

Methods of Proteomic Analysis

Protein-CyDye Labeling

Protein labeling with CyDye was performed using the CyDye technology developed by GE Healthcare. After adjusting sample solution to pH 8.5, each protein sample was covalently linked to a different CyDye fluorophore, such as Cy2 to reference (a mixture of equal amounts of protein extracts from all samples presenting in this project), Cy3 to control, and Cy5 to NEC. In each case 100 ug of protein was labeled with 400 pmol CyDye for 30 min in dark in cold, then quenched with 1 ul of 10 mM lysine.

2D-DIGE Experimental Design

To ensure statistical significance for data obtained from control and NEC cases, the following experimental design was applied. Equal amounts (100µg) of Cy3-control, Cy5-NEC, and Cy2-reference mixture were loaded per gel, resulting of 300 ug total protein loaded in each gel. For biological variation evaluation, three separate gels each including different set of sample were performed to evaluate the protein abundance variation statistically. To minimize dye difference if there was any, Cy3/Cy5 dye swaping between control and NEC was performed also.

2-D Gel Electrophoresis

Three different CyDye labeled samples were mixed and brought up volume to 500 ul with IEF buffer (8 M urea 2M thiourea 4% CHAPS 100 mM DDT 0.5% IPG buffer pH 3 to 11) before passively rehydrate a IPG pH 3 to 11 non linear strip (GE

Healthcare). 3 strips were used to separate proteins at 19 °C on an IPGphor Unit (GE Healthcare) with voltage ramping up to and held at 10000 Volt for a total 60 kWh.

After completion of IEF, the strips were first equilibrated with reducing buffer (50 mM Tris-HCl (pH 6.8), 6 M Urea, 30% (v/v) glycerol, 2% (w/v) SDS 100 mM DTT), then was equilibrated in alkylation buffer (50 mM Tris-HCl (pH 6.8), 6 M Urea 30% (v/v), glycerol 2%, SDS 2.5% idoacetamide), both equilibration was held at room temperature in dark for 15 minutes. After equilibration, the strip was transferred and mounted on top a 24 x 24 cm 8 to 16% Tris Glycine polyacrylamide gel (Jule) under a layer of warm 0.5% agarose made in SDS electrophoresis running buffer. Electrophoresis was carried out in Ettan Daltsix Unit (GE Healthcare) at 12C at 10 mA/gel for one hour then overnight at constant current at 12 mA/gel and a limit of 150 V until the dye front reached the bottom of the plate.

Image Acquisition and Data Analysis

Immediately after gel electrophoresis, CyDye labeled proteins in gel were scanned using a Typhoon 9400 Variable Mode Imager (GE Healthcare). The excitation/emission wavelengths for Cy2, Cy3 and Cy5 were 488/520, 532/580 and 633/670 nm respectively. Three images per gel (internal standard, control and experimental) were acquired.

The digital image information acquired was then analyzed with DeCyder 2D software, version 7.0, (GE Healthcare). All spots present in all images in each individual gel were co-detected, matched, and normalized with the DIA (Differential In-Gel Analysis) Module within the software. Information from replicate gels was analyzed with the BVA (Biological Variation Analysis) Module. In the BVA, a master gel was created

with information from all gels, including matching multiple images from different gels to provide statistical data on differential protein expression levels between control and experimental groups. There were 2000 spots detected and matched. Interesting spots were selected by setting the fold difference threshold to 1.5 folds (any protein spots from the experimental group that was expressed above or below 1.5 folds when compared with spots from the control group were selected). These spots are listed in Table 1. A pick list was made after filtering the spot information based on matching quality, appearance in all gels (6 out of 9 images in this case), and statistical confidence when Student's t-test p value was less than 0.05.

Protein Spot Excision

The ordinance information obtained from DeCyder software for each interesting protein spot was transferred to an automated ProPic spot picker (Genomic Solutions) using the pick list. The spots then were excised by the picker and transferred to a collecting plate and were submitted for protein identification.

Protein Identification

Protein identification was performed using LC-MS/MS. The enzymatically digested samples were injected onto a capillary trap (LC Packings PepMap) and desalted for 5 min with a flow rate of 3 μ l/min of 0.1% v/v acetic acid. The samples were loaded onto an LC Packing® C18 Pep Map nanoflow HPLC column. The elution gradient of the HPLC column started at 3% solvent A, 97% solvent B and finished at 60% solvent A, 40% solvent B for 30 minutes for protein identification. Solvent A consisted of

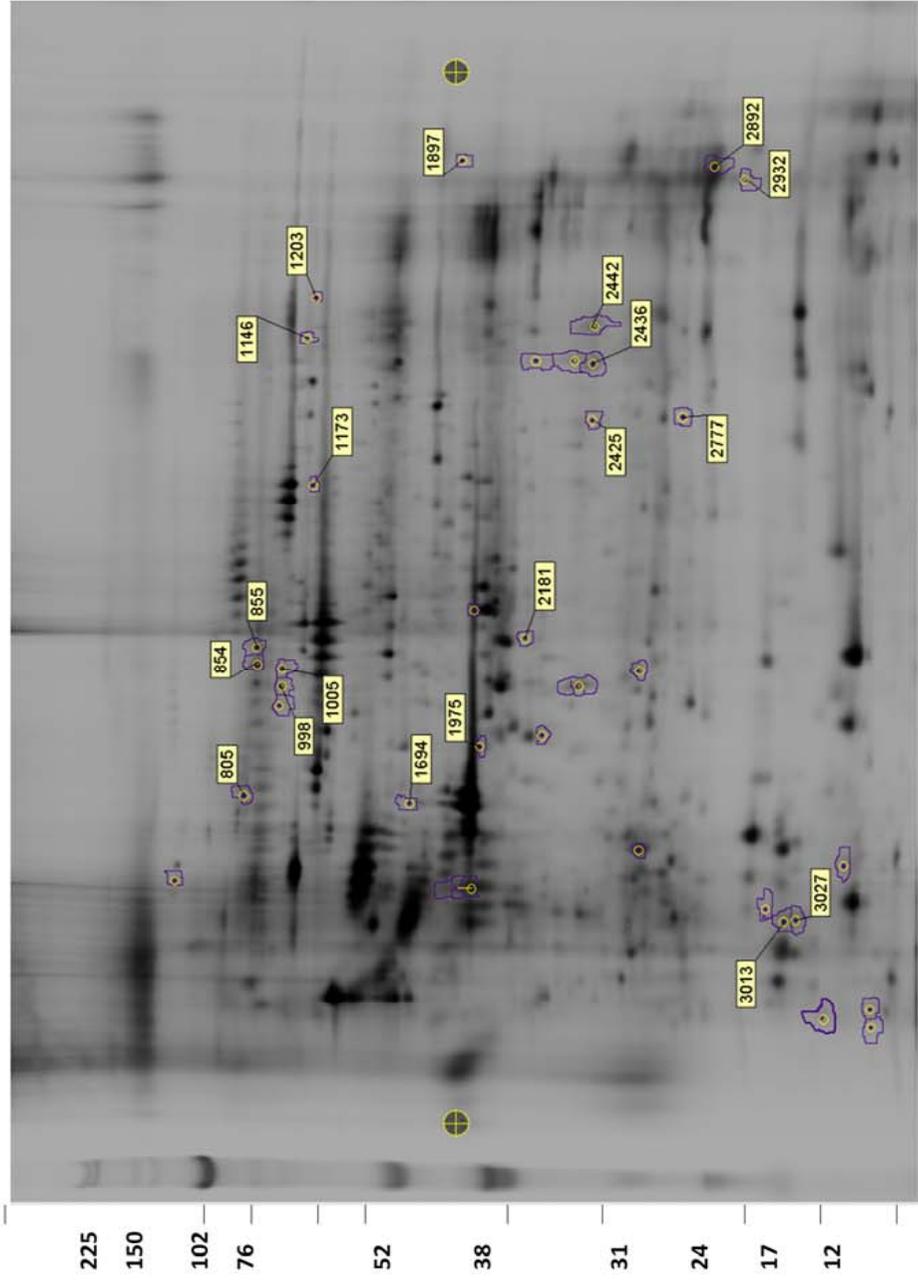
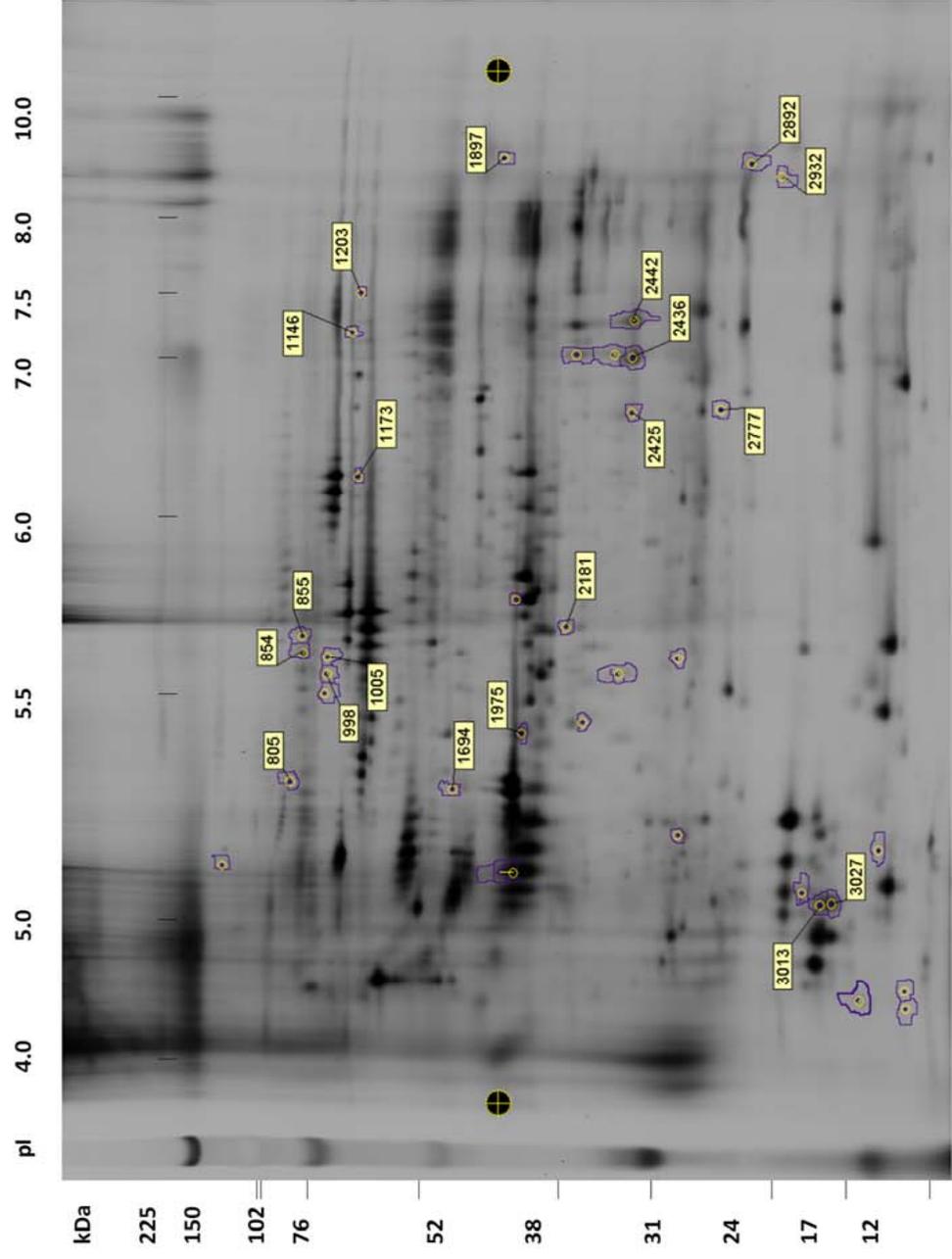
0.1% v/v acetic acid, 3% v/v ACN, and 96.9% v/v H₂O. Solvent B consisted of 0.1% v/v acetic acid, 96.9% v/v ACN, and 3% v/v H₂O.

LC-MS/MS analysis was carried out on a LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Bremen, Germany). The instrument, under Xcalibur 2.07 with LTQ Orbitrap Tune Plus 2.55 software, was operated in the data dependent mode to automatically switch between MS and MS/MS acquisition. Survey scan MS spectra (from m/z 300 – 2000) were acquired in the orbitrap with resolution R=60,000 at m/z 400. The five most intense ions were sequentially isolated and fragmented in the linear ion trap by collisionally induced dissociation (CID) at a target value of 5,000 or maximum ion time of 150 ms. Dynamic exclusion was set to 60 seconds. Typical mass spectrometric conditions include a spray voltage of 2.2 kV, no sheath and auxiliary gas flow, a heated capillary temperature of 200 degrees C, a capillary voltage of 44V, a tube lens voltage of 165V, an ion isolation width of 1.0 m/z, a normalized CID collision energy of 35% for MS₂ in LTQ. The ion selection threshold was 500 counts for MS₂. An activation q = 0.25 and activation time of 30 ms were set.

Protein Search Algorithm

All MS/MS spectra were analyzed using Mascot (Matrix Science, London, UK; version 2.2.2). Mascot was set up to search the International Protein Index (IPI) human database assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.50 Da and a parent ion tolerance of 15 ppm. Iodoacetamide derivative of Cys, deamidation of Asn and Gln, oxidation of Met, are specified in Mascot as variable modifications. Scaffold (version Scaffold-02-03-01, Proteome Software Inc.,

Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified unique peptides. Protein probabilities were assigned by the Protein Prophet algorithm.



Differential protein expression of baby buccal swab as revealed by 2-D DIGE analysis. Protein extract from NEC and normal baby buccal swabs were labeled with Cy3 and Cy5, respectively. A mixed internal reference combining all the proteins from both NEC and normal and their replicates was labeled with an additional Cy2 dye, was included in all gels. Non linear IPG strips (3 to 11) were used for F and 8 to 16% Tris-glycine SDS PAGE for the second dimension. Both normal (upper panel) and NEC (low panel) image show the spot map on which circled spots marked with spot number indicate those proteins identified with protein abundance changes when comparing NEC to normal with 95% confidence level. Numbers in the figure correlate with those included in Table 2.