

# The ZOOM® IPGRunner™ System: A versatile apparatus for the analysis of complex, hydrophobic proteins

Karen M. Dobos<sup>1\*</sup>, John T. Belisle<sup>1</sup>, Bradley Scott<sup>2</sup>, and Regina Rooney<sup>2</sup>

<sup>1</sup>Mycobacteria Research Laboratories, Dept. of Microbiology, Immunology, and Pathology, Colorado State University, Ft. Collins, CO, 80523 and

<sup>2</sup>Invitrogen Corporation, Carlsbad, CA, 92008

\* kdobos@colostate.edu

## Abstract

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) maps and databases have been widely used to evaluate gene expression profiles under different environmental conditions, assess global changes associated with specific mutations, and define drug, diagnostic, and vaccine targets of bacterial pathogens. Moreover, the proteome holds secrets that are not readily obtained or proven by direct analysis of the genome, including post-translational modifications. The definition of serodiagnostic, T-cell, and glycosylated secreted *Mycobacterium tuberculosis* proteins was previously established through rigorous biochemical and molecular technologies. In contrast, the description of similar proteins that define the cellular envelope of *M. tuberculosis* is lacking. The study of these proteins is hindered by their poor solubility and recovery from mycobacteria lysates (restricting their availability for study) and the lack of technologies to study these macromolecules in standard IPG systems. In this report, traditional biochemical methods to extract and isolate mycobacterial cell wall proteins were partnered with the ZOOM® IPGRunner™ System to afford high-throughput analysis of this complex mixture of proteins. The application of Concanavalin-A (Con-A) enrichment of this protein mixture and separation of products using narrow range ZOOM® strips (pH range 4.5-5.5) provided further separation of glycolipoproteins, permitting their identification and routes to further study of these complex macromolecules. These methods can be universally applied to the elucidation of poorly soluble proteins from other bacterial pathogens as well as other complex biomolecule mixtures.

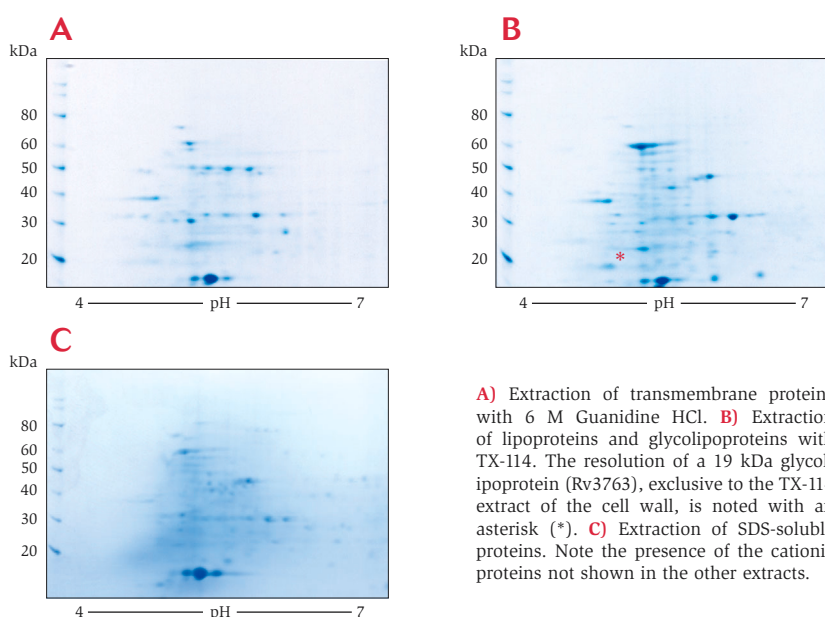
## Introduction

The ability to identify proteins from pathogens is key to understanding the physiology of an organism and defining effective diagnostic or vaccine candidates. In the past, most studies employing exploitation of the bacterial proteome were directed toward antigen discovery. The advent of whole genome sequencing, and the availability of several fully annotated bacterial genomes has profoundly altered the basic experimental approach other studies (1,2). DNA microarray technology has been partnered with proteomics to identify all of the genes that are potentially involved in a specific cellular process (3-5). While the two main proteomic technologies, two-dimensional

polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometry (MS) of peptides, have been around for decades (6,7), innovative software for the interrogation of MS data to identify proteins of interest (8) has reduced the burden of data analysis. Thus, these technologies can now be applied to high-throughput global efforts of bacteriology. In this report, we utilized these methodologies for the global analysis of the *Mycobacterium tuberculosis* cell wall in an effort to define glycosylated proteins.

Previously, 2D-PAGE and MS were used to define the population of proteins secreted from *M. tuberculosis* (9) and characterize the first *O*-glycosylated protein in *M. tuberculosis* (10). Similarly, the combined use of 2D-PAGE, Western blot

**Figure 1** – Two dimensional gel electrophoresis of three cell wall extracts using the ZOOM® IPGRunner™ System and ZOOM® strips of 4-7



**A)** Extraction of transmembrane proteins with 6 M Guanidine HCl. **B)** Extraction of lipoproteins and glycolipoproteins with TX-114. The resolution of a 19 kDa glycolipoprotein (Rv3763), exclusive to the TX-114 extract of the cell wall, is noted with an asterisk (\*). **C)** Extraction of SDS-soluble proteins. Note the presence of the cationic proteins not shown in the other extracts.

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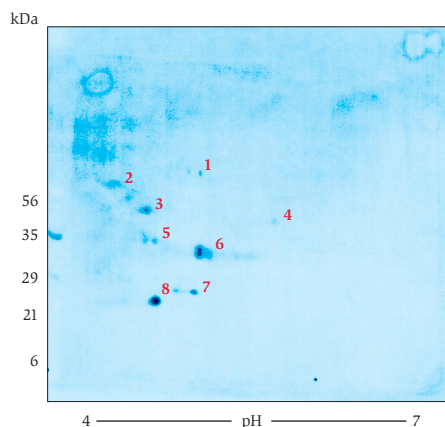
analysis, and liquid chromatography-mass spectrometry (LC-MS) was used to define 26 proteins of *M. tuberculosis* that reacted with patient sera, and three of these subsequently were determined to have significant potential as serodiagnostic reagents (11,12). Such studies have not been conducted for proteins from the cellular envelope of *M. tuberculosis*, research that is crucial to understanding the physiology of the bacilli. The most significant obstacle to the study of these proteins is their poor solubility and aggregation potential. Recently, we used complex buffer systems to solubilize these proteins and rehydrate IPG strips to resolve this mixture of proteins by 2D-PAGE. Use of traditional systems was incompatible with these buffers due to an inability to achieve the proper conductivity for isoelectric focusing. This barrier was overcome through the use of the ZOOM® IPGRunner™ System.

### Materials and Methods

*Preparation of subcellular fractions.* Whole cell lysate was generated by disruption of bacilli using a French Press cell (13). The cell wall was purified by centrifugation of the whole cell lysate at 27,000 x g, and the cell wall pellet was washed 3X with PBS (3:1 vol:vol pellet). The washed cell wall was extracted with three different buffer combinations:

1. The cell wall was extracted with 6 M guanidine HCl. Soluble proteins were exchanged into 10 mM ammonium bicarbonate. A uniform suspension of the material was obtained to estimate the protein concentration.
2. The cell wall was extracted with 1% SDS. SDS was removed by paired-ion

**Figure 2** – Con-A reactive TX-114 proteins



Eight proteins with masses of 58 (1, 2), 48 (3), 46 (4), 38 (5), 32 (6), 26 (7), and 19 kDa (8) were identified. The 38 (Rv0934; spot 5) and 19 (Rv3763; spot 8) kDa proteins were previously identified as putative glycoproteins.

extraction with triethylamine:acetic acid:acetone (5:5:85 per 5 vol of sample) followed by centrifugation at 27,000 x g. The precipitate was admixed with 10 mM ammonium bicarbonate and a uniform suspension of the material obtained to estimate protein concentration.

3. Cell wall pellet was extracted with 6% Triton X-114 and partitioned. The detergent phase was washed with PBS and detergent removed by precipitation of cold acetone. The material was admixed with 10 mM ammonium bicarbonate and a uniform suspension obtained to estimate protein concentration.

*Two-Dimensional gel electrophoresis:* ZOOM® strips pH 4-7. An aliquot of 100 µg of each cell wall preparation was solubilized in 8 M deionized Urea, 1% CHAPS, 1% ASB-14, 0.5% NP-40, 20 mM DTT, and 0.5% ampholytes (0.4% ZOOM® carrier ampholytes, pH range 4-7, and 0.1% ZOOM® carrier ampholytes, pH range 3-10). Samples were rehydrated at

RT for 14 hr, and cathode and anode wicks were doped with 1 mM lysine and 1 mM Phosphoric acid, respectively. Isoelectric focusing was performed using the following program:

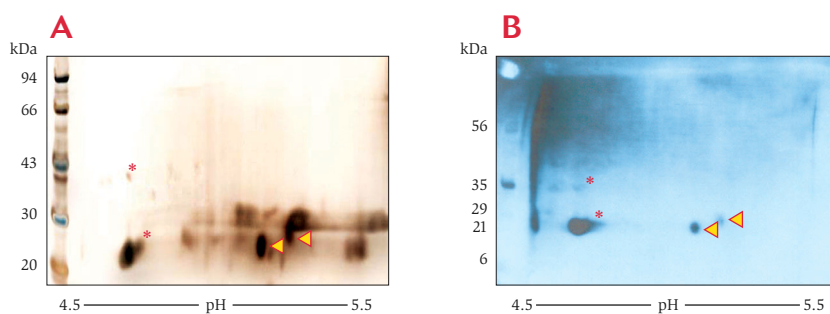
1. 200 V for 15 min (current limit can be set for 4 mA; actual mA should rapidly decrease during this step to less than 2 mA).
2. 450 V for 15 min (current limit can be set for 2 mA; actual mA should rapidly decrease during this step to 1 mA or less).
3. 750 V for 15 min (set current limit to no greater than 1 mA from this step onward).
4. 2000 V for 75 min (more focusing time may be required for complex mixtures containing non-proteinaceous biomolecules).

Strips were equilibrated per standard protocol (see ZOOM® IPGRunner™ Manual for details) and loaded onto NuPAGE® 4-12%

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**Figure 3** – Two dimensional gel electrophoresis of Con-A bound proteins from the TX-114 extract of *M. tuberculosis* cell wall using narrow range ZOOM® strips (pH 4.5-5.5)



**A)** Proteins visualized by staining with silver. **B)** Proteins reactive by probing with Con-A HRP. The previously identified glycolipoproteins (Rv0934 and Rv3763) are noted with asterisks (\*). The arrows annotate two putative glycoproteins that are closely related by size and pI, and were obscured from identification and isolation using the broad range ZOOM® strips.

Bis-Tris gels (Invitrogen) and proteins separated using MES running buffer. Proteins were visualized by staining with SimplyBlue™ SafeStain (Invitrogen).

*Enrichment of M. tuberculosis cell wall glycoproteins.* TX-114 extracted cell wall proteins were solubilized in 25 mM phosphate buffer (pH 5.7), 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>, 10 mM DTT and sonicated until a uniform mixture was obtained. The suspension was applied to an open column of Con-A Sepharose 4B. The column was extensively washed and proteins bound to the Con-A beads eluted by addition of 1 M  $\alpha$ -methyl mannoside to the base buffer. The resultant pool of Con-A bound proteins was exchanged into 10 mM ammonium bicarbonate. A uniform suspension of this material was obtained for estimation of protein concentration.

*Identification of Concanavalin A reactive products.* Triton X-114 extracted cell wall proteins and Con-A bound proteins

resolved by 2D-PAGE were transferred to nitrocellulose. The membrane was probed with Concanavalin-A HRP, and developed onto x-ray film after exposure with chemiluminescent substrate.

*Two-Dimensional gel electrophoresis: Narrow range Zoom® strips pH 4.5-5.5.* An aliquot of 20  $\mu$ g of the Con-A bound protein preparation was solubilized in 8 M deionized Urea, 1% CHAPS, 1% ASB-14, 1% NP-40, 20 mM DTT, and 0.5 % ampholytes (0.4% ZOOM® carrier ampholytes, pH range 4-6, and 0.1% ZOOM® carrier ampholytes, pH range 4-7). Samples were rehydrated at RT for 14 hr, and cathode and anode wicks were doped with water. Isoelectric focusing was performed using the following program:

1. 175 V for 15 min (current limit can be set for 4 mA; actual mA should rapidly decrease during this step to less than 2 mA).
2. 500 V for 15 min (current limit can be

set for 2 mA; actual mA should rapidly decrease during this step to 1 mA or less).

3. 1000 V for 15 min (set current limit to no greater than 1 mA from this step onward).
4. 1500 V for 15 min.
5. 2000 V for 120 min (more focusing time may be required for complex mixtures containing non-proteinaceous biomolecules).

Strips were equilibrated per standard protocol (see ZOOM® IPGRunner™ Manual for details) and loaded onto NuPAGE® 4-12% Bis-Tris gels and proteins separated using MES running buffer. Proteins were visualized by staining with the SilverQuest™ Silver Staining Kit (Invitrogen).

## Results and Discussion

Cellular envelope proteins from each extraction were resolved by 2D-PAGE using the ZOOM® IPGRunner™ System and methodology described. Extraction with 6 M guanidine was used to solubilize trans-membrane proteins (Figure 1A), while extraction with TX-114 was used to solubilize lipoproteins and glycolipoproteins (Figure 1B). SDS extraction was used to solubilize cellular envelope proteins indiscriminately, however, lipoproteins were not readily extracted basic proteins, including histone-like proteins, were solubilized in this buffer (Figure 1C).

The TX-114 extracted protein sample was transferred to nitrocellulose and the membrane probed with Con-A conjugated to HRP for identification of  $\alpha$ -mannosylated proteins. Eight spots were identified (Figure 2). Two of these spots had been previously described as

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glycolipoproteins, namely, the 38 kDa protein (RV0934; spot #5) and the 19 kDa protein (RV3763; spot #8). Confirmation of the identity of the 26 kDa protein, as well as identification of the other Con-A reactive proteins, was obscured due to either their low abundance or co-resolution with other proteins in the sample.

The TX-114 extracted sample was applied to a column of Con-A Sepharose 4B in order to enrich for glycoproteins and glycolipoproteins and enable their identification. The Con-A bound proteins were resolved by 2D-PAGE using narrow range ZOOM® strips (pH 4.5-5.5), and either stained with silver or transferred to nitrocellulose and probed with Con-A HRP. This method afforded high resolution of the proteins of interest (Figure 3A and B), including identification of two Con-A reactive proteins with apparent molecular masses of 26 kDa; differing only in the isoelectric point (Figure 3B). Identification of these proteins will be readily accomplished by established LC-MS/MS techniques coupled with MS/MS database analysis of the annotated TB genome.

Historically, 2D-PAGE used a low-crosslinked polymer of acrylamide and application of the protein sample into a mobile gradient within this gel (6). Isoelectric focusing was accomplished by exposing one end of the gel to an acidic solution, the other to a basic solution, and applying a high voltage across the gel. While the methodology was rigorous and could accommodate most any sample, the accumulation of gas at the cathode, leakage of base into the anodic end of the system, poor gel-to-gel reproducibility, and laborious procedure impeded the use of 2D-PAGE for global protein analysis.

The advent of immobilized pH gradients (IPG) to IEF gels, and further development of “dry” IEF systems reduced the burden of performing 2D-PAGE, eliminated problems associated with the use of corrosives, and initiated a reproducible format for the global analysis of proteins by 2D-PAGE (14). However, this technology also highlighted limitations of analysis of samples containing hydrophobic proteins, including those with transmembrane domains or modified by acylation. Extraction of these proteins from the cell is generally accomplished using detergents, restricting their ability to be studied by traditional biochemical methods, including 2D-PAGE (15). These poorly soluble proteins will not focus using standard IPG systems due to the amperage limitations and absence of a buffering system to quench ions present in the protein sample. In addition, proteins that aggregate in solution and require a stronger pH gradient for separation will also resolve poorly unless the rehydration buffer is supplemented with additional ampholytes.

The ZOOM® IPGRunner™ System permits the use of additional agents in the rehydration buffer, such as salts and zwittergents. These solutes are taken up into the ZOOM® strips with the proteins of interest. Excess ions are quenched from the strip during the initial 15 minutes of focusing, and become adsorbed into the wicks present at the cathode and anode ends of the ZOOM® strip cassette. The combined chemistry of the ZOOM® strips and design of the ZOOM® IPGRunner™ System therefore affords the study of complex, hydrophobic protein mixtures. Resolution of these samples by 2D-PAGE renders them accessible to other proteomics analysis

technologies, such as in-gel digestion (16,17) coupled with analysis by LC-MS/MS (18). Further, the separation of closely related aggregates across a narrow pH range can enhance the study of closely related proteins, or the study of protein families and enzymatic complexes.

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