

Operating ProPic II with 2D DIGE for Mass Spectrometry.

INTRODUCTION



Figure 1. ProPic II Gel Imaging and Picking System (PRO51100)

Equine protozoal myeloencephalitis (EPM) is a common and costly neurological disease of horses caused by *Sarcocystis neurona*, a protozoan parasite, infecting the nervous system. Confirmation of the clinical diagnosis is a challenge for veterinarians because the current immunological test (Western blot against *S. neurona*) lacks specificity. The interpretation of the standard EPM test is based on a subset of proteins; however, the identity of the proteins associated with this pathology is unknown and hence, warrants investigation.

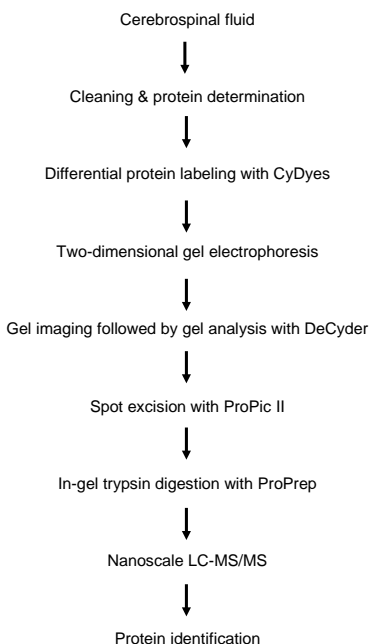
In this study, a multiplexing strategy approach including two-dimensional fluorescence difference gel electrophoresis (2D DIGE) and mass spectrometry was employed to identify differential protein expression associated with EPM. The identification of the proteins will provide important information on EPM pathogenesis and assist in the characterization of possible biomarkers for early diagnosis or potential targets for drug treatment of the neurological disease.

MATERIALS AND METHODS

1. The **ProPic II** (Figure 1) is the most reliable and accurate currently available automated system designed for picking protein spots from a gel, and the only spot-picking platform with a high resolution 16-bit line scanning CCD camera (imaging and picking area: 25 cm x 28 cm). It allows direct imaging and picking from 1D, 2D and DIGE gels stained with all most common fluorescent or visible protein dyes (Coomassie, SYPRO, DeepPurple, Flamingo, Pro-Q, Silver) with high positional accuracy (10 μ m at the picking tip, 1 μ m at encoder). It operates at high speed (120 spots/hour) and excellent efficiency (100% at picking, >95% at harvesting). The robot is completely enclosed for light-tight and keratin-free operation. ProPicII is the best robotic instrument to be used to prepare samples for further treatment in ProPrepII and downstream Mass Spectroscopic applications. Picking parameters used in this study are summarized in the Table 1.
2. The experimental design used in this study is shown in the flow chart below. Cerebrospinal fluid (CSF) samples were collected from normal and EPM horses. EPM was diagnosed if neurological signs were present, Western blot against *S. neurona* on the CSF was positive and other neurological diseases were excluded by ancillary tests and/or necropsy. CSF was collected under general anesthesia, at the atlanto-occipital site to avoid blood contamination. The CSF proteins were desalted with a Bio-Gel P2 spin column (Bio-Rad) or precipitated with ethanol, labeled with CyDye™ DIGE Fluor minimal dyes (GE Healthcare) and separated by 2D gel electrophoresis using pH 3-11 IPG strips, followed by 10% SDS-PAGE on an Ettan DALTsix Electrophoresis Unit (GE Healthcare). The 2D gels were scanned with a Typhoon™ multi-wavelength scanner (GE Healthcare), and digitized images were analyzed with DeCyder™ 2D Differential Analysis Software (GE Healthcare). The resulting DeCyder spot list was translated into a ProPic II pick list with DIGE software (both -

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Digilab). Differentially expressed proteins were then excised with the ProPic II spot excision robot directly from the DIGE gel without post staining (see Table 1 for optimized picking parameters from glass-backed gels). After in-gel trypsin digestion on the ProPrep II digestion robot (Digilab), the resulting peptide mixtures were analyzed with a linear ion-trap mass spectrometer (LTQ, Thermo Electron Corporation) equipped with a nanoscale liquid chromatography system (Ettan™ MDLC, GE Healthcare) with a Zorbax C18 column (Agilent Technologies) delivering a flow rate of ca. 200 nL/min online to the electrospray ionization interface of the LTQ. MS and MS/MS data were used to search the NCBI database using BioWorks software (version 3.2, Thermo Fisher Scientific).

**Experimental Flow Chart**

Parameter	Setting
Picking Tip	Special Picking Tip 1.8mm i.d.; 0.7mm cavity depth; 1.0mm shoulder
Wash Volume	400 µl
Gel Pre-wet Volume	40 µl
Air gap Volume	80 µl
Pick Volume	120 µl
Dispense into Collection Plate Volume	300 µl
Prewash Height	5 mm
Picking Depth	5 mm
Picking Shift	0.9 mm

Table 1: Optimized picking parameters for glass-backed gels

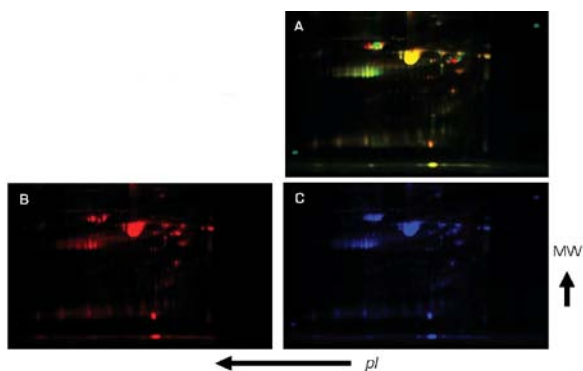


Figure 2. Images of a DIGE gel scanned with the Typhoon at excitation wavelengths of each of the three CyDyes. Image (A) is an overlay view of the three dyes Cy2 (yellow, standard), Cy3 (red, disease) and Cy5 (blue, normal). Images B and C are single channel views of the disease sample (labeled with Cy3) and the normal, control sample (labeled with Cy5), respectively.

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PROCEDURE

Digitized versions of the 2D images of the gel (Figure 2) were analyzed with DeCyder for differential protein expression (see Figure 3). Some of the proteins that showed at least 2-fold increase (blue spot labeling) or decrease (red spot labeling) in expression compared to normal CSF were subjected to further analysis. Figure 4 shows additional 3D views of spots 1, 2, and 3 in the control (normal) and EPM (infected) sample. Protein spots 1 and 2 were decreased in the sample from EPM horse, while spot 3 was increased in the corresponding sample. Following differential analysis the protein spots of interest were automatically directly excised (without post-staining) using the translated DeCyder pick list on the ProPic II. DIGE software from Digilab enables automatic translation of spot coordinates created with DeCyder into ProPic II coordinates for accurate spot excision directly from a DIGE gel without the need for post-staining. A custom algorithm is used to correlate the locations of the reference spots from the DIGE image (analyzed using DeCyder) and the ProPic II image of the DIGE gel. The software uses this correlation to calculate the location of the gel spots in the ProPic II image and generates a compatible pick list. Following spot excision (Figure 5), proteins in the gel plugs were subjected to automated in-gel trypsin digestion and resulting peptide mixtures were analyzed by nano-LC-MS/MS. Figure 6 shows the base peak chromatogram (A) and a mass spectrum (RT 31.99 min, B) of spot 3. The ion with m/z 655.51 was subjected to fragmentation. Figure 7 shows the resulting MS/MS spectrum, identifying a precursor ion (m/z 1965.16) from the peptide mixture resulting from the digestion of the protein in spot 3. Spot 3 was identified to be Serotransferrin (see Table 2).

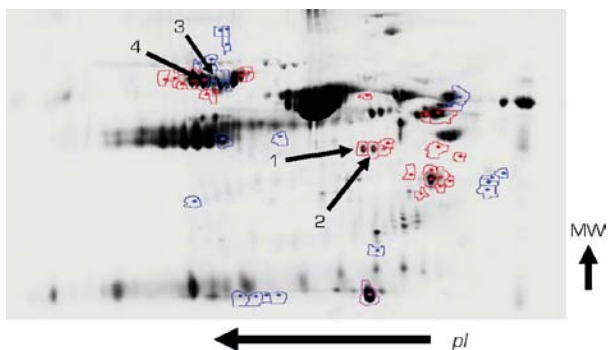


Figure 3: Digitized versions of the 2D images analyzed with DeCyder for differential protein expression. Some of the proteins that showed at least 2-fold increase (blue spots) or decrease (red spots) in expression compared to normal CSF were analyzed further (see Figure 5).

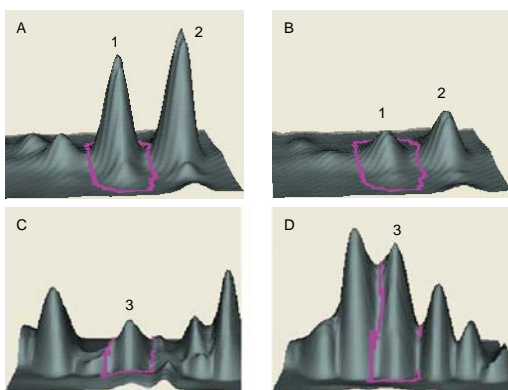


Figure 4: 3D views of differentially expressed spots 1 & 2 (A, B), and 3 (C, D). Control sample (healthy animal, A, C) and a sample from *S. neurona* infected horse (B, D).

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Figure 5. Images of the 2D gels that were taken right before and after the picking process in order to assess the harvesting success and picking accuracy. 5A, 5B: single channel and multiple channel overlay views, respectively, of the 2D gel images before spot excision. After spot excision with the ProPic II, the same gel was rescanned with Typhoon (compare images 5C and 5D, single channel and multiple channel overlay views), to confirm that the correct spots were accurately picked.

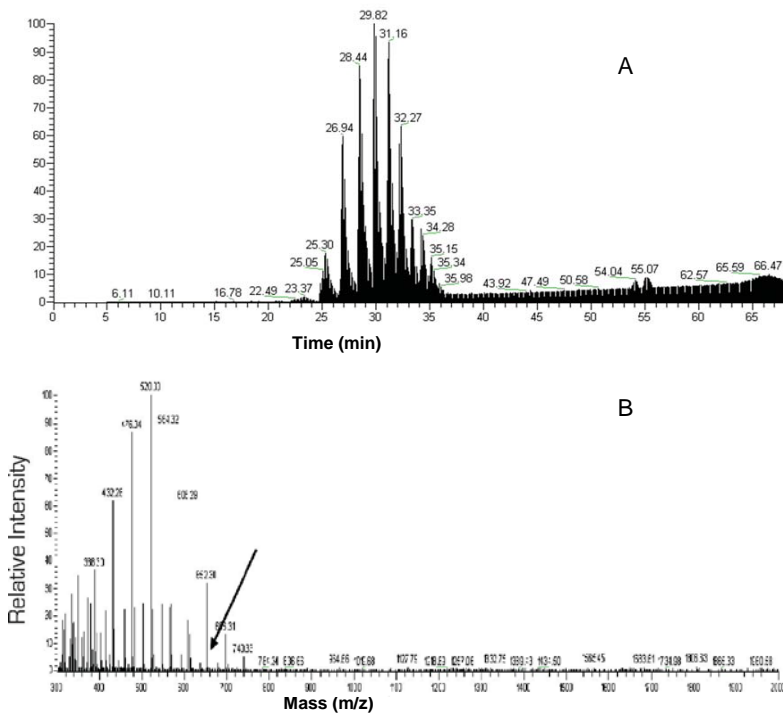
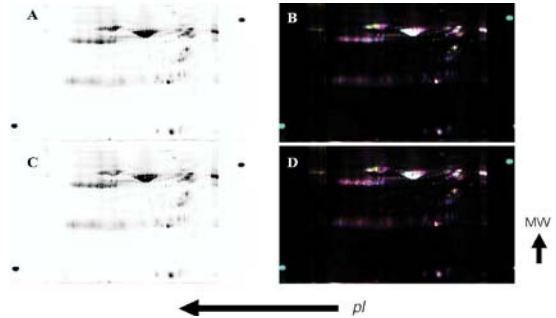


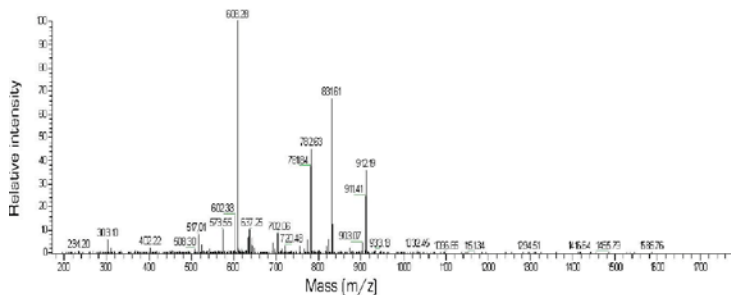
Figure 6: Base peak chromatogram (A) and mass spectrum RT 31.99 min (B). The arrow points to m/z 655.51 that has a charge of three.

Spot #	Protein Name	P(prot)/P(pept)	Score	Coverage Δ Cn	MW kDa	Accession #	Peptide (Hits) Ions
3	Serotransferrin	9.8×10^{-8}	382.2	52.5	78.04	136190	169 (49 39 29 26 26)
4	Serotransferrin	5.5×10^{-8}	608.2	67.3	78.04	136190	294 (104 58 38 53 41)

Table 2: Differentially expressed proteins identified in this experiment

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Figure 7: MS/MS spectrum of a precursor ion (m/z 1965.16) from the spot 3 mass spectrum in Figure 6B. This is the triple charged peptide m/z 655.5



CONCLUSIONS

- The CSF of horses affected with EPM showed a decrease in some isoforms of serotransferrin compared to the CSF of normal horses.
- Specific isoforms of serotransferrin may be associated with EPM, and characterization of additional proteins that were found to be differentially expressed may lend insight into the pathogenesis and diagnosis of EPM.
- With the ProPic II, it is possible to pick directly and accurately from a DIGE gel without post-staining.

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