Purity Adjusted Total Protein

VaxArray® Imaging and Analysis System

Overview

Purity adjusted total protein analysis is a common method employed to assign protein-specific concentrations to complex mixtures of proteins, such as virus or vaccine mixtures. This method can be used to perform initial calibration of internal standards. This can be especially beneficial during strain changes and emerging pandemics when common reference antigens from WHO regulatory laboratories are not yet available.

In this technical note, we describe a common method for implementing purity adjusted total protein known as the paBCA assay.

Background and Principle

Total protein assays such as the BCA (bicinchoninic acid) assay are accurate, well understood assays that provide a good measurement of the total protein concentration for a sample. Unfortunately, total protein assays cannot distinguish between individual proteins within a mixture.

SDS-PAGE analysis of a protein mixture can be a powerful way to separate proteins by molecular weight. With appropriate treatment, individual proteins can be separated sufficiently enough to determine relative abundance. However, the SDS-PAGE analysis of absolute protein concentration is difficult and often inaccurate.

The purity adjusted total protein analysis couples both of these assays together to overcome their drawbacks. A total protein assay is utilized to determine the total protein concentration, while an SDS-PAGE analysis is used to determine the relative abundance of the protein of interest in the sample. Coupling these two analyses together results in a final concentration of the specific protein(s) of interest.

Total Protein Assay

Total protein assays are well understood and commonplace in many laboratories. Each assay varies slightly in its sensitivity, replicability, and protein-protein variability and each laboratory should determine the best assay for their needs.

Total protein assays generally utilize a linear regression made by analyzing standards composed of bovine serum albumin. Samples with unknown protein content are analyzed in parallel. Each standard and sample are incubated with reagents sensitive to protein concentration. By plotting absorbance of each standard against its known concentration, a standard curve is generated, against which all samples of interest are quantitated.

Each sample of interest should be analyzed in triplicate at three separate dilutions to account for effects/variance due to varying regions of the standard curve. This also helps to ensure that a sample of unknown concentration will have at least one dilution fall within the standard curve.

A final dilution factor-adjusted protein concentration can be determined from all

InDevR, Inc
2100 Central Ave, Suite 106, Boulder, CO 80301
www.indevr.com | 303.402.9100 | sales@indevr.com

analyzed replicates and the appropriate error calculated.

For this step InDevR, Inc. uses the Thermo Fisher Micro BCATM Protein Assay Kit (Thermo Fisher catalogue number 23235) according to the procedure provided by Thermo Fisher.

Deglycosylation of Samples

In order to identify proteins of interest and determine their relative abundance, samples are separated by electrophoretic current on a polyacrylamide gel. Many proteins are expressed in glycosylated forms which can make them run as diffuse bands on the gel. Not only can this make defining the boundary of the band difficult, it can also cause bands to overlap, further complicating the analysis. Deglycosylation treatment of samples can help overcome this issue. Common glycosidases such as PNGase F work very well in these applications. We suggest denaturing the sample at 95°C with 10% Sodium Dodecyl Sulfate (SDS) and a reducing agent, cooling to room temperature, adding a non-ionic detergent such as Triton as well as Tris-HCl to preserve glycosidase activity, and finally incubating with a small volume of glycosidase overnight at 37°C. This process will remove any N-linked glycoforms from proteins in the mixture and result in compact bands on the gel.

For this step InDevR, Inc. uses Promega PNGase F (Promega catalogue number V4831). First, we incubate the sample(s) at 95°C with a final concentration of 0.38% SDS and 54 mM DTT. After 5 min at 95°C we allow the solution to cool and add Tris-HCl to a concentration of 59 mM, Triton X-100 to a concentration of 1.2%, and PNGase F to a concentration of 0.58 u/µL. We then incubate at 37°C overnight.

SDS-PAGE and Densitometric Analysis

After deglycosylating the samples of interest, analysis by SDS-PAGE is performed under reducing conditions. At these conditions HA will run as two separate bands: HA1 at ~40 kDa (varies slightly depending upon subtype) and HA2 at ~25 kDa. It is important to include a protein standard 'ladder' in the gel. These 'ladders' help to assign molecular weights to specific bands in samples. Following electrophoretic separation on the gel, the gel should be stained with a Coomassie blue stain. For this step InDevR, Inc. uses SimplyBlue™ SafeStain (Thermo Fisher catalogue number LC6060) according to the procedure provided by Thermo Fisher.

Samples should be analyzed at sufficiently high concentrations to ensure the stain provides strong enough bands to evaluate accurately. This should be evaluated individually at each laboratory, but InDevR, Inc. has found that samples analyzed with no dilution (full concentration) yield the best results.

After staining, each protein band in the sample lane can be identified based upon that proteins molecular weight using the protein standard 'ladder' as a reference.

For the densitometric analysis we use the software ImageJ. This software allows the creation of a graph of the pixel intensity in each lane of a gel. The user can then identify the bands that make up HA1 and HA2. A relative concentration of HA0 for the sample, expressed as a percentage of total protein, is determined by adding together the area under the curves for the HA1 and HA2 bands and comparing that area with the area under the curves for all bands. It is important to exclude the band for

InDevR, Inc
2100 Central Ave, Suite 106, Boulder, CO 80301
www.indevr.com | 303.402.9100 | sales@indevr.com

PNGase F from this analysis as it was added to the sample during a previous step and is not an inherent part of the total protein measurement. After the relative concentration of HA0 has been calculated, the total protein measurement can be multiplied by this value in order to determine the total concentration of HA0.

InDevR, Inc
2100 Central Ave, Suite 106, Boulder, CO 80301
www.indevr.com | 303.402.9100 | sales@indevr.com