Processing Human Serum for Rapid and Reproducible N-Glycan Mass Profiling

APPLICATION NOTE CL0110

KEYWORDS:
Automation, Biomarkers, Glycans, Glycomics, GX-274 ASPEC®, Human Serum, Mass Profiling, Mass Spectrometry, MALDI-FTICR, MS, N-Glycan, N-linked glycans, Serum, Solid Phase Extraction, SPE

This study was performed by Scott R. Kronewitter, Carlito B. Lebrilla and other scientists in the Department of Chemistry, University of California-Davis and UC Davis Cancer Center, Sacramento, California, USA (Kronewitter, S.R. et al., 2010)

INTRODUCTION

Glycans consist of polymers of monosaccharides joined together by glycosidic linkages. Glycans are complex in structure and can be either branched or linear (Figure 1). Glycans are commonly attached to proteins to form glycoproteins. N-linked glycans (N-glycans) are the most common glycans bound to proteins in eukaryotic cells (Apweiler, R. et al., 1999; Kronewitter, S.R., 2010). N-glycans play a major role in regulating biochemical pathways and in the communication at the cellular level between cells, tissue and organs (Shriver at al., 2004). As such, there is a great deal of interest in the study of glycosylation and N-glycans as biomarkers for a variety of diseases (Lebrilla, C.B., 2009; deLeoz, M.L.A. et al., 2008; Turnbull, J.E. et al., 2007).

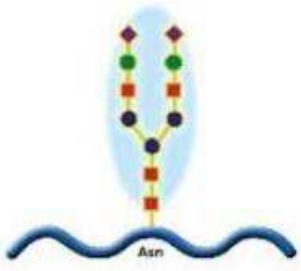

Figure 1
Example of Glycoprotein Structure

Biomarker discovery relies on the reproducible release and quantitative analysis of glycans from tissue. Typically, there are several steps required to characterize these compounds. The first step is to release the glycans from the glycoproteins. The released glycans are then purified/enriched followed by analysis by mass spectrometry. For biomarker discovery, repeatability of each step is critical for success. The reliability of the markers relies on each of the steps to be highly reproducible.

This application note describes a high throughput sample preparation procedure for global glycan mass profiling. Microwave-assisted enzymatic N-glycan release followed by an automated solid phase extraction (SPE) purification procedure utilizing the Gilson GX-274 ASPEC System (Figure 2) were employed to improve sample throughput and stabilize sample processing . A matrix-assisted laser desorption/ionization Fourier transform ion cyclotron resonance (MALDI-FTICR) mass spectrometer was used to rapidly scan the glycan sample and capture high mass accuracy high resolution mass spectra.

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Figure 2
Gilson GX-274 ASPEC® System

EXPERIMENTAL CONDITIONS

Samples

Human serum samples were purchased from Sigma Aldrich and the Gynecological Oncology Group (GOG) Tissue Bank. The number of technical replicates in the case of Sigma serum, and number of individual serum samples from GOG are shown in Table 1. The pertinent processing parameters are also included in the table.

Table 1:
Sample breakdown. Set A,B,C came from a single human serum sample purchased from Sigma Aldrich. Set D consists of 48 different control normal individuals purchased from GOG.

	SAMPLES	SOURCE	RELEASE METHOD	SOLID PHASE EXTRACTION
Sample Set A	4	Sigma	Water Bath	Vacuum Manifold
Sample Set B	4	Sigma	Microwave	Gilson GX-274
Sample Set C	1	Sigma	Microwave	Gilson GX-274
Sample Set D	48	GOG	Microwave	Gilson GX-274

Serum proteins were denatured under mild conditions by mixing equal parts of human serum and an aqueous solution of 200 mM ammonium bicarbonate and 10mM dithiothreitol. This solution is easy to remove during the solid phase extraction step. Thermal denaturation included four cycles alternating between boiling water and room temperature for 15 seconds each.

Rapid Enzymatic Glycan Release and Ethanol Precipitation

Global enzymatic release of N-glycans was achieved using a CEM Microwave Reactor (CEM Corporation, Matthews, NC, USA) to enhance the enzymatic activity of PNGase F. A constant power setting at 20W for 10 minutes yielded the best glycan library coverage and glycan abundances.

Chilled ethanol was used to precipitate residual proteins prior to the SPE purification step. After addition of chilled ethanol, the solution was frozen for 1 hour at -75°C. Samples were then centrifuged for 30 minutes at 13,200 rpm and supernatant containing the glycans was collected.

Automated Graphitic Carbon SPE Step

The Gilson GX-274 ASPEC System was configured as follows:

DESCRIPTION	PART NUMBERS
GX-274 ASPEC	2614010
Four 10 mL Syringes	(4) 25025345
10 mL Plumbing Package for GX-274 ASPEC	2644705
Four 221 x 1.5 x 1.1 BV Tapered Probes and Guide Package for 1.5 mm Probes (GX-274)	(4)27067374 and 2604641
GX-274 Rinse Station and Rinse Station Riser	260440002 and 26045103
Three Custom SPE racks to hold 12 3 mL SPE cartridges and 36 13x100mm collection tubes (Holds Alltech™ Carbograph columns part nos. 210142 or 510142)	(3) Special 1318: Custom Rack and 210630CR, TLH Rack File for Special 1318
Disposable sealing caps for 3 mL SPE cartridges, package of 1000	2954699
Locator Tray for five 20-Series Racks, GX-274	26041032
Plumbing pkg, GX274 ASPEC Air-gas and SPE Pressure Regulator Assembly	2644707 and 25051376
GX Rinse Pump Assembly, 2 Channel and Tubing Kit, 2.0 mm ID Pharmed Tubing	261452 and 26035221
Custom Rack to hold 54 1.5 mL Eppendorf Vials with tethered cap	Special 1392: Custom Rack and 210630CR, TLH Rack File for Special 1392
Custom Rack to hold 44 15 mL Falcon™ tubes	Special 1316: Custom Rack and 210630CR, TLH Rack File for Special 1316
Solvent Reservoir Tray Insert for 700 mL bottles and pkg of four 700 mL solvent bottles	260440005 and 543701700
Viton tubing, .313 ID x .438 OD, 20 ft	4701438630
System Organizer, GX27X	21050000
Safety Shield Assembly, GX27X	2604706
TRILUTION® LH Software Package	21063024

The fractionation procedure used 150 mg/ 4mL Alltech Carbograph™ Cartridges (Grace Davison Discovery Sciences, Deerfield, IL, USA). The cartridges were sealed using Gilson 3 mL Sealing Caps.

The fractionation protocol is entirely automated using the Gilson GX-274 ASPEC system. The SPE steps are summarized with the general schematic provided in the GX-274 ASPEC control software, TRILUTION LH (Figure 3).

The summary of each step are as follows:

- Initialization Step: Gilson Mobile SPE Racks are moved above the waste rack (Figure 3)
- Condition the cartridge with 6 mL nanopure water
- Condition the cartridge with 6 mL 0.1% trifluoroacetic acid (TFA) in 80% acetonitrile (ACN)/H^20 (v/v)
- Condition the cartridge with 6 mL of nanopure (E-Pure, Barnstead) water
- Load glycan solution onto the graphitized carbon cartridge at a low flow rate
- Wash the cartridge with 12 mL of nanopure water at a flow rate of 1 mL/min for desalting
- Move the Gilson Mobile SPE Rack over the collection tubes
- Elute the first fraction with 4.5 mL of 10% ACN/H^20 (v/v) with 0.05% TFA
- Move the cartridges to the next set of collection tubes (Fractionate task) and elute with 4.5 mL of 20% ACN/H^20 with 0.05% TFA
- Move the cartridges to the next set of collection tubes (Fractionate task) and elute with 4.5 mL of 40% ACN/H^20 (v/v) with 0.05% TFA
- Collect the fractions and dry using a centrifugal evaporator apparatus
- Reconstitute each sample in nanopure water prior to MS analysis

Figure 4
Gilson Mobile SPE Rack

MALDI FT-ICR Mass Spectrometry

Mass spectra were recorded on an external source MALDI FT-ICR instrument (HiResMALDI, IonSpec Corporation, Irvine, CA, USA) equipped with a 7.0 T superconducting magnet and a pulsed Nd:YAG laser 355 nm. A solution of 2,5-dihydroxybenzoic acid (DHB)/NaCl was used as the matrix for all oligosaccharide analysis (5 mg/mL in 50% ACN, 25% 0.01M NaCl, and 25% water). For the negative mode analysis, the 0.01M NaCL solution was replaced with nanopure water.

MALDI spotting was performed on disposable MALDI plates to prevent contamination from previous samples. Each MALDI spot was thoroughly mixed and dried rapidly under vacuum. The instrument is equipped with a hexapole for ion accumulation allowing for multiple laser shots to be accumulated prior to loading the ICR cell. Fifteen 355 nm Nd:YAG laser pulses were accumulated then scanned. Three to five spectra were collected from different parts of the MALDI spot and averaged together during data analysis.

The coefficients of variation (CV, standard deviation/ mean) were calculated for the abundance of each glycan and averaged across several areas within the MALDI sample spot.

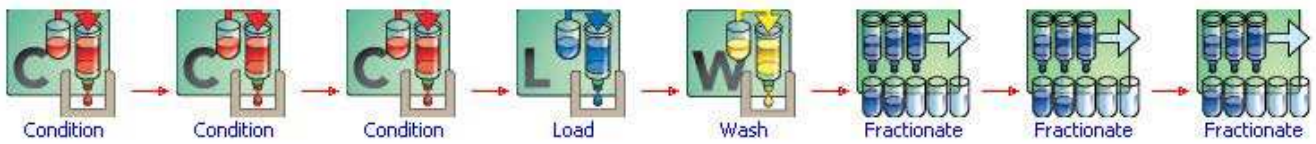

Figure 3
TRILUTION LH Basic SPE Tasks for Fractionation of Glycans.

RESULTS

Reproducibility

A set of replicate samples derived from a single serum sample processed with a microwave reactor and the Gilson GX-274 ASPEC was examined. Five spectra were collected from each MALDI spot and their glycan profiles were elucidated. See Figures 5 and 6 below.

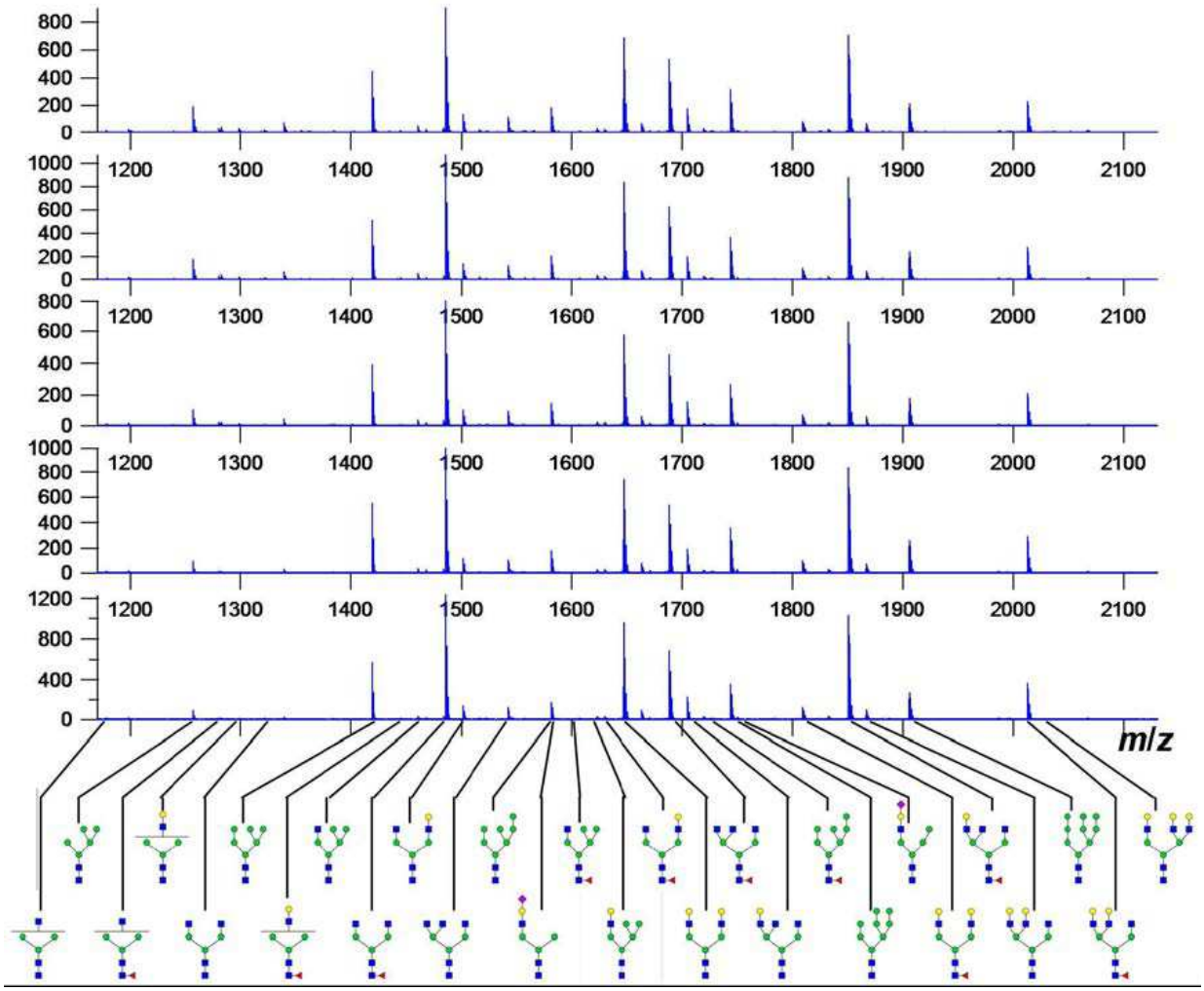

Figure 5

Replicate mass spectra from a single MALDI spot from the 10% ACN elution fraction. The relative distribution of the glycans is highly conserved between the technical replicate spectra. Annotated putative structures are depicted based on common serum glycobiology.

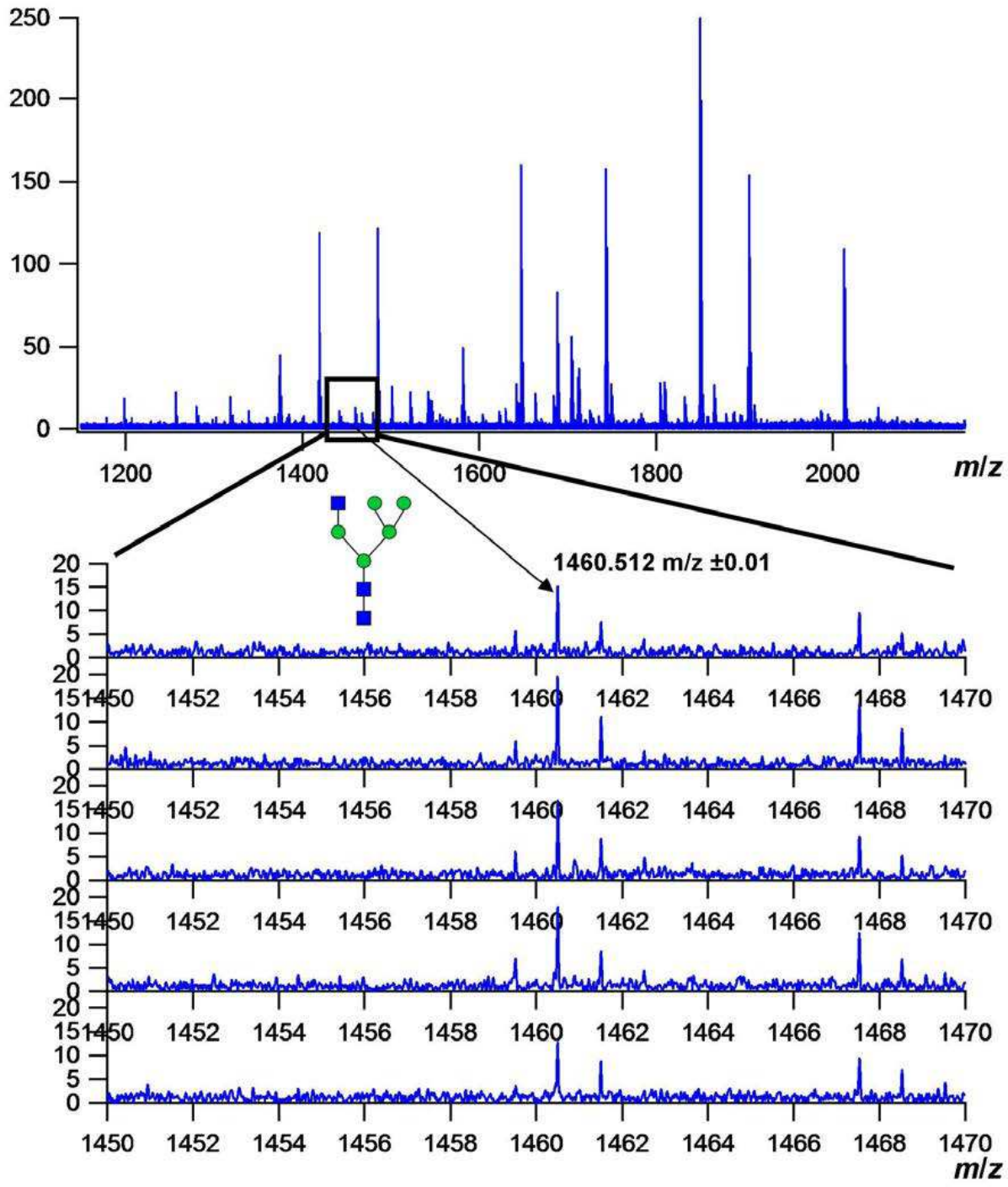

Figure 6
Expanded view of low abundance glycans detected in technical replicate spectra from the same MALDI spot from the 10% glycan fraction.

Variation between different MALDI spots were studied by comparing spectra collected from separate MALDI spots on the probe. The reproducibility of the MALDI spots were examined by comparing the spectra from eight different MALDI spots from a single serum sample (10% ACN fraction). The average coefficient of variation was 10%.

Comparison of the Standard Glycan Enrichment and Release Method with the Microwave Release and GX-274 ASPEC Enrichment Method

Three criteria were used to compare the two methods (Set A = standard digestion and vacuum manifold SPE; Set B = microwave digestion and GX-274 ASPEC Method): number of glycans detected and annotated with the theoretical retrosynthetic N-glycan network library (Figure 7), average glycan abundances (Figure 8), and the mean coefficient of variation (CV) of the glycan abundances.

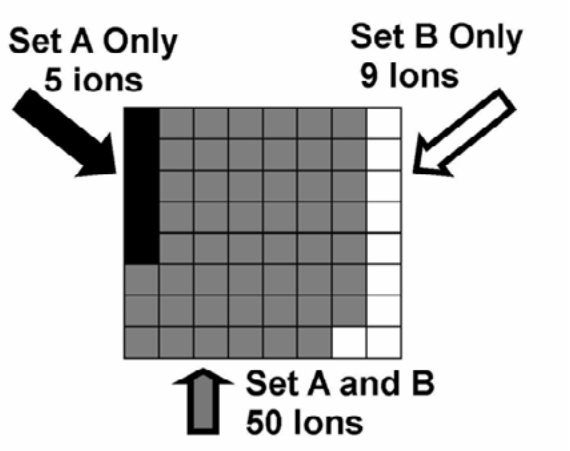

Figure 7
This grid diagram represents the number of glycans detected that are unique and in common between sample Set A and sample Set B. Fifty glycans were detected in both sets while 5 were only detected in Set A and 9 were only detected in Set B.

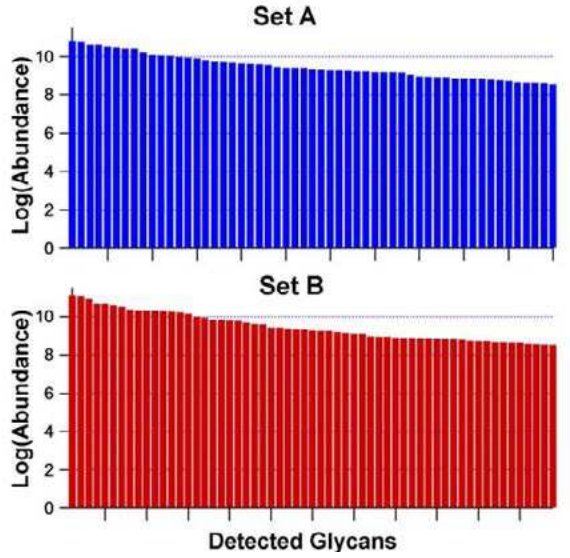

Figure 8
Specific glycan log-abundances from Set A and B are compared. Each bar represents a different glycan. Both plots are sorted by decreasing abundance.

Set A and Set B had a mean coefficient of variation (CV) of 23% and 25%, respectively. This includes all CV values from each ACN fraction. Along with the data above, this suggests good correlation between the manual process and the automated process.

Application of the Automated Method for 48 Serum Samples

The average frequency of detection of each glycan in the serum samples (n=48) is plotted in Figure 9. The gradual decrease in detection frequencies can either be attributed to sample process variability or biological diversity in the sample set. This sample set contained 31 ions detected in 100% of the spectra and the average frequency of detection is 67%. Across all samples, 81 N-glycan compositions were identified from the sample set and half were detected in at least 95% of the samples.

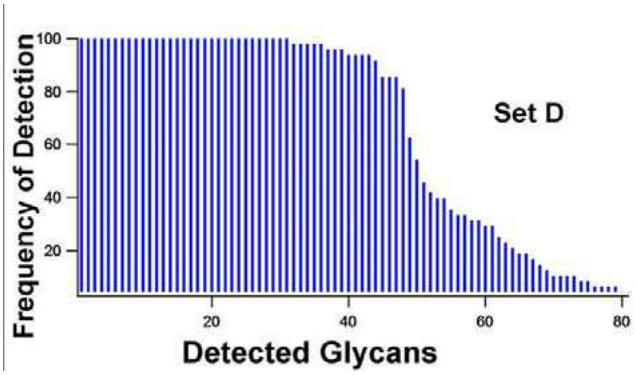

Figure 9
Frequency of detection of glycans in Set D (48 serum samples from GOG)

CONCLUSION

A rapid and reproducible serum glycan purification pipeline was described. The faster pipeline allows for 96 serum samples to be processed and ready for mass spectrometry analysis in one week. This is faster and more reproducible compared to traditional sample preparation methods. This method is reproducible and applicable for large studies of healthy control and patient populations.

Substituting the manual SPE method, which uses vacuum manifolds, with automated SPE using the Gilson GX-274 ASPEC imparted the benefits of both positive pressure displacement and large sample processing capacity. The automation of the solid phase extraction also creates free time for the researcher to perform concurrent tasks. By expediting, automating and standardizing the procedure, the coefficients of variation were decreased while maintaining glycan average

abundances and profiles. The operator variability was minimized allowing for new operators to learn the procedure and help process larger sample sets (Kronewitter, S.R. et al., 2010).

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