying protein in solution, however, avoiding reagents which interfere can be difficult.

Instead of TCA precipitation and dialysis, a ProSorb sample preparation cartridge (Figure 7 on page 18) can be used to concentrate your protein from chromatography fractions. Excess liquid volume including salts, buffers, and other reagents which may interfere with protein sequencing simply pass through the ProBlott PVDF membrane which binds the protein but not the salts. The ProBlott membrane with adsorbed sample can then be rinsed and loaded directly into the protein sequencer.

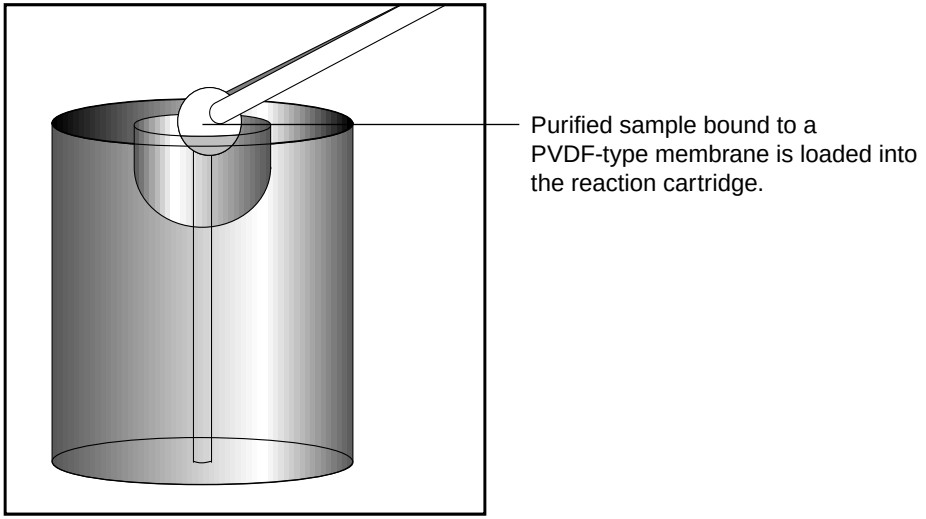
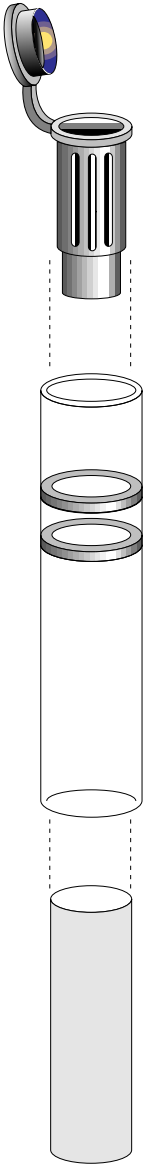


Figure 6. Upper Block of the Blott Cartridge for Protein Sequencing

Once your sample has been purified and bound to a support, it is loaded into a reaction cartridge and placed in an automated protein sequencer. In this figure, the sample was purified and bound to a ProBlott PVDF membrane using a ProSorb Sample Preparation Cartridge (shown in Figure 7 on page 18).

The membrane in this cartridge is designed for easy removal and direct loading into the Perkin-Elmer Blott Cartridge—a specially designed reaction cartridge for protein sequencing. Figure 6 illustrates the upper block of the Blott Cartridge. Figure 8 on page 27 provides a more complete illustration of the Blott Cartridge.



The Perkin-Elmer ProSorb Sample Preparation Cartridge provides a simple, one-step method for concentrating, desalting and recovering protein on PVDF membrane.

Samples can be collected directly from HPLC, open column chromatography or automated electrophoresis systems. Contaminating salts, amines and detergents are centrifuged away as the sample is bound to a ProBlott PVDF membrane. Unlike glass fiber filters, no precycling is required.

ProSorb enables extensive sample washing with minimal sample loss, and is compatible with a wide variety of sample matrices.The ProBlott PVDF membrane inside the ProSorb cartridge is designed for direct transfer to the Blott Cartridge reaction vessel (see Figure 6).

Figure 7. ProSorb Sample Preparation Cartridge

5 Preparing Samples by Electrophoresis

Gel electrophoresis followed by electroblotting onto PVDF membrane is currently the most popular way to prepare proteins for sequencing. Electroelution of a sample from a gel is an inefficient method of sample preparation, and is not recommended. In the United States, more than one third of the core labs which offer protein sequencing services also offer electrophoresis and electroblotting services. In Japan and Europe, electroblotting services are not yet commonly available.

Quite often, the use of electrophoresis and electroblotting can replace a number of conventional purification steps such as ammonium sulfate precipitation and chromatography. Sodium dodecylsulfate-polyacrylamide gel electrophoresis, commonly referred to as SDS-PAGE, can separate partially purified or even crude mixtures of proteins into highly purified bands with relatively little investment in equipment and training. With the development of more effective techniques for digesting proteins separated on gels, electrophoresis has also become a powerful method for preparing fragments for internal sequence analysis.

Although one- and two-dimensional (2-D) electrophoresis can be used to prepare samples for protein sequencing, 2-D electrophoresis has proven to be the most effective of the two. The following examples illustrate the success which has been achieved using 2-D electrophoresis.

- Outer membrane fractions from *Pseudomonas aeruginosa* containing 2.5 mg of protein were separated on sucrose gradients. This was sufficient material to sequence and identify 100 proteins when loaded onto a 2-D gel.[9]

- The cytosolic fraction from a single high-speed centrifugation of whole tissue homogenate[10] or a crude cell lysate[11], can be directly purified by 2-D electrophoresis. Proteins present in the crude extracts in low microgram or even sub-microgram quantities, provide sequence information after 2-D gel electrophoresis and electroblotting.
- Pooling material from approximately 10 preparative 2-D gels can provide sufficient material for sequencing the 200 most abundant proteins in a crude cell lysate.[12]
- Samples have been concentrated 200–fold with yields higher than 80% by collecting protein from pieces of many 2-D gels onto a concentration gel.[13]

Once the protein of interest has been isolated on a gel, it can be electroblotted onto a PVDF-type membrane such as the ProBlott or Mini ProBlott (Perkin-Elmer P/N 400994 or P/N 401194 respectively), then washed free of salts, buffers, and other interfering substances. The membrane can then be loaded directly into the automated protein sequencer.

PVDF is inert to the solvents and reagents used in protein sequencing and other protein chemistry protocols which use acetonitrile, trifluoroacetic acid (TFA), ethyl acetate, and a strong organic base. Common solvents to which PVDF is not stable include dimethylformamide (DMF) and dimethylsulfoxide (DMSO). Applied Biosystems protein sequencing User Bulletin #58, *SDS-PAGE and Electroblotting*, contains standard recipes and protocols for sample preparation.[14]

It is important to note that nitrocellulose and nylon will degrade when exposed to the reagents used for Edman degradation. Therefore, these materials should never be used for protein sequencing supports.

Maximizing the Yield of Electrophoresis

Listed below are suggestions for preparing samples by electrophoresis which will yield the best sequencing results. Suggestions on gel preparation and setup for maximizing resolution and sharpening bands are listed on page 22.

Suggestions for Preparing Samples by Electrophoresis
♦ Load as much protein onto as little PVDF-type membrane as possible.
♦ Load crude samples onto the gel as soon as possible in the purification process. This will help preserve as much of the protein of interest as possible. Separate protein components by SDS-PAGE.
♦ Salt cuts or high speed centrifugation for pre-gel enrichment may help increase the proportion of the protein of interest in the sample loaded onto a gel. Caution should be exercised, however, since too many prior purification steps may result in too great a loss of the protein of interest.
♦ Load the gel to the limit of resolution. If sample is dilute, load the wells and run bromophenol blue into the stacking gel. Use a syringe to flush glycerol out of the sample well, and load a second aliquot. Repeat this procedure two to three times until the blue dye reaches the stacking/main gel interface.
♦ Load as much sample into as few lanes as possible, using deeper wells instead of thicker gels. Although thicker gels hold more sample, some resolution will be sacrificed. Furthermore, thicker gels are likely to reduce the efficiency of electroblotting, perhaps because more SDS dissociates from the protein during the longer transfer times required.[15]

Suggestions for Gel Preparation and Setup
♦ De-gas acrylamide solutions before pouring gels. This will result in a more uniform matrix.
♦ Use fresh sample buffer, or add fresh beta-mercaptoethanol to sample buffer which has been frozen.
♦ The resolution of Laemmli-type gels depends on the glycine/chloride/pH boundary between the stacking gel and the running gel. The boundary can be restored in previously-cast gels by loading stacking buffer into the wells and incubating without an electric field. Allow approximately one half hour for equilibration. In pre-run gels, the boundary can be established by: 1. Casting the stacking gel with running buffer, then 2. Pre-running the gel with stacking buffer in the upper reservoir.[16] See Chapter 8, *Avoiding N-terminal Blockage*, for more information.
♦ If standard gels do not provide good resolution, the sample may be too salty. Salty samples run better on modified Laemmli systems (developed by Fling and Gregerson). This system calls for twice the amount of Tris and glycine in the tank buffer, and twice as much Tris in the resolving gel buffer.

Electroblotting Conditions

Any published set of electroblotting conditions can be used as a starting point for achieving good results with many proteins. Slight adjustments to these conditions can sometimes yield better results with particular proteins. The recovery of any particular protein on the membrane may be as little as 40% or as much as 90%.[17]

The selection of electroblotting conditions necessarily involves a trade-off. Although SDS encourages protein to leave the gel, too much SDS can cause the protein of interest to be lost in the buffer. Methanol dissociates SDS from the protein, and encourages the protein to bind to the membrane. Too much methanol, however, can precipitate the protein of interest in the

gel. Although the high binding capacity of ProBlott PVDF membranes usually results in good recovery of both high and low molecular weight proteins at the same time, varying the amount of methanol used with different proteins is an easy way to favor recovery of the protein of interest.

The *Troubleshooting Guide for Electroblotting* on pages 30 to 34 lists suggestions for solving a variety of problems which can be encountered when electroblotting.

Varying the Amount of Methanol Used

Varying the amount of methanol used for different proteins is an easy way to favor recovery of the protein of interest onto PVDF-type membranes. The recommended amounts of methanol shown below are based on the molecular weight of the protein.

Molecular Weight of Protein	% of Methanol Recommended
Proteins above 80 kilodaltons (kDa)	5%
Proteins between 20 kDa and 80 kDa	10%
Proteins less than 20 kDa	20%

Changing the pH of the Transfer Buffer

Changing the pH of the transfer buffer can improve electroblotting efficiency for a particular protein because a protein does not leave the gel when its pI is similar to the pH of the transfer buffer.

Extremely basic proteins can actually travel towards the cathode instead of the anode. If you suspect this might be occurring, simply place a PVDF membrane on both sides of the sample. Thus, the protein will be captured regardless of the direction in which it travels.

A four-part buffer system[18] has been developed for the semi-dry electroblotting of proteins that:

1. Have limited solubility,
2. Are smaller than 20 kDa, or
3. Are larger than 70 kDa.

This system maintains 1) the SDS concentration and an acidic pH on the gel-side of the membrane, and 2) the methanol concentration and a basic pH on the other side of the membrane.

Staining Blotted Proteins

A number of different stains can be used to visually locate proteins on PVDF-type membranes. This section briefly discusses the use of Coomassie Brilliant Blue, Ponceau S, amido black, colloidal metals and transillumination.

Gels should not be stained before electroblotting because exposure to acetic acid and methanol will fix the protein in the gel. This will reduce the amount of protein transferred out of the gel during electroblotting. Staining a gel is useful for quantitation purposes, for instance, when preparing an analytical gel. After electroblotting, the PVDF membrane can be stained before sequencing as long as the stain is compatible.

Coomassie Brilliant Blue R-250 (CBB R-250) is versatile and sensitive enough to detect sequenceable amounts of protein.[19]

Ponceau S and amido black[20] are less sensitive than CBB R-250. Blots stained with Ponceau S or amido black can be destained with water. Amido black, however, contributes a UV-absorbing contaminant which appears in the hydrophobic region of chromatograms from either peptide mapping or PTH-amino acid analysis.[21]

Colloidal metals such as gold and silver are generally more sensitive than the stains listed above. Unfortunately, these stains cannot be used to prepare sequenceable samples. Therefore, colloidal metals should not be used to stain proteins bound to PVDF-type membranes.[22]

An easy, but not very sensitive, way to visualize protein bands is through transillumination. First, dry the PVDF membrane on filter paper at room temperature. Then re-wet the membrane by floating it on top of 20% methanol. View the blot in front of white light, or place the blot in a glass tray and set the tray on a light table. The membrane remains opaque except where protein is bound; the protein bands appear translucent—wetter and shinier than the surrounding membrane.

When using stains, remember to prepare them fresh every time, and discard them after one use. Contaminants, especially glycine and Tris from the gel, will become concentrated in stains that are reused.

Sequencing Suggestions for Blotted Proteins

Perkin-Elmer automated protein sequencers are shipped with two types of reaction cartridges: *standard* cartridges, and Blott Cartridges. Standard reaction cartridges are recommended for sequencing samples prepared in solution by methods such as HPLC or CE. The Blott Cartridge is specifically designed for use when sequencing samples bound to PVDF-type membranes.

In the Blott Cartridge (illustrated in Figure 8 on page 27), the membrane is oriented parallel to the solvent flow. The width of the sample chamber is very narrow to avoid trapping bubbles, and to ensure that solvent is channeled through the membrane instead of around it. When used instead of the standard reaction cartridge for PVDF-bound samples, the extraction of ATZ-amino acids and by-products is improved. For example, better recovery of histidine and arginine residues can be expected. Increases in tryptophan yield and decreases in sequencing by-products have also been observed when using the Blott Cartridge.[23]

Although different instruments and different cycle programs may well achieve different results, some general characteristics of the Blott Cartridge can be noted. Users have reported[23] that up to three pieces of membrane can be stacked inside the Blott Cartridge to increase the amount of protein available for sequencing without causing any adverse effects. Too many

pieces of membrane in the reaction chamber, however, will impede reagent flow and can result in lag. Refer to Chapter 3, *Edman Degradation*, for more information on lag. To achieve the best sequencing results, load the smallest amount of PVDF which will provide enough protein for reliable sequencing.

Before loading the membrane into the automated protein sequencer, rinse it alternately in HPLC-grade water and 50% methanol. This removes residual salts and dust, and any organic contaminants. If the membrane is heavily stained with Coomassie Brilliant Blue, follow these steps before loading it into the sequencer.

1. Rinse the membrane with water.
2. Soak it in 1 mL of 50% methanol with 2 µL of triethylamine (Pierce catalog #25108) until the color is gone.
3. Rinse the membrane with 100% methanol.

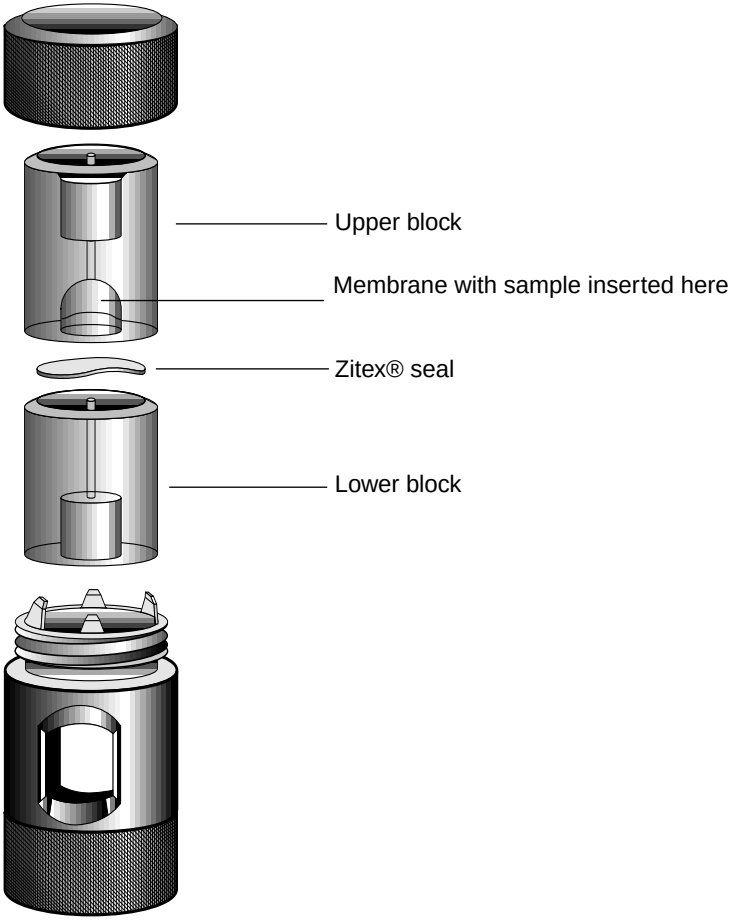


Figure 8. Perkin-Elmer Blott Cartridge

The Blott Cartridge is specially designed for membrane-bound samples, and provides more efficient sequencing because reagents can cross-flow over both sides of the membrane. The PVDF membrane (ProBlott) incorporated in the Perkin-Elmer ProSorb Sample Preparation Cartridge is designed for easy removal and insertion directly into the Blott Cartridge. For more information, see Figure 6 on page 17 and Figure 7 on page 18.

Estimating the Likelihood of Successful Sequencing

Sample loss is unavoidable despite one's best efforts and fastidious technique. The number of amino acid residues identified during sequencing is dependent on the sequence and the amount of sample analyzed. In general, sequencing is likely to be successful when at least 25 pmol of protein has been concentrated onto one or two pieces of PVDF-type membrane.

To determine the amount of starting material needed, consider the probable yields of the various steps in your protocol. Two examples for calculating the necessary amount of sample are presented on page 29. Back-of-the-envelope calculations like these will help ensure you prepare enough protein for successful sequencing.

A quick check can be run on a small amount of sample to determine whether enough protein is present in the sample before committing it to sequencing analysis. This method is described in Chapter 7, *Measuring the Protein in a Sample*.

As much as 90% of the sequencing yield can be lost due to partial blockage of the N-terminus during sample preparation. N-terminal blockage, when not present in the original native protein, can be caused by impurities in the gel. These impurities can be eliminated by running the gel prior to loading the sample, and by including antioxidants such as thioglycolate with the sample. For more information on N-terminal blockage, refer to Chapter 8, *Avoiding N-terminal Blockage*.

Calculating the Amount of Starting Material Required For Successful Sequencing

Assumptions for Electrophoresis and Electroblotting (Methods 1 and 2)

- Only 80% of the protein of interest is actually concentrated into the band of the gel.
- Only 50% of the protein of interest transfers to the PVDF membrane, and remains there after staining and destaining.

Method #1

If…

The protein of interest is 10% of the total protein loaded onto the gel,

Then…

The amount of protein loaded onto the sequencer will be only 4% of the total protein loaded onto the gel.

Formula: 0.1 X 0.8 X 0.5 = 0.04

(% protein of interest loaded onto gel) X

(% concentrated onto band of gel) X

(% transferred to and remaining on PVDF membrane)

Method #2

If…

a. The protein of interest is 25% of the total protein loaded onto the gel, and

b. 20 µL of 1 mg/mL solution of the protein mixture is loaded into each of 5 gel lanes (100 µL total),

Then…

All 5 pieces of PVDF membrane together will provide 100 pmol of a 100 kDa protein, or 200 pmol of a 50 kDa protein.

Troubleshooting Guide for Electroblotting

Problem	Suggestion
The membrane does not wet evenly.	♦ PVDF-type membranes are very hydrophobic. They must be immersed in 100% methanol until completely wet, then moved quickly into electroblotting buffer for equilibration.
Coomassie staining the gel after electroblotting shows that the protein of interest has not completely left the gel.	♦ Reduce the concentration of methanol. ♦ Reduce the amount of SDS that is removed from the gel by incubating the gel in electroblotting buffer no longer than 5 minutes prior to transfer. ♦ Increase the current, while keeping the transfer sandwich as close to room temperature as possible. Electroblotting for a longer time period is not likely to help because the protein of interest may be unable to leave the gel after the SDS has been driven away. ♦ If the resolution of the protein of interest from other proteins in the mixture is sufficient, use a lower acrylamide concentration in the separating gel. For proteins around 100 kDa, 8% acrylamide is usually sufficient. For 10 to 70 kDa proteins, 12% acrylamide is often needed. ♦ Try an electroblotting buffer with a higher (or lower) pH in case the isoelectric point of the protein of interest happens to be near the pH of the transfer buffer first used.

(Continued)

Troubleshooting Guide for Electroblotting (Continued)

Problem	Suggestion
Although the gel contains little or no protein after electroblotting, the stained membrane also shows little or no protein.	♦ Proteins, especially small ones, may transfer through some PVDF-type membranes. When this occurs, a second sheet of membrane placed behind the first sheet in the transfer sandwich may bind some of the protein which is *blown through.* To avoid this problem, use PVDF-type membranes with a high binding capacity, such as ProBlott membranes. ♦ Reduce the SDS concentration, equilibrate the gel in electroblotting buffer for a longer time prior to transfer, and/or increase the methanol concentration of the electroblotting buffer. ♦ Since PVDF-type membranes are very hydrophobic, they must be immersed in 100% methanol before they are equilibrated with buffer. Protein will not bind well to dry PVDF.
Blank patchy areas on the membrane remain after staining.	♦ Remove any air bubbles which are trapped between the layers of the transfer sandwich because they can prevent transfer from occurring in small regions. The condition of the Scotch brite sponges is critical. They must be springy to properly compress the gel/PVDF sandwich. Always shake sponges; never squeeze them. Discard compressed sponges immediately, and replace with new sponges. ♦ Use only high quality PVDF-type membranes. Poor quality membranes may contain irregularities. ♦ After complete immersion in methanol, the entire membrane must be thoroughly wetted with buffer. Protein will not bind well to areas of PVDF that are not properly wetted.

Troubleshooting Guide for Electroblotting (Continued)

Problem	Suggestion
No signal results from sequence analysis.	♦ Run a sample evaluation gel to re-assess the amount of protein in the gel prior to electroblotting. Or, if the amount of material allows, measure the amount of protein in an aliquot of the sample by amino acid analysis. If an insufficient amount of protein was delivered to the sequencer, prepare a new sample following the suggestions offered in *Maximizing the Yield of Electrophoresis* on page 21. ♦ Fragment the unsuccessful sample on its sequencing matrix with CNBr treatment and attempt sequencing again. If the sample was N-terminally blocked, several signals will be apparent. Prepare a new sample following the suggestions in Chapter 8, *Avoiding N-terminal Blockage*. ♦ Prepare fragments for internal sequencing.
More than one signal results from sequence analysis.	♦ The primary signal from the predominant protein can sometimes be distinguished from the secondary signals of contaminating proteins. ♦ Improve resolution of the protein of interest on a gel of different acrylamide concentration, a gradient gel, or a 2-D gel. ♦ Protect the sample from heat and acid, which can cause non-specific cleavage of internal peptide bonds.

6 Preparing Samples in Solution

The design and operating characteristics of most automated protein sequencers require that samples in solution be adsorbed to a support or matrix, either a glass fiber filter or a piece of PVDF-type membrane. A 9 mm glass fiber disk can absorb no more than 15 µL of liquid at one time. PVDF-type membranes can absorb only 1 to 3 µL at one time. Additional aliquots of sample can be applied to PVDF membranes one at a time after the previous aliquot has dried. When dealing with large volumes, the use of ProSorb sample preparation cartridges is recommended.

The sample should be in a volatile solvent such as water, acetic acid, trifluoroacetic acid, triethylamine, or acetonitrile. To avoid contaminants which leave residues upon drying, all reagents and solvents must be of the highest purity available, for example ultra- or analytical-grade. Molecular biology-grade reagents and solvents are not suitable. The sample should contain no more than 0.01% SDS, since larger amounts can wash the protein from the filter and/or cause bubbling in instrument tubing. Commonly-used reagents which interfere with protein sequencing must be avoided or removed. (See Chapter 4, *Sample Purity: Pitfalls to Avoid*.)

HPLC Methods

Many core facilities which offer protein sequencing services in the United States also offer high-performance liquid chromatography (HPLC) purification of proteins as a service. When working with more than 50 µg of material, HPLC methods such as reverse-phase, ion exchange, and gel filtration usually offer good resolution in sequencing-compatible solvents. Figure 9 on page 35 illustrates the basics of HPLC.

Although HPLC methods can offer good resolution, the cost of equipment and training for HPLC is likely to be much higher than for SDS-PAGE and electroblotting methods. Furthermore, large proteins with hydrophobic regions, such as receptors and other membrane proteins, are not easy to purify on reverse-phase columns. The variability of results from column-to-column is another disadvantage. Peaks eluted from any column should be analyzed by SDS-PAGE or capillary electrophoresis to evaluate purity.

Finally, specific conditions for every new separation problem must be developed through trial and error. Although the intuition of experienced operators can expedite the method development process considerably, some amount of precious sample is always lost during this process.

Perkin-Elmer will soon be offering a new sample preparation system based on capillary liquid chromatography—the 173A cLC Microblotter System. This system will provide users with an integrated protein / peptide mapping system for research requiring high sensitivity and high recoveries, especially for protein sequence analysis.

173A cLC Microblotter System

Soon to be released by Perkin-Elmer, the 173A cLC Microblotter System is a capillary liquid chromatography sample preparation system. It separates and prepares very small amounts of sample in one step with no significant sample loss. The eluted protein/peptide fractions at picomole or subpicomole levels are collected onto a PVDF membrane which can then be directly subjected to sequence analysis.

The PVDF surface area onto which the fractions are collected is very small—2 to 3 mm i.d. spots. This eliminates the need for further concentration or transfer steps, thus minimizing possible sample loss.

If you would like more information on the 173A system, contact Perkin-Elmer.

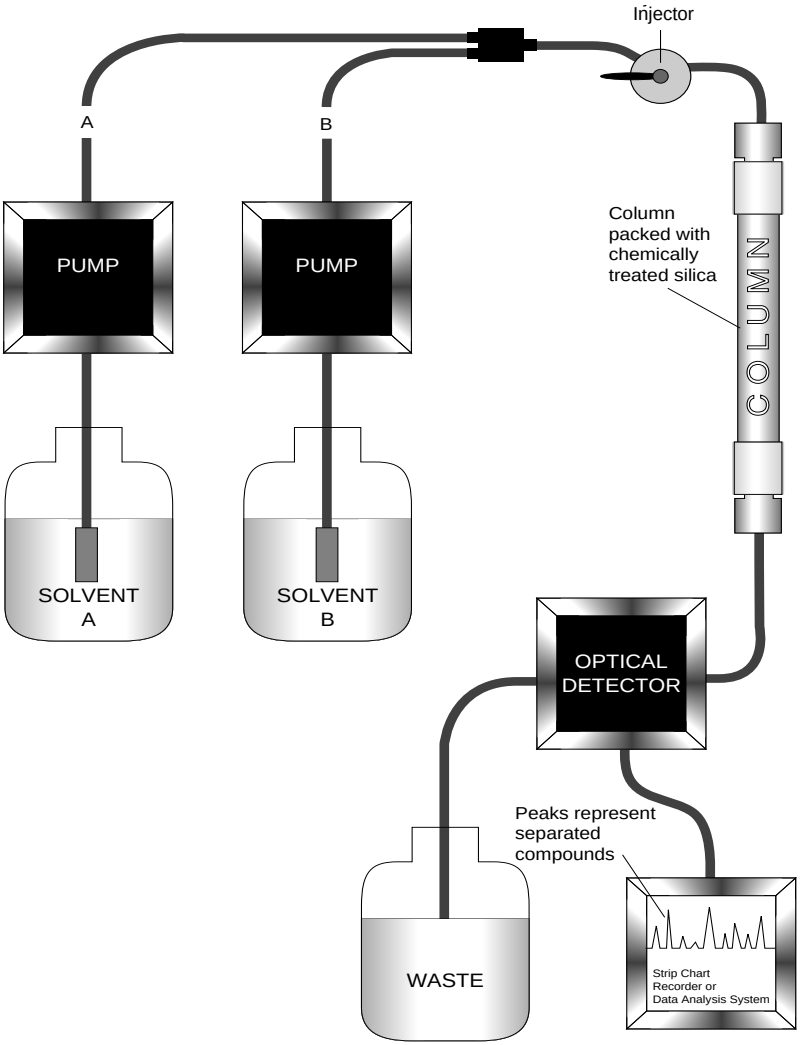


Figure 9. High Performance Liquid Chromatography (HPLC)

A mixture of compounds within a sample are separated by passing the sample through a column packed with chemically treated silica. An optical detector identifies the different concentrations of samples within the solvent. Peaks recorded on the chromatograph represent the separated compounds.

Minimizing the Loss of Proteins in Solution

Low- and sub-microgram amounts of protein require careful handling. Reducing the number of steps in your sample preparation procedure will minimize the opportunities to lose material. The final concentration and buffer exchange steps often employed in conventional purification strategies are frequently the cause of significant material loss. Such steps include trichloroacetic acid precipitation, reverse-phase HPLC, gel filtration, dialysis, and volume reduction under a stream of N_2. These techniques are obviated by using SDS-PAGE and electroblotting.

An alternative to the final concentration and buffer exchange steps is the Perkin-Elmer ProSorb sample preparation cartridge (illustrated in Figure 7 on page 18). ProSorb cartridges can be used to collect proteins in solution onto ProBlott PVDF membranes. When using ProSorb cartridges, the recovery of proteins larger than 10 kDa is up to 90%. Buffers and other interfering reagents can be washed away before the membrane is put into the sequencer. Before loading the sample, detergent micelles must be broken up either by:

1. Dilution with water until the concentration of detergent is below the critical micelle concentration (CMC), or
2. The addition of an organic solvent such as acetonitrile. Enough solvent should be added to bring the final solvent concentration up to 10% to 20%.

Protein samples can be concentrated by performing multiple loads of the same ProSorb sample preparation cartridge. The following are suggestions for keeping sample loss to a minimum when preparing samples in solution.

Keeping Sample Loss to a Minimum when Preparing Samples in Solution

- Use only polypropylene or polyethylene containers. Polystyrene and glass containers (even siliconized glass) adsorb proteins—especially hydrophobic ones.
- Minimize surface losses by using the smallest container possible, and by avoiding transfers from tube to tube.
- Use either acetonitrile, 1% acetyl glucoside, or up to 0.01% SDS to help minimize adsorption.
- To prevent proteins, particularly membrane proteins, from sticking to tubing, coat tubes with analytical grade polypropylene glycol-4000 (10 µL per liter overnight followed by three rinses with water). No sequencing artifacts will be produced.
- Avoid vacuum concentrating or lyophilizing samples completely, since even highly soluble proteins are unlikely to be reconstituted from complete dryness. Greater than 80% recovery after lyophilizing or vacuum concentration would be unusually good.

7 Measuring the Protein in a Sample

Because protein loss is unavoidable, measuring the protein content in your sample is most meaningful when performed immediately prior to sequencing—not before a long storage period, or before the final steps of sample preparation. Initial sequencing yields will be unexpectedly low when the amount of protein in a sample is overestimated. Appendix B contains detailed instructions on how to convert picomoles to micrograms, including formulas and a conversion chart.

A variety of techniques can be used to measure the amount of protein in a sample; some are more accurate than others. Staining intensity is the most common method used, particularly for complex mixtures of proteins. Estimates obtained from stained gels, also referred to as sample evaluation or analytical gels, are more reliable than estimates obtained from stained membranes. The following is a simple method which utilizes silver stain and requires only a small amount of protein.

1. Run 5% of your sample on a mini-gel.
2. Apply silver stain.
3. If a band appears, enough protein is *probably* present in your sample for sequencing.

This quick check requires very little sample because of the sensitivity of silver stain, and is useful when preparing a sample from tissue cultures. If enough protein is not present, more cells can be grown.

Other more accurate methods for measuring the amount of protein in a sample are spectrophotometric assays and amino acid analysis. These methods are only effective, however, when the sample is purely the protein of interest—not a complex mixture of proteins.

Sample Evaluation Gels

A sample evaluation (analytical) gel can be used to estimate the concentration of a protein in solution. A measured volume of sample is loaded onto a gel with four or five lanes containing known amounts of molecular weight standards from 0.1 to 20 µg. The gel is then stained with Coomassie Brilliant Blue (CBB R-250). The intensity of the standard proteins' CBB R-250 staining is used to estimate the number of micrograms of the protein of interest.

Keep in mind the following important facts.

- CBB R-250 stains some proteins better than others.
- CBB R-250 stained gels give more reliable estimates than CBB R-250 stained membranes.
- Once a sample on a gel has been stained, it cannot be electroblotted. A second gel without stain must be used for electroblotting.

Spectrophotometric Assays

Spectrophotometric assays of protein concentration can be more reliable than estimating the amount of protein by staining. The sample, however, must be in solution, and must contain *only* the protein of interest.

Four commonly used spectrophotometric methods are the:

- Lowry method—based on copper binding[24]
- Bradford method—based on Coomassie Blue binding[25]
- Absorbance method (A_{280} / A_{260})—relies on the aromatic rings of certain amino acids[26]
- BCA method—based on copper binding[26]

The BCA method is the most tolerant of buffer components such as 1% SDS and 3.0 M urea. However, only the absorbance method can be used with protein solutions containing beta-mercaptoethanol or dithiothreitol. The sensitivity of the Micro BCA* Assay available from Pierce (catalog #23235) is rated at 0.5 µg per mL. Protein concentrations determined by spectrophotometric assays are relative to whatever standard is used, usually immunoglobulin (IgG) or bovine serum albumin (BSA).

Amino Acid Analysis

Amino acid analysis is the only reliable method of protein quantitation. The protein of interest is completely hydrolyzed, and the amino acid products are separated and quantitated by HPLC. Amino acid analysis may be obtained from a sample in solution, or bound to ProBlott or other PVDF-type membranes.[27]

The disadvantage of amino acid analysis is the amount of protein required. A 1991 survey of core facilities offering this service found that the average amount of a 30,000 Da protein required to obtain an amino acid composition accurate to within 8% is 6.6 µg, or 193 pmol.[6] Some laboratories, however, can perform amino acid analysis on as little as 0.5 to 1.0 µg of protein.

8 Avoiding N-terminal Blockage

As discussed in Chapter 2, *Using Protein Sequence Information*, the first step in protein sequencing by Edman degradation is the coupling of the free N-terminus of the protein with PITC. Some proteins, however, naturally undergo post-translational modification resulting in N-terminal blockage with acetyl, formyl, or pyroglutamyl groups. This blockage prevents the N-terminus from coupling with PITC.

Naturally occurring N-terminal blockage is especially common in eukaryotic cells. For example, 50% of the proteins from human myocardial tissue prepared by electroblotting 2-D gels were found to be unsequenceable.[28] Since Edman degradation cannot be initiated on the N-termini of such naturally blocked proteins, internal sequencing may be an option. Refer to Chapter 9, *Internal Sequencing*, for more information.

If the protein of interest is not naturally blocked, care must be taken to avoid chemical modification of the N-terminus during sample preparation. Acrylamide monomer can react with the free N-termini of proteins. A recent report states the reaction can be prevented by pre-washing the gel with 100 mM cysteine. The report also demonstrates the formation of a cysteine-acrylamide adduct, Cys-S-β-Propionamide (Cys-S-Pam), with no appreciable N-terminal blockage.[29] The modification of cysteine with acrylamide is actually beneficial in preserving this labile residue during sequencing. The PTH-derivative of Cys-S-Pam can be identified with PTH-amino acid chromatography.[30] A better approach may be to reduce and alkylate the cysteines prior to gel electrophoresis when possible.

To determine whether an unexpectedly low sequencing signal was caused by chemical modification during electrophoresis and electroblotting, sequence a Beta-lactoglobulin standard (Perkin-Elmer P/N 400979)

electrophoresed in and electroblotted from an adjacent lane on the gel. If a reasonable amount of Beta-lactoglobulin, but not the protein of interest, can be sequenced, then chemical modification occurred prior to electrophoresis.

Avoiding N-terminal Blockage

The following practices[31] can be adopted to help minimize the chemical modification of samples in acrylamide gels.

- Use HPLC grade glycerol in the sample buffer. Heat only to 37 ˚C for 10 minutes.
- Use 0.1 mM to 1 mM sodium thioglycolate, or 10 mM reduced glutathione in Laemmli running buffer.
- Allow the running gel to age overnight, or pre-electrophorese it before adding the stacking gel.
- Use Towbin buffer if the protein of interest is reduced and alkylated; do not use a pH 11 electroblotting buffer.

Deblocking Samples that will not Sequence

Attempting to deblock a sample can be a triumphant or disappointing experience. A number of different methods for removing N-terminal blocking groups have been reported. To date, none of these methods have proven to be very efficient or generally applicable. The following reported methods are listed by the group(s) they remove.

Formyl group removal—Incubating a stained protein band on a PVDF-type membrane with 0.6 N HCl at 25 ˚C for 24 h is reported to remove formyl groups from N-terminal amino acids.[32]

N-terminal acetylserine or acetylthreonine removal—Brief incubation at 45 ˚C with trifluoroacetic acid followed by drying, and a subsequent 16 h incubation at 60 ˚C or 65 ˚C is reported to remove N-terminal acetylserine or acetylthreonine from many different proteins which are either absorbed on a glass fiber filter[33] or electroblotted onto a ProBlott PVDF membrane.[34] However, some cleavage of internal serines and threonines may also occur.

N-terminal pyroglutamic acid removal—Pyroglutamate aminopeptidase (Boehringer Mannheim catalog #1420 445, or TaKaRa catalog #7321) is reported to remove N-terminal pyroglutamic acid from proteins bound to PVDF membranes[35] or adsorbed to glass fiber filters.[36]

Acetylated N-terminal amino acid removal—Acylamino acid releasing enzyme (Boehringer Mannheim catalog #1370502) is reported to remove acetylated N-terminal amino acids. (For example, from peptide fragments recovered in solution from in situ trypsin digestion.[37])

Enzymatic removal of N-terminal blocking groups is more efficient for some samples than for others. The efficiency of the deblocking method can sometimes be improved if the sample is first reduced and alkylated.

9 Internal Sequencing

Edman degradation can be performed on the N-termini of individual peptide fragments from a specific protein. The various methods involved in this process are collectively referred to as *internal sequencing*. Internal sequencing is useful when:

- The N-terminus of a protein is blocked
- N-terminal sequence data provides little discrimination between members of a family of proteins having extensive N-terminal homology (for example, immunoglobulins)

Internal sequences can also:

- Provide more information for the definitive identification of newly isolated proteins
- Provide new options for designing hybridization probes and PCR primers
- Be used to help verify the authenticity of putative positive clones

Because proteins differ so greatly from one another, the development of a generalized procedure for sample preparation is difficult, and is currently an area of intense research activity. Approximately one half of the core facilities in the United States which offer protein sequencing services also offer the fragmentation of proteins for internal sequencing.

Most of the methods currently used to prepare proteins for internal sequencing are based on the following scheme.

1. Cysteine residues are reduced and alkylated.
2. The protein is cleaved into peptide fragments by chemical or enzymatic digestion.
3. The peptide fragments are separated by electrophoresis or reverse-phase HPLC.
4. Peptide fragments are individually subjected to N-terminal sequencing in an automated protein sequencer.

The challenge and goal of internal sequencing is to achieve cleavage (digestion) at particular sites in a large percentage of the available protein molecules. Low yields of particular fragments due to incomplete cleavage reduces the likelihood of successful sequencing. A survey of core facilities providing internal sequencing services found that the average amount of a 30 kDa protein required for isolating and sequencing an average 15-residue tryptic peptide is approximately 684 pmol.[6] Therefore, measuring the amount of protein contained in an aliquot of your sample by amino acid analysis is recommended before committing the time and effort involved in preparing peptide fragments.

Planning your strategy for sample preparation is largely a matter of personal preference. This chapter provides an overview of the methods which can be used for each of these steps.

Preparation of Proteins in Solution for Cleavage

Proteins in solutions which do not contain glycerol, SDS, or salts can be concentrated under a stream of N_2 or by vacuum-centrifugation. Samples must *never* be completely dried before concentration; otherwise, significant sample loss is likely to occur.

A protein in a solution containing SDS, glycerol, ampholines, or salts may be prepared by precipitation with either trichloroacetic acid (TCA) or acetone.[38] For efficient cleavage, the amount of SDS, glycerol, ampholines, and salt in the sample must be very low.

For efficient precipitation, the protein concentration should be at least 100 µg/mL, and the glycerol concentration must be less than 15%. To remove glycerol before precipitation, add Triton X-100 to a final concentration of 0.02%, and concentrate several times by ultrafiltration to 50 µL.[39]

The precipitated protein pellet can be resuspended in 8 M urea for subsequent reduction, alkylation, and cleavage.

Reduction and Alkylation of Cysteine Residues

The reduction and alkylation of cysteine residues is required for their positive identification in the chromatograms used for sequence analysis. Reduction breaks disulfide bonds, while alkylation covalently modifies the sulfhydryl group and prevents disulfides from reforming. The resulting unfolded state of the protein increases the efficiency of cleavage because the cleavage sites are more accessible.

Many reagents have been used to modify cysteine residues before sequencing. *Carboxymethylation,* the traditional method, results in Carboxymethyl cysteine (CM-Cys). PTH-CM-Cys is difficult to resolve from PTH-glutamine with standard PTH-amino acid chromatography methods. Another commonly used reagent is *4-vinylpyridine,* which gives a pyridylethylated product—PE-cysteine. PTH-PE-cysteine is resolved in PTH chromatography by using Premix buffer.

After reduction at 65 ˚C in 2% SDS and 0.1 M DTT, alkylation can be performed in either:

1. Laemmli buffer containing 0.5% beta-mercaptoethanol and 1.5% 4-vinylpyridine[11], or
2. 2 M acrylamide.[30]

The use of *3-bromopropylamine* as a cysteine alkylation reagent results in aminopropylcysteine[40]. The retention time of the resulting PTH derivative is unaffected by changes in the ionic strength of the chromatography solvents. Methods for performing the cysteine alkylation directly in the

sequencer have been developed. One such method[41], which uses vinylpyridine to form the PE-cysteine derivative, avoids problems with sequencing preview observed with other on-sequencer methods.

Sample loss inevitably occurs when excess reagents and the by-products of alkylation are removed from proteins in solution. For example, when using size exclusion or reverse-phase HPLC for the purification of alkylated peptides, an initial sample quantity of at least 2 nmol has been recommended.[42] To reduce sample loss during alkylation, ProSorb sample preparation cartridges can be used to collect alkylated proteins in solution onto ProBlott PVDF membranes. By-products and excess reagents can then be washed away.

Alternatively, enzymatic digests may be performed in situ directly following carboxamidomethylation without an intervening purification step.[38]

Cleavage

Cleavage of proteins can be achieved through the use of enzymes or chemicals. For enzymatic cleavage, several sequencing-grade enzymes with different cleavage site-specificities are available. Trypsin and Lys-C are the most common enzymes used to prepare fragments for internal sequencing. Trypsin is most likely to deliver completely cleaved peptide fragments, even from insoluble proteins and proteins bound to membranes. Lys-C cleaves only at Lys residues, thus producing fewer peptides of approximately 30 residues. Peptides produce by Lys-C cleavage tend to be easier to purify, and are a better size for sequencing.[43]

When selecting enzymes, choose recombinant enzymes engineered to reduce autolysis.

Enzymatic cleavage is most efficient when:

- The concentration of protein in the sample exceeds 500 µg/mL
- The amount of SDS, glycerol, ampholines and salt is very low
- Cysteine residues are alkylated

To minimize trypsin autolysis, use as little enzyme as possible. More enzyme, however, is required for smaller amounts of protein on a molar ratio basis. For example, a 1:25 ratio weight-by-weight (w/w) of enzyme to protein is usually sufficient for the cleavage of 1 to 5 μg of a protein smaller than 60 kDa. For larger proteins, a 1:50 (w/w) ratio of enzyme to protein is usually sufficient if the molar ratio of substrate to enzyme is at least 10:1. For 5 to 100 μg samples, use 1:50 or even 1:100 (w/w).

A peptide map of the enzymatic digestion without the substrate protein can be used to help identify peptide peaks resulting from autolysis of the protease. Alternatively, you can search for sequences from peptide fragments in a smaller database of protease sequences before undertaking a full search. Thus, it is advantageous to use enzymes whose sequences are in the database.

Although enzymatic cleavage is likely to be more complete than chemical cleavage, the peptides resulting from enzymatic cleavage may be too small to purify easily. In such cases, chemical cleavage may be preferred. Two of the methods used to perform chemical cleavage are cyanogen bromide (CNBr) and 2-(2-nitrophenylsulfenyl)-3-methyl-3-bromoindolenine (BNPS-skatole). Specifically, the CNBr method cleaves proteins at methionine residues[44], while the BNPS-skatole method cleaves proteins at tryptophan residues.[7]

In Situ Cleavage Methods

Proteins bound to PVDF-type membranes can be cleaved in situ, either enzymatically[45] or chemically.[44] The quantity of blotted protein, and the amount of enzyme required for in situ digestion can be estimated from the initial yield of N-terminal sequencing analysis performed on protein blotted from another lane of the same gel. In situ cleavage can be performed by following these steps.

1. Remove Coomassie Blue stain with cold acetone; remove Amido Black stain with water.
2. Treat the membrane with 5 M CNBr.
3. Wash the resulting peptides off the membrane with acetonitrile.[38]

The cleavage step in this method has traditionally been performed in formic acid. However, a recent report indicates that incubation in formic acid may result in N-terminal formylation. Therefore, the use of 6 N HCl or aqueous TFA may be preferred.[46]

Polyvinylpyrrolidone (PVP-40) has been used[11, 47] to prevent the binding of protease to the membrane, and to promote the release of fragments from the membrane, especially for large proteins. Unfortunately, even extensive washing cannot remove the UV-absorbing material which obscures the hydrophobic region of HPLC peptide maps when using PVP-40. A recent report indicates that 1% actyl glucoside or PVP-360 can be successfully used in place of PVP-40 in digestion reactions. Besides eliminating PVP-40 contamination, fewer artifacts are produced.[21]

The amount of sample recovered from membranes after in situ fragmentation is typically less than 80%—often much less.

In-Gel Cleavage Methods

Many methods have been reported to cleave proteins within pieces of acrylamide gels. These methods include trypsin digestion[48]; CNBr and BNPS-skatole treatments; *S. aureus* V_8 *protease*; chymotrypsin digestion[50]; and lysyl-endopeptidase (or endoproteinase LysC) digestion.[51]

The piece of gel containing the digested mixture can be loaded directly onto a second gel for separating the peptide fragments.[50] Alternatively, a small portion of the resulting peptide fragments can be recovered in the digestion supernatant. Better recovery has been reported when the gel is either:

1. Centrifuged through a fine nylon mesh after digestion, and extracted with 5 to 10 volumes of buffer,[51] or
2. Macerated with a Kontes-type homogenizer with pestle before digestion, and separated from the gel pieces by ultrafiltration through a 0.22 µ filter.

Peptide Fragment Separation

The preparation of peptides is very dependent on the solvent composition. Peptide fragments can be separated by SDS-PAGE and electroblotted onto PVDF-type membranes. Peptides as small as 1000 Da can be resolved on Tris Tricine gels.[52] Peptides this small, however, will go through the PVDF during electroblotting.

A more common way to separate peptides is to use reverse-phase HPLC on C-18[53] or C-4[54] columns with linear gradients of acetonitrile with 0.1% TFA. Ion exchange on a DEAE pre-column can be used to remove SDS from peptide mixtures.[53]

Peptides can be concentrated for sequencing with ProSorb sample preparation cartridges.

10 C-terminal Sequencing

C-terminal sequencing is the sequential cleavage and identification of the amino acids of a protein starting at the C-terminus. The C-terminal end of a peptide chain is that which carries the free alpha∝carboxyl group of the last amino acid. The automation of this process is an area of active research.

The information obtained from C-terminal sequencing can be an important supplement to the information generated with automated Edman degradation (N-terminal and internal sequencing). C-terminal sequencing information can be used to:

- Determine whether N-terminal sequencing has proceeded to the last residue of a peptide or small protein
- Reveal heterogeneity and truncations in recombinant proteins
- Verify the absence of frame-shifting errors in cDNA sequence analysis

At present, the most common approach for obtaining information about the C-terminus is to measure the amount of amino acids released at precisely timed intervals during carboxypeptidase digestion. This is done by removing an aliquot of reaction mixture, halting digestion, and performing amino acid analysis without hydrolysis. The amount of sample available at each time point is usually very small. The following is an example of the requirements for C-terminal sequencing necessary to obtain accurate sequence data for several C-terminal residues.

Sample C-Terminal Sequencing Requirements

Amino Acid Analyzer

An amino acid analyzer which can analyze less than 20 pmol of amino acid.

Time Points

Analysis of 5 time points.

Sample Type and Quantity

A total of 250 pmol of a 13 residue tryptic fragment purified by reverse-phase HPLC[55] .

A Do's and Don'ts of Sample Preparation

Suggestions for Successful Sample Preparation

Do

- Do prepare at least 100 pmol of sample whenever possible.
- Do use the purest grade reagents—not molecular biology grade.
- Do use the smallest polypropylene tubes possible.
- Do use SDS-PAGE and electroblotting whenever possible.
- Do dissolve proteins which have not been electroblotted in water, acetic acid, formic acid, trifluoroacetic acid, triethylamine, acetonitrile, or propanol, or

  Do use ProSorb sample preparation cartridges to remove contaminants, or to concentrate proteins in solution.
- Consult with Service Lab experts as early as possible.

Suggestions for Successful Sample Preparation (Continued)

Don't

- Don't use Tris, glycine or other free amino acids, guanidine, glycerol, sucrose, ethanol amine, ammonium sulfate, Triton X-100, Tween, or more than 0.01% SDS without removing these interfering reagents prior to sequencing.
- Don't manipulate samples or transfer them to new containers unless absolutely necessary.
- Don't dry protein solutions to dryness.
- Don't use tubes or pipet tips from open boxes. This may introduce dust or other contaminants into the sample.
- Don't rely on an assay of protein quantitation which was performed weeks earlier, or prior to subsequent purification steps or other manipulation.

B Microgram/Picomole Conversion Guide

For most purposes, proteins are measured in units of micrograms (µg). For the purpose of protein sequencing, however, proteins are measured in terms of picomoles (pmol) because of the stoichiometric relationship of the protein and its Edman degradation products. For sequencing, half as many micrograms of a 50,000 Da protein is required than of a 100,000 Da protein. This is because a given number of micrograms of a 50,000 Da protein contains twice as many picomoles (that is, twice as many N-termini) as the same number of micrograms of a 100,000 Da protein. (See Figure 10 on page 58.)

General Formula

The following general formula can be used for picomole / microgram conversions.

1000 / molecular weight of protein in kDa = number of pmol/µg

Given the molecular weight of the protein, quick estimates can be made using this mnemonic device.

100 kDa:	100 µg = 1 nmol	10 µg = 100 pmol
50 kDa:	50 µg = 1 nmol	5 µg = 100 pmol
25 kDa:	25 µg = 1 nmol	2.5 µg = 100 pmol
10 kDa:	10 µg = 1 nmol	1 µg = 100 pmol

From Micrograms to Picomoles

The number of picomoles in a particular sample is equal to the number of micrograms divided by the molecular weight of the protein times 10^{-6}.

To convert micrograms of protein to picomoles

1. Calculate the number of micrograms per picomole of a particular protein by multiplying the protein's molecular weight in Da by 10^{-6}.
2. Divide the measured or estimated number of micrograms by the number of micrograms per picomole.

   The following formula can be used.

   $M / (W \times 10^{-6}) = P$

   Where. . .

   M = the number of micrograms of sample available

   W = the molecular weight of the protein

   P = the number of picomoles of sample available

For example

A 45,000 Da protein weighs 0.045 µg per picomole ($45{,}000 \times 10^{-6}$). Therefore, 2.5 µg is equal to 55.6 pmol (2.5 / 0.045).

Similarly, a band in a gel estimated to contain 4.0 µg of a 45,000 Da protein provides about 90 pmol for sequencing (4.0 / 0.045 = 88.9).

From Picomoles to Micrograms

The number of micrograms of protein needed for a sample consisting of a specified number of picomoles is equal to the number of picomoles multiplied by the molecular weight times 10^{-6}.

To convert picomoles of protein to micrograms

1. Multiply the molecular weight of the protein by 10^{-6}.
2. Multiply the sum from step 1 by the number of picomoles desired.

In other words, to determine the amount of protein required to prepare a 50 pmol sample for sequencing, multiply the number of micrograms per picomoles by 50.

The following formula can be used.

$P(W \times 10^{-6}) = M$

Where . . .

M = the number of micrograms of sample required

W = the molecular weight of the protein

P = the number of picomoles desired

For example

A 60,000 Da protein weighs 0.06 µg per picomole ($60{,}000 \times 10^{-6}$). Therefore, the preparation of a 50 pmol sample for sequencing requires 3 µg (0.06×50).

Similarly, preparing a 50 pmol sample of a 30,000 Da protein for sequencing requires 1.5 µg (0.03×50).

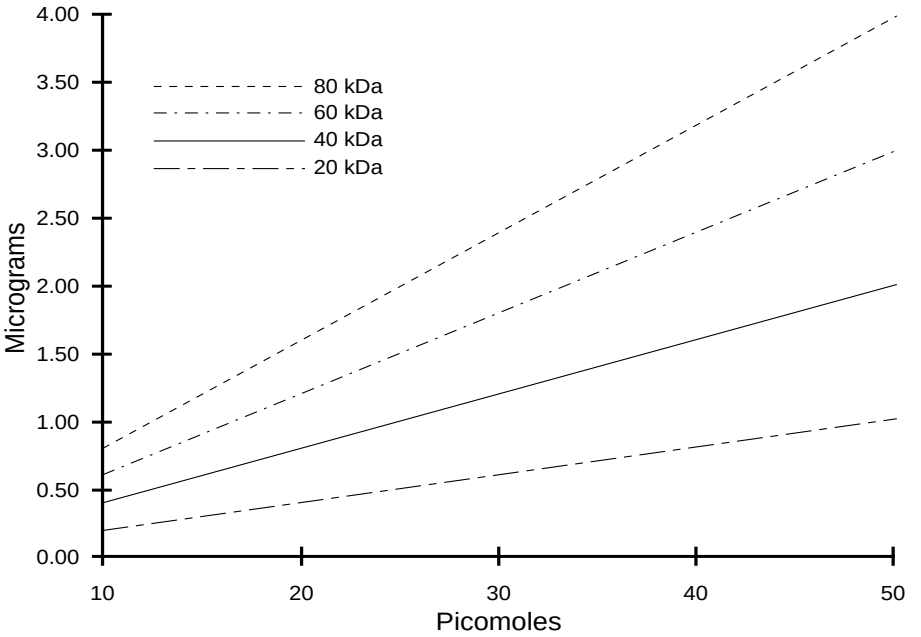


Figure 10. Microgram/Picomole Conversion Chart

Glossary

Acid Hydrolysis the decomposition or alteration of a chemical substance by water catalyzed by acid.

Adduct a chemical compound that forms from chemical addition of two species. For example, reaction of butadiene with styrene forms an adduct, 4-phenyl-1-cyclohexene.

Amino Acid any of the organic compounds that contain one or more basic amino groups and one or more acidic carboxyl groups, and that are polymerized to form peptides and proteins. Only 20 of the more than 80 amino acids found in nature serve as the building blocks for proteins.

Antibody a protein produced by lymphoid cells (plasma cells) in response to foreign substances (antigens). An antibody is capable of coupling specifically with its homologous antigen (the one that stimulated the immune response), or with substances that are chemically very similar to that same antigen. Can produce immunity in an organism against certain microorganisms and their toxins by neutralizing them.

Antigen 1) a foreign substance that, upon introduction into a vertebrate animal, stimulates the production of homologous antibodies; a complete antigen or immunogen. A complex antigenic molecule may carry several antigenically distinct sites (determinants). 2) A substance that is chemically similar to certain parts of an immunogen and can react specifically with its homologous antibody, but is too small to stimulate antibody synthesis by itself; an incomplete antigen or hapten.

Assay quantitative or qualitative analysis of a substance.

Autolysis self-digestion or decomposition. Also, an enzyme cleaving itself.

Background the level of PTH-amino acid signal that is not attributable to a residue in the protein.

Blotting (electroblotting) the general name given to methods by which electrophoretically or chromatographically resolved RNAs, DNAs or proteins can be transferred from the support medium (e.g. gels) to an immobilizing paper or membrane matrix. Blotting can be performed by two major methods: 1) capillary action (e.g. Southern or Northern blotting), or 2) electroblotting, which involves the transfer of molecules by electrophoresis.

Chromatograms see chromatography

Chromatography a technique used for separating and identifying the components from mixtures of molecules having similar chemical and physical properties. The population of different molecules is dissolved in an organic solvent miscible (capable of being mixed in all proportions) in water, and the solution is allowed to migrate through a stationary phase. Since the molecules migrate at slightly different rates, they are eventually separated. In *thin layer chromatography*, the stationary phase is a thin layer of absorbent silica gel or alumina spread on a flat glass plate. Chromatograms/graphs are the records produced from chromatography (for example, a picture of a multi-colored gel).

Clone genetically engineered replicas of DNA sequences.

Cloned DNA any DNA fragment that passively replicates in the host organism after it has been joined to a cloning vector.

Cloning vector (DNA vector) a replicon, such as a small plasmid or bacteriophage, that can be used in molecular cloning experiments to transfer foreign nucleic acids into a host organism in which they are capable of continued propagation.

Codon each set of three bases of the RNA sequence; the nucleotide triplet in messenger RNA that specifies the amino acid to be inserted in a specific position in the forming polypeptide during translation. There are also codons which have the function of starting and stopping the protein synthesis process. A complementary codon resides in the cistron, specifying the mRNA in question.

Crossing Over the exchange of genetic material between homologous chromosomes. Crossing over is characterized by positive interference and by reciprocality; that is, wild-type and double mutants are produced simultaneously in the same tetrad from a trans heterozygote.

Dalton a unit equal to the mass of the hydrogen atom.

Digest (partial) a technique in which a restriction endonuclease is not permitted to cleave at every recognition site in every DNA molecule, but instead is allowed to cleave only some targets. Usually there is not enough time or enzyme to cleave all the sites. One way to obtain a partial digest is to stop a portion of the reaction early. This allows the restriction recognition sites of a single enzyme to be ordered. For example, if there are fragment sizes of 2100 bp and 1400 bp in a complete digest, and a fragment size is 3500 bp in the partial digest, this might suggest that the 2100 bp piece and the 1400 bp piece are adjacent to each other.

Edman degradation cyclical process by which the amino acids of a protein or peptide are sequentially separated from the chain one-by-one, starting from the N-terminus, and identified.

Elution the removal of adsorbed species from a porous bed or chromatographic columns by means of a stream of liquid or gas.

Electropherogram the printed image resulting from gel electrophoresis—a technique used to separate substances by electric charge and size using an electric field; usually applied to a solid, porous matrix. (See *gel electrophoresis*.)

Heterozygote a diploid or polyploid individual that has inherited different alleles at one or more loci and therefore does not breed true.

Homozygote an individual or cell characterized by homozygosity (the condition of having identical alleles at one or more loci in homologous chromosome segments.)

Homogenous of the same or similar nature or kind.

Homolog(ue) in evolution: characteristics that are similar in different species because they have been inherited from a common ancestor. In cytology: see homologous chromosomes

Homologous 1) referring to structures or processes in different organisms that show a fundamental similarity because of their having descended from a common ancestor. Homologous structures have the same evolutionary origin although their functions may differ widely, for example, the flipper of a seal and the wing of a bat. 2) corresponding or similar in position, value, structure or function.

Homologous chromosomes chromosomes that pair during meiosis. Each homologue is a duplicate of one of the chromosomes contributed at syngamy by the mother or father. Homologous chromosomes contain the same linear sequence of genes and as a consequence, each gene is present in duplicate.

HPLC high-performance liquid chromatography.

Hydrolysis the splitting of a molecule into two or more smaller molecules with the addition of the elements of water.

Hydrophobic water repelling; referring to molecules or functional groups in molecules (such as alkyl groups) that are poorly soluble in water. Populations of hydrophobic groups form the surface of water repellent membranes.

Hydrophilic strongly attracted to water.

Labile (labile residue) unstable residue which remains unstable until it reacts with something else.

Lag the partial removal of an amino acid during Edman degradation, resulting in an out-of-phase signal in subsequent degradations.

Lambda a microliter (μL).

Lambda cloning vector a lambda phage that is genetically engineered to serve as a receptor for foreign DNA fragments in recombinant DNA experiments. Vectors that have a single target site at which foreign DNA is inserted are called insertion vectors, those having a pair of sites that span a DNA segment that can be exchanged with a foreign DNA fragment are called replacement or substitution vectors.

Ligase an enzyme that catalyzes the repair of nicks in double-stranded DNA. Also used in cloning to join DNA fragments.

Lyophilize to dry (as tissue or serum) in a frozen state under high vacuum for preservation; to freeze-dry; to dry by sublimation of solvent from frozen sample.

Meiosis the doubling of the gametic chromosome number (which accompanies syngamy) is compensated for by halving the resulting zygotic chromosome number at some other point during the life cycle.

Micelle a spherical array of amphipathic molecules in which the nonpolar tails form a hydrocarbon microdroplet enclosed in a shell composed of the polar heads.

Monomer a simple compound from which, by repetition of a single reaction, a polymer is made. For example, uridylic acid (U) can be polymerized to form polyuridylic acid (UUU...).

N-terminus protein sequences are conventionally written with the amino (NH_2) end to the left. The assembly of amino acids into a polypeptide starts at the N-terminal end.

Nucleotide one of the monomeric units from which DNA or RNA polymers are constructed, consisting of a purine or pyrimidine base, a pentose, and a phosphoric acid group. The nucleotides of DNA are deoxyadenylic acid, thymidylic acid, deoxyguanilic acid and deoxyctidylic acid. The corresponding nucleotides of RNA are adenylic acid, uridylic acid, guanylic acid and cytidylic acid.

The building blocks of nucleic acids composed of three parts: a base, a sugar, and a phosphate. The sugars and phosphates form the backbone of the nucleic acid, while the bases lie flat like the steps of a staircase. DNA is composed of four different kinds of nucleotides represented by the letters C (cytosine), G (guanine), A (adenine) and T (thymine). U (uracil) replaces thymine in RNA.

Oligonucleotides nucleic acid molecules formed by the joining of a small number of nucleotide bases. A short sequence of single-stranded DNA or RNA. A linear sequence of up to 20 nucleotides joined by phosphodiester bonds.

Oligonucleotide probes short, synthetic single-stranded DNA fragments (for example, fragments made on Perkin-Elmer DNA synthesizers like the ABI 392/4 or 391). Used in gene analysis to detect single base changes in DNA (e.g. gene analysis for the base mutation that causes sickle-cell anemia).

Peptide 1) a compound formed of two or more amino acids. 2) a protein with relatively few amino acids (30 to 50).

Peptide Bond a covalent bond between two amino acids formed when the amino group of one is bonded to the carboxyl group of the other and water is eliminated.

Plasmid an extrachromosomal genetic element found in a variety of bacterial species that generally confers some evolutionary advantage to the host cell (such as resistance to antibiotics and production of colicins.) Plasmids are double-stranded, closed DNA molecules ranging in size from 1 to 200 kb. Plasmids whose replication is coupled to that of the host so that only a few are present per bacterium are said to under *stringent control*. Under *relaxed control*, the number of plasmids per host cell may be from 10 to 100.

Plasmid cloning vector a plasmid used in recombinant DNA experiments as an acceptor of foreign DNA. Plasmid cloning vectors are generally small and replicate in a relaxed fashion. They are marked with antibiotic resistance genes and contain recognition sites for restriction endonucleases in regions of the plasmid that are not essential for its replication. One widely used plasmid cloning vector is pBR322.

Polymerization 1) the bonding of two or more monomers to produce a polymer. 2) Any chemical reaction that produces such a bonding.

Polypeptide a polymer made up of amino acids linked together by polypeptide bonds.

Polymer 1) a substance made of giant molecules formed by the union of simple molecules (monomers). 2) A macromolecule composed of a covalently bonded collection of repeating subunits or monomers linked together during a repetitive series of similar chemical reactions. Each strand of DNA is a linear polymer of nucleotide monomers. A linear polypeptide chain is a polymer of amino acid monomers.

Protein a molecule composed of one or more polypeptide chains, each composed of a linear chain of amino acids covalently linked by peptide bonds. A class of long, chain-like molecules containing hundreds or thousands of subunits called amino acids. Proteins have a variety of important functions. As enzymes, they control the rate of chemical reactions. As structural elements, they provide the cell with its shape. Proteins also may have secondary (cross-links) and tertiary (spatial form) structure.

Proteolytic causing the digestion of proteins into simpler units called proteolytic fragments.

Proteolytic fragments see proteolytic.

Putative generally regarded as such; supposed.

Recombinant 1) the new individuals or cells arising as the result of recombination. 2) recombinant DNA or a clone containing recombinant DNA.

Recombination the occurrence of progeny with combinations of genes other than those that occurred in the parents due to independent assortment or crossing over.

Recombinant DNA a composite DNA molecule created in vitro by joining a foreign DNA with a vector molecule.

Recombinant DNA technology techniques that unite foreign RNA molecules or splice different RNAs from the same species. For example, a heterologous RNA sequence can be constructed by ligation of two or more different RNA molecules with T4 RNA ligase.

Restriction enzymes enzymes which cut DNA stands at specific sites. Over 400 restriction enzymes are known today.

SDS-PAGE sodium dodecylsulfate-polyacrylamide gel electrophoresis. The inherent high resolution of SDS-PAGE combined with the blotting efficiency of PVDF membranes allows protein chemistry laboratories to routinely obtain useful sequence information on low picomole quantities of isolated proteins.

Stoichiometric (stoichiometry) the numerical relationship of elements and compounds as reactants and products in chemical reactions.

Supernatant the fluid lying above a precipitate in a centrifuge following the centrifugation of a suspension.

Syngamy the union of the two nuclei of two gametes following fertilization to produce a zygote nucleus.

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Index

Click the letter to the right of each page to go to it.

Then click any entry to go to it.

A

B

C

D

E

M

N

O

P

R

S

Click here to see more “S” entries

T

U