**NMR Metabolomics Methods for Breast Tissue Processing**

Individually weighed breast tissue samples were provided frozen in tubes containing ceramic homogenization beads. Aliquots of 50-120 mg of breast tissue were mixed with degassed 3:3:2 Acetonitrile:Isopropanol:Water solution at a concentration of 100 mg/mL. Samples were homogenized in a MagNA Lyser Bead Homogenizer (Roche) for two pulses at 6500 rpm for 20 sec and placed in a cooling station (chilled at -20°C) for one minute in between the pulses. Homogenates were transferred into new labeled tubes. Samples were centrifuged again at 4°C for 5 minutes at 12000 rcf, and a volume of the homogenate corresponding to 50 mg breast tissue was transferred to a new set of tubes for further processing. Additionally, 100-250 µL of the doubly centrifuged homogenates were transferred to larger tubes based on phenotypes (Reduction, DCIS, HER2+, Luminal A, Luminal B, and Basal) to create phenotypic QC pool samples by combining equal volume aliquots from each sample in the phenotype. Equal volumes of the phenotypic pooled samples were then combined in another larger tube to create a pooled QC sample for the entire study (Total Pool 1). Pooled samples were aliquotted into analytical pool sample tubes and handled the same as the experimental sample tubes containing volumes corresponding to 50 mg of each sample. The samples were taken to dryness on the vacuum centrifuge and mixed with a 50:50 solution of Acetonitrile:Water. Samples were vortexed and centrifuged for 5 minutes at 4°C. The supernatant was removed and placed into a new tube. Samples were completely dried by vacuum centrifuge and were reconstituted by adding 630 µL of D2O (Aldrich) and 70 µL of Chenomx ISTD solution plus Imidazole as the chemical shift reference (Chenomx, Edmonton, Alberta, Canada). The samples were vortexed and centrifuged at 12000 rcf for 2 minutes. 600 µL of sample was transferred into 5mm NMR tubes.

1H NMR spectra of tissue extract samples were acquired on a Bruker Avance III 700 MHz NMR spectrometer (located at the David H. Murdock Research Institute at Kannapolis, NC, USA) using a 5 mm cryogenically cooled ATMA inverse probe and ambient temperature of 25℃. A 1D NOESY presaturation pulse sequence (noesypr1d, [recycle delay (RD)-90°-t1-90°-tm-90°-acquire free induction decay (FID)]) was used for data acquisition. For each sample 256 transients were collected into 65k data points using a spectral width of 14.01 kHz (20.14 ppm), 2 s relaxation delay, 100 ms mixing time, and an acquisition time of 2.324 s. The water resonance was suppressed using resonance irradiation during the relaxation delay and mixing time. Spectra were zero filled, and Fourier transformed after exponential multiplication with line broadening factor of 0.5. Phase and baseline of the spectra were manually corrected for each spectrum. Spectra were referenced internally to the DSS signal. The quality of each NMR spectrum was assessed for the level of noise and alignment of identified markers and assessed for missing data and quality.

NMR spectra were processed using ACD 1D NMR Processor 12.0 (ACD Labs, Toronto, CA). NMR bins (0.20-9.00 ppm) were made after excluding water (4.75-4.95 ppm) using intelligent binning width of 0.04 ppm and 50% looseness factor. Integrals of each of the bins were normalized to total integral of each of the spectrum.