Protein Sequencing Service

GUIDELINES FOR SAMPLE SUBMISSION

Automatic N-terminal sequencing of proteins both in solution or blotted onto PVDF membrane is carried out on an Applied Biosystems protein/peptide automated sequencer Procise Model H49. This instrument is employing the gas-phase chemical procedure derived from the degradation method originally developed by Edman.
At the end of each Edman degradation cycle, the PTH-amino acid is loaded onto a reverse-phase C-18 column, separated from reaction by-products and identified by its retention time on the HPLC chromatography. A standard mixture containing 19 standard PTH-amino acids is used for each analysis to obtain the standard retention times of the amino acids.
All HPLC chromatograms are collected using a computer data analysis system. Identification of the correct amino acid released at each Edman cycle is obtained by comparison of the chromatogram corresponding to the same cycle with that obtained in the previous cycle. Quantitative analysis of the HPLC peak from each cycle is provided by tha data analysis software. The procedure is repeated sequentially to provide the experimental N-terminal sequence of the protein/peptide submitted to analysis.

Conditions for N-terminal Protein Sequencing

N-terminal sequencing by automated Edman degradation can be performed on samples in solution or from proteins electroblotted onto PVDF membranes.

Sample purity:

PVDF blotted Sample: well separated bands.
Sample in solution or lyophilized: ≥ 90% purity
In any case, samples should contain only one protein or peptide. Sample quality is critical. Any impurity that interferes with Edman's chemistry will lead to poor results.

A) Sample in solution or lyophilized:

Single purified protein is preferentially provided in aqueous solution or organic solvents (i.e. Acetonitrile, TFA). Pure lyophilized protein is also acceptable. Purification by RP-HPLC usually gives good samples for sequencing. In any case, a free additional clean up is applied to all incoming samples by loading onto a pro-sorb device before N-terminal sequencing analysis.
Please, indicate the last step in sample preparation. Provide information on protein concentration, buffer composition and protein molecular mass, if known.

B) Sample blotted onto a PVDF membrane (preferred):

The protein should be electroblotted from 1D or 2D gels onto a PVDF membrane, using CAPS buffer and staining with Comassie Blue (R250). Following staining/destaining washes, blotted membrane should be rinsed thoroughly with deionized water.

For details, see below for the PVDF membrane blotting protocol.

Samples should be as concentrated as possible before electrophoretic run (15 to 50 pmol/lane). Only two-three little PVDF lanes can be loaded into the sequence cell and submitted to N-terminal sequencing analysis at most.
Please, send a copy of the gel or membrane image, identifying the bands or spots of interest. Do not excise protein bands from the membrane. Send the intact membrane between 2 pieces of Whatman paper, at room temperature.

Requested amount of sample

The amount of protein sample for N-terminal sequencing is strictly depending on the number of requested cycles. Indicative amounts consists of 50-300 pmol of lyophilized or soluble protein or one-three well visible Coomassie stained PVDF bands. Sometimes, a lower amount is also acceptable.

Important for sample submission

Primary and secondary amines (i.e. Tris buffer) react with PITC and should be avoided.
Please, avoid high concentration of detergents and Glycerol or non volatile salts. These contaminants affect Edman degradation reaction, contaminate the instrument or affect PTH amino acid detection.
Peptides and proteins with blocked N-terminal amino groups (e.g. acetylated, formylated, pyroGlu) will not provide sequence by Edman degradation. If a blocked N-terminal is suspected, de-blocking procedures or other options can be discussed and performed.
Unmodified Cys and some modified residues will give a blank cycle during the Edman sequencing. Other modified amino acids will give unidentifiable anomalous peaks.

Analysis time

Usually 5-10 working days from the arrival of the sample.

PROTOCOL for Electroblotting of proteins onto PVDF membrane for N-terminal sequencing

The electroblotting onto PVDF membrane procedure is carried out as usual western blot procedure with some precautions taken into account for N-terminal sequencing.
Important: The gel is run in the normal manner but it is NOT STAINED prior to the blotting. Blotting should be performed as soon as possible after the separation is complete.

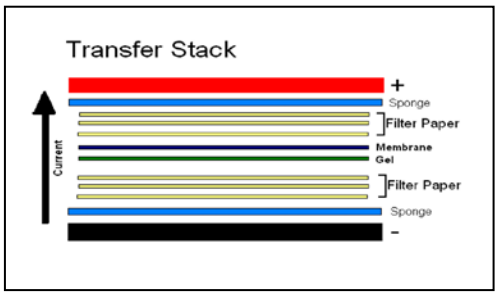
Solutions and materials:

Stock solution of Transfer Buffer: 100mM CAPS (10X):
22.1g CAPS (Sigma) in 900 ml H_2O MilliQ
Adjust pH to 11 with NaOH 2M (~20 ml)
Adjust volume to 1l with H_2O MilliQ
Transfer buffer: 10mM CAPS (1X) with 10% CH_3OH:
800ml H_2O +100 ml CH_3OH+100 ml 100mM CAPS (10X):
Staining Solution: 0.25% Coomassie Blue R250:
2.5g Coomassie R250, 500 ml CH_3OH
Adjust volume to 1l with H_2O MilliQ
**Coomassie and Colloidal Coomassie stains also acceptable.*
Destaining Solution:
50% CH_3OH in H_2O
PVDF membrane
(DO NOT USE NITROCELLULOSE)
Other material:
Six pieces Whatman filter papers; two sponges; blotting apparatus.

Procedure:

1. *During the last step of electrophoresis, prepare the necessary materials and solutions.*
2. *After sample electrophoresis, soak gel in transfer buffer for 5-10 min.*
3. *Prewet the pieces Whatman filter papers and the sponges in the transfer buffer.*
4. *Prewet PVDF membrane with 100% methanol (HPLC grade), then soak membrane in H_2O MilliQ and then in transfer buffer for 5-10 min.*
5. *Assemble Blot apparatus as following described:*

(-) Plastic plate
3 pieces Whatman filter paper
Gel
PVDF membrane
3 pieces Whatman filter paper
(+) Plastic plate



6. *Roll out air bubbles with a plastic pipette between each step.*
7. *Once the stack is prepared, put it in the transfer system and blot for 1-3 hrs at 50 volts at 4°C with stirring of buffer.*

(Time length depends on protein MW. Larger proteins may require transferring overnight at 40 volts in the cold).

8. *After blotting, stain the membrane with Coomassie R250 (or similar) for 10-15 min.*
9. *Destain with 3-4 changes of destain solution. Background staining can be reduced by rinsing the blot with 100% methanol (HPLC grade).*
10. *Store the intact membrane sandwich between 2 pieces of Whatman paper at room temperature or at 4°C.*
11. *After blotting, stain the gel with Colloidal Coomassie Brilliant Blue to ensure that a sufficient quantity of material has been transferred.*

Notes:

For blotting larger (above 80 kDa) and/or apolar proteins, we suggest to add 0.1% SDS to the buffer (because of SDS increases the mobility of the proteins) or to remove MeOH from the CAPS buffer (because of MeOH strips SDS from the protein).