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Lessons in Proteomics in Quality Assurance and Quality Control

This brief is the summary of a presentation given by Sushmita Mimi Roy at the Brooks Life Science Systems Corporate Symposium at ISBER 2015 on May 5, 2015.

Introduction

Proteomics can contribute significant data about quality control and assurance when it comes to the viability of biological samples. Proteomics is defined as a branch of science where people study protein structure, function, and interactions, and how proteins are organized in cells, tissues, and organisms. For the sake of simplicity, this paper will focus on technologies that have been used previously to annotate and measure the variety and amounts of different proteins in complex matrices such as human blood specimens.

Proteomics is a good indicator of sample quality. It shows if some proteins are going out of solution, what proteins are precipitating, which ones are staying in solution, and how much change has occurred. It tells us if some proteins are increasing and the relative amount of increase, the change in disulphide bonds, and whether proteolysis or deamadation is occurring. These are all changes that mass spectrometry has been successfully utilized to quantify.

When analyzing biological samples with mass spectrometry, the most important thing analytical chemists worry about is analytical variation. Is the mass spec calibrated? Is the sample prep good? In any good proteomics lab or biobanking facility, a good quality management system is essential for best-practice performance.

To quote a colleague, "The costs of repeating a process due to a non-conformity (error) is 3 times the cost of performing the test correctly in the beginning. There is the cost of the initial activity, the cost of redoing the activity, and the cost of the lost opportunity."[1]

That is why we there is such tremendous interest in studying pre-analytical as well as analytical variation. Some of the pre-analytical factors that affect analytical results are as follows:

- At baseline, samples can have variation due to the use of drugs, antibiotics, and anesthesia plus the nature of the sample itself (proteases in gut or RNAse in placenta).
- Conditions during sample handling, processing, storing, and distribution
- Time at room temperature
- Room temperature
- Additives/fixatives (EDTA, Formalin, protease inhibitors)
- Freezing conditions (rate, final temperature, thaw events)
- Time in freezer
- Thawing conditions
- Aliquot size (freeze/thaw rates, uniformity)
- Type of storage tube (plastics/container leaching)
- Restocking (number of freeze/thaw cycles)
- The not-so-perfect human: while there are SOPs, training, and oversight, differences do occur in the way technicians prepare and handle samples before and after analysis.

The Study

The study was conducted as part of 4-year contract from the National Cancer Institute (NCI Contract No. HHSN261200800001E). The goal was to test and develop highly specific quality assessment resources, tools, and guidelines for the collection, manipulation, and storage of human blood-derived biospecimens for protein analysis by proteomics.

The workflow included:

- Developing computer-aided clinical sample collection
- Characterizing protein content and stability in blood samples after collection and induction of probable sources of variation
- Providing recommendations on how to process samples
- Developing a multiplexed assay to assess sample quality and history

The clinical blood sample collection was done at the Palo Alto Medical Foundation. More than 200 subjects donated samples. These were prostate and breast cancer patients as well as age- and sex-matched controls. An application was developed that incorporated the design of experiment, and samples were processed and data was collected with computer-based prompts. All of the sample process times, sample IDs, tube types, etc. were measured electronically using bar codes.

In the set of experiments, several variables were studied:

- Five different tube types
- Hours the blood or plasma samples sat on the bench (30 minutes to 4 days) and their temperatures (4° C, 20° C, 37° C)
- Number of freeze-thaw cycles (1 to 5 cycles)
- Time (0, 6, 12 months) and temperature (-20° C, -80° C) in freezer

The Results

The study covered three laboratory scenarios that might lead to sample degradation — room temperature exposure, multiple freeze-thaw cycles, and long-term freezer storage.

The Effects of Room Temperature Exposure on Sample Quality

The first test involved evaluating room temperature exposure on sample quality. Something new was revealed that had not been previously reported. A group of proteins, called the S100 family, showed a significant increase in peptide intensity in the EDTA tubes when incubated on the bench at room temperature for 24 hours. These proteins are held together with calcium, and they bind to the surface of white blood cells. When

there is EDTA in the tube (which is present in the EDTA and P100 tubes), the EDTA chelates the calcium, the complex proteins then fall off of the surface of white cells into the plasma, and therefore these proteins start to be observed at higher intensities in the plasma.

This result was seen consistently in all of the 2,000 samples in different conditions. The data was verified with a different method using an independent set of samples. S100 proteins proved to be a good predictor that blood samples had degraded due to excessive exposure to ambient temperature on the bench.

The Effects of Freeze-Thaw Cycles on Sample Quality

The next test simulated when a freezer stops working, or the electricity goes out, and samples stored at -80° C start to thaw. Five different freeze-thaw cycles were simulated. Representative samples were selected from each of the four cohorts and were exposed to room temperature for 1 hour. They were then returned to the freezer at -80° C for 24 hours. This scenario was repeated up to five times. There were 10 subjects from each of the four cohorts and the five tube types for each of the groups were used.

When the data from the samples that went through one freeze-thaw cycle was compared to the samples that went through three, four, and five freeze-thaw cycles, the protein damage increased significantly. Fibronectin peptides decreased while actin peptides increased.

Different results between tube types also were found. In order of increasing number of significant changes observed, the tube

types were serum, P-100, heparin, EDTA and EDTA+PI. The serum tube was the only one that didn't show an increase of cytoskeleton proteins (actin, tropomyosin), suggesting that the removal of blood cells may be more efficient with this tube type. The serum tube, and to a lesser degree the P-100 tube, also appear to offer some protection against the precipitation of cryofibrinogen.

When the proteins most affected by the freeze-thaw cycles were studied, it was observed that entire families of apolipoproteins dramatically decreased in all of the tubes (see figure 1). Some of the fibrinogens plus fibronectin proved particularly sensitive in the heparin tube.

However, not every protein tested was affected by the multiple freeze-thaw cycles. A large number of proteins remained stable. Only about 5% to 10% of the proteins measured actually changed.

When the different tube types were compared, similarities occurred from the cycle 1 sample to the cycle 5 sample. In the P100 and serum tubes, all of the data aligned, indicating the overall global changes were less. In the heparin and EDTA tubes, both with or without protease inhibitor, the samples transitioned through significant changes by cycle 5.

The results achieved the goal of identifying a panel of protein markers that would indicate if samples had been through too many freeze-thaw cycles and should be excluded from pertinent studies. This final set of markers were validated and verified using a different method and an independent set of samples. The proteins included the following:

Apolipoprotein C-II
Apolipoprotein C-III
Apolipoprotein C-IV
Apolipoprotein E
Apolipoprotein M
von Willebrand factor
Fibronectin

In addition, it was found that P100 and serum tube types protect samples against degradation better than EDTA and heparin tubes. It was also clear there was gradual sample degradation with every freeze-thaw cycle. And finally, there was an impact on innocent samples during sample retrieval.

The Effects of Long-Term Freezer Storage on Sample Quality

The next test involved studying sample quality during long-term storage. Blood collected in EDTA, P100, serum, and heparin tubes was evaluated. The serum and plasma samples were stored at -20° C or -80° C and frozen for 0 versus 6-month, 12-month, and 18-month intervals. Again, proteomics was used to do the assessment.

The result for IgGFc binding protein was dramatic (see figure 2). The samples stored at -20° C were significantly degraded compared to those stored at -80° C. That's because at -20° C there isn't enough energy for reactions or precipitation to happen. The opposite also occurred, which is lysis. Long-term storage can lead to more lysis of cells. This behavior at both -80° C and -20° C was observed.

It is clear that protein stability is a function of its biophysical characteristics —its sequence, how it folds, and how much of it is exposed. It can be very different for each protein. And yet there are some proteins that were not affected. For example, the biomarker cadherin exhibited minimal degradation after 18 months in storage at both -80° C and -20° C.
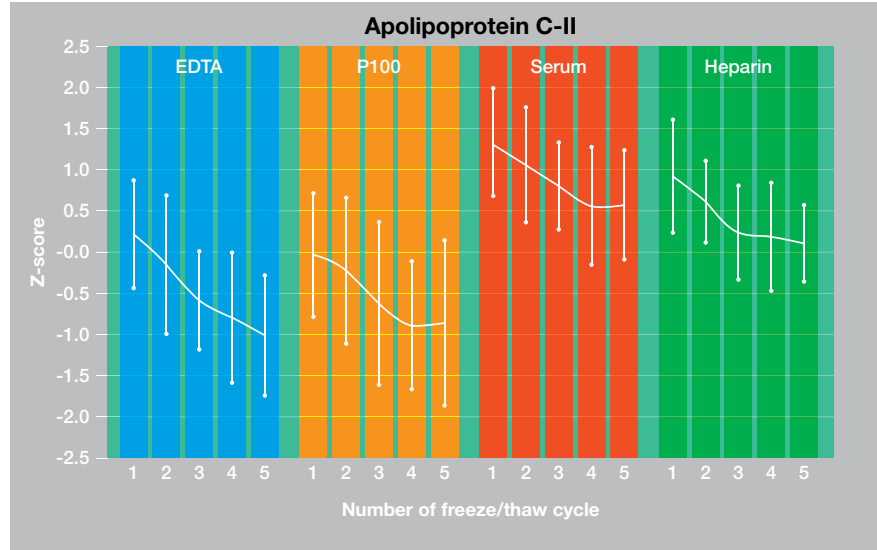

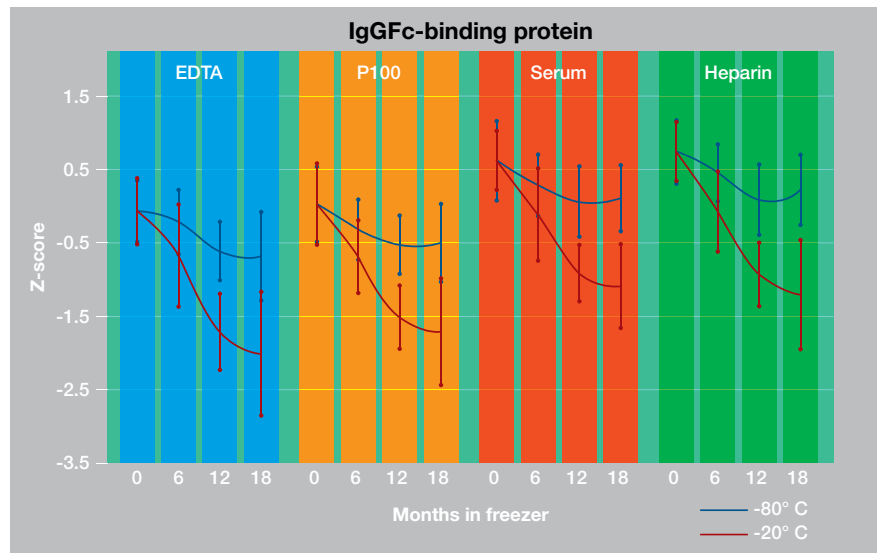Figure 1: Protein degradation over freeze/thaw cycle

Figure 2: 6 vs. 12 vs. 18 month in freezer Greater impact of -20° C vs. -80° C on protein degradation

In addition, sample changes as a result of long-term freezer storage were greater in the EDTA tubes than the serum tubes. This may be because in the serum tubes, the unstable proteins, such as the clotting proteins, are being removed. There is a physical separator in the serum tubes. So the separation between the cells and serum is much better.

A final panel of proteins was developed that represented markers indicating long-term storage damage to samples. The protein list includes Apolipoprotein E, Antithrombin-III, Vitamin D-binding protein, Apolipoprotein C-III and L1, and several others.

The long-term freezer storage test confirmed there are protein markers that signal sample degradation, precipitation,

and cell lysis. Evidence also was found of cell denaturation. There was greater impact of protein degradation and lysis at -20° C than at -80° C. Several proteins showed changes at 6 months, and then experienced smaller changes at the later time points. Others were stable at 6 months, but then showed changes at 6 months to 12 months. Some proteins demonstrated unstable behavior in both the freeze-thaw experiments and in the time-in-freezer studies.

Summary

Overall, the study achieved the following results:

1. Operator prep and sample storage characteristics are very important and should be recorded, because correlations may be found after analyzing the samples.

2. The protein damage due to a number of freeze-thaw cycles is incremental. Consideration of exposure of innocent samples during sample retrieval is very important.

3. The P100 and serum tubes outperform the other tubes for time on bench, multiple freeze-thaw cycles, and time in freezer. Note that the P100 tube is much more expensive, so a cost-benefit analysis is warranted if use is considered. The serum tube is not as expensive, and is also a good performer. The proteomics community adopted plasma tubes because they thought proteins should not be thrown away. However, it appears that serum tubes are superior for these types of experiments.

Overall, evidence-based best practices are very important. Proteomics can be a great tool to study complex samples and verify if they are fit for the purpose.

References

[1] Roberto Salgado, EORTC Pathobiology Group

About the Presenter

Sushmita Mimi Roy is managing partner at iON Bioservices LLC. She previously was senior director and USA site head at Caprion Proteome Inc. and principal scientist/director analytical chemistry at Sutro Biopharma Inc. Ms. Roy holds a Ph.D. in Chemistry from Princeton University, a M.Sc. in Biochemistry from the University of Sydney, and a B.Sc. in Chemistry from St. Stephens College. She is the author of numerous papers and publications on bioscience and bioengineering.

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