The Whole Gel Eluter in the Public Domain

Selected references and abstracts demonstrating the versatility of the whole gel eluter system

Thoren K, Gustafsson E, Clevnert A, Larsson T, Bergstrom J, Nilsson CL
Proteomic study of non-typable *Haemophilus influenzae*
J Chromatogr B Analyt Technol Biomed Life Sci 782, 219–226 (2002)

Non-typable *Haemophilus influenzae* (NTHi) are small, gram-negative bacteria and are strictly human pathogens, causing acute otitis media, sinusitis and community-acquired pneumonia. There is no vaccine available for NTHi, as there is for H. influenzae type b. Recent advances in proteomic techniques are finding novel applications in the field of vaccinology. There are several protein separation techniques available today, each with inherent advantages and disadvantages. We employed a combined proteomics approach, including sequential extraction and analytical two-dimensional polyacrylamide electrophoresis (2D PAGE), and two-dimensional semi-preparative electrophoresis (2D PE), in order to study protein expression in the A4 NTHi strain. Although putative vaccine candidates were identified with both techniques, 11 of 15 proteins identified using the 2D PE approach were not identified by 2D PAGE, demonstrating the complementarity of the two methods.

Gustafsson E, Thoren K, Larsson T, Davidsson P, Karlsson KA, Nilsson CL
Identification of proteins from *Escherichia coli* using two-dimensional semi-preparative electrophoresis and mass spectrometry
Rapid Commun Mass Spectrom 15, 428–432 (2001)

Escherichia coli is a gram-negative bacterium that causes sepsis and infections of the nervous system, and the digestive and urinary tracts. The availability of the complete nucleotide sequence encoding the *E. coli* K-12 genome has made this organism an excellent model for proteomic studies. Semi-preparative two-dimensional electrophoresis, including liquid phase isoelectric focusing (IEF), one-dimensional sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and gel elution, have for the first time been used in combination with matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOFMS), electrospray tandem mass spectrometry and database searching for rapid separation of proteins from a uropathogenic strain of *E. coli.* The identity of 30 proteins, including the membrane protein nmpC, was obtained using this approach.

Hesse C, Nilsson CL, Blennow K, Davidsson P
Identification of the apolipoprotein E4 isoform in cerebrospinal fluid with preparative two-dimensional electrophoresis and matrix assisted laser desorption/ionization-time of flight-mass spectrometry
Electrophoresis 22, 1834–1837 (2001)

Apolipoprotein E (apoE) was isolated from human cerebrospinal fluid (CSF) from control individuals and patients with Alzheimer's disease (AD). The purification was performed with preparative two-dimensional electrophoresis (2-DE), involving liquid-phase isoelectric focusing (IEF) in the Rotofor cell in combination with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electroelution in the Mini Whole Gel Eluter. ApoE was characterized by matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS) analysis of tryptic digests. The known change of Cys to Arg in position 112 of the apoE4 isoform was identified. This was detected in CSF from AD patients, reflecting the increased frequency of the apoE4 allele in this population. This peptide was not detected in CSF samples from healthy control individuals. The use of this rapid electrophoretic separation in proteomic studies of CSF proteins provides single proteins, such as apoE, of high purity in yields sufficient for characterization by MALDI-TOF-MS. Characterization of proteins and their modifications (amino acid substitutions, glycosylation or phosphorylation) in CSF will be a useful tool in the investigation of the pathophysiology of brain disorders such as AD.

[*] Nilsson CL, Larsson T, Gustafsson E, Karlsson KA, Davidsson P
Identification of protein vaccine candidates from *Helicobacter pylori* using a preparative two-dimensional electrophoretic procedure and mass spectrometry
Anal Chem 72, 2148–2153 (2000)

Helicobacter pylori is an important human gastric pathogen for which the entire genome sequence is known. This microorganism displays a uniquely complex pattern of binding to complex carbohydrates presented on host mucosal surfaces and other tissues, through adhesion

[*] A reprint of this article is available in the whole gel eluter technical folder.

molecules (adhesins) on the microbial cell surface. Adhesins and other membrane-associated proteins are important targets for vaccine development. The identification and characterization of cell-surface proteins expressed by H. pylori is a prerequisite for the development of vaccines designed to interfere with bacterial colonization of host tissues. However, identification of membrane proteins is difficult using a traditional proteomics approach employing 2D-PAGE. We have used a novel approach in the identification of microbial proteins that employs a rapid preparative two-dimensional electrophoretic separation followed by mass spectrometry and database searches. No pre-enrichment of bacterial membranes was required. The entire process, from sample preparation to protein identification, can be completed in less than 18 hours, and the presence of proteins can be monitored after both the first- and second-dimensional separations using mass spectrometry. We were able to identify 40 proteins from a detergent-solubilized *H. pylori* preparation; over one-third of these were membrane or membrane-associated proteins. A functionally characterized low-abundance membrane protein, the Leb-binding adhesin, was found in this group. The use of this rapid 2D electrophoretic separation in proteomic studies of *H. pylori* is expected to speed up the identification of expressed virulence proteins and vaccine targets in this and other microbial pathogens.

Weldingh K, Hansen A, Jacobsen S, Andersen P
High resolution electroelution of polyacrylamide gels for the purification of single proteins from *Mycobacterium tuberculosis* culture filtrate
Scand J Immunol 51, 79–86 (2000)

Culture filtrate from *Mycobacterium tuberculosis* contains protective molecules which have been used successfully in experimental vaccines against tuberculosis. Despite an increasing number of mycobacterial proteins being characterised, a major effort is still needed to get an overview of the many potentially interesting molecules in culture filtrate. In this study we describe a high throughput method for purification and biological evaluation of protein components in complex protein mixtures. The method presents a new application of the recently developed Mini Whole Gel Eluter and employs this apparatus for the high resolution electroelution of selected molecular mass fractions of protein mixtures previously separated in large polyacrylamide gels. Two novel *M. tuberculosis* culture filtrate proteins (CspA and TB18.6) were purified by this method, their N-terminal sequences were determined and the open reading frame encoding each of the proteins identified. The immunological recognition of the molecules were evaluated in tuberculosis infected mice and guinea pigs. Both proteins induced DTH responses in guinea pigs and IFN-gamma release from spleen lymphocytes isolated from infected mice.

Davidsson P, Nilsson CL
Peptide mapping of proteins in cerebrospinal fluid utilizing a rapid preparative two-dimensional electrophoretic procedure and matrix-assisted laser desorption/ionization mass spectrometry
Biochim Biophys Acta 1473, 391–399 (1999)

A quick two-step procedure involving liquid phase isoelectric focusing in the Rotofor cell in combination with electroelution in the Mini whole cell gel eluter has been used for purification of proteins from human cerebrospinal fluid (CSF). Fractions, each highly enriched in a single protein band and virtually free of other proteins, were selected for characterization by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOFMS). Six CSF proteins, transferrin, alpha1-acid-glycoprotein, Zn-alpha2-glycoprotein, apolipoprotein A1, apolipoprotein E and beta-trace were identified by MALDI-TOFMS analysis of the tryptic digests. These results demonstrate that the combination of liquid phase IEF and electroelution is a rapid preparative two-dimensional separation which can provide single proteins of high purity, in yields sufficient for characterization by MALDI-TOFMS. Characterization of such brain-specific proteins in CSF will be useful in the investigation of the pathophysiology of different brain disorders.

Wang WQ, Merriam DL, Moses AS, Francis GA
Enhanced cholesterol efflux by tyrosyl radical-oxidized high density lipoprotein is mediated by apolipoprotein AI-AII heterodimers
J Biol Chem 273, 17391–17398 (1998)

Myeloperoxidase secreted by phagocytes in the artery wall may be a catalyst for lipoprotein oxidation. High density lipoprotein (HDL) oxidized by peroxidase-generated tyrosyl radical has a markedly enhanced ability to deplete cultured cells of cholesterol. We have investigated the structural modifications in tyrosylated HDL responsible for this effect. Spherical reconstituted HDL (rHDL) containing the whole apolipoprotein (apo) fraction of tyrosylated HDL reproduced the ability of intact tyrosylated HDL to enhance cholesterol efflux from cholesterol-loaded human

fibroblasts when reconstituted with the whole lipid fraction of either HDL or tyrosylated HDL. Free apoAI or apoAII showed no increased capacity to induce cholesterol efflux from cholesterol-loaded fibroblasts following oxidation by tyrosyl radical, either in their lipid-free forms or in rHDL. The product of oxidation of a mixture of apoAI and apoAII (1:1 molar ratio) by tyrosyl radical, however, reproduced the enhanced ability of tyrosylated HDL to induce cholesterol efflux when reconstituted with the whole lipid fraction of HDL. HDL containing only apoAI or apoAII showed no enhanced ability to promote cholesterol efflux following oxidation by tyrosyl radical, whereas HDL containing both apoAI and apoAII did. rHDL containing apoAI-apoAIImonomer and apoAI-(apoAII)2 heterodimers showed a markedly increased ability to prevent the accumulation of LDL-derived cholesterol mass by sterol-depleted fibroblasts compared with other apolipoprotein species of tyrosylated HDL. These results indicate a novel product of HDL oxidation, apoAI-apoAII heterodimers, with a markedly enhanced capacity to deplete cells of the regulatory pool of free cholesterol and total cholesterol mass. The recent observation of tyrosyl radical-oxidized LDL in vivo suggests that a similar modification of HDL would significantly enhance its ability to deplete peripheral cells of cholesterol in the first step of reverse cholesterol transport.

Rosenkrands I, Rasmussen PB, Carnio B, Jacobsen S, Theisen M, Andersen P
Identification and characterization of a 29-kilodalton protein from *Mycobacterium tuberculosis* culture filtrate recognized by mouse memory effector cells
Infect Immun 66, 2728–2735 (1998)

Culture filtrate proteins from *Mycobacterium tuberculosis* induce protective immunity in various animal models of tuberculosis. Two molecular mass regions (6 to 10 kDa and 24 to 36 kDa) of short-term culture filtrate are preferentially recognized by Th1 cells in animal models as well as by patients with minimal disease. In the present study, the 24- to 36-kDa region has been studied, and the T-cell reactivity has been mapped in detail. Monoclonal antibodies were generated, and one monoclonal antibody, HYB 71-2, with reactivity against a 29-kDa antigen located in the highly reactive region below the antigen 85 complex was selected. The 29-kDa antigen (CFP29) was purified from *M. tuberculosis* short-term culture filtrate by thiophilic adsorption chromatography, anion-exchange chromatography, and gel filtration. In its native form, CFP29 forms a polymer with a high molecular mass. CFP29 was mapped in two-dimensional electrophoresis gels as three distinct spots just below the antigen 85 complex component MPT59. CFP29 is present in both culture filtrate and the membrane fraction from *M. tuberculosis,* suggesting that this antigen is released from the envelope to culture filtrate during growth. Determination of the N-terminal amino acid sequence allowed cloning and sequencing of the cfp29 gene. The nucleotide sequence showed 62% identity to the bacteriocin Linocin from *Brevibacterium linens*. Purified recombinant histidine-tagged CFP29 and native CFP29 had similar T-cell stimulatory properties, and they both elicited the release of high levels of gamma interferon from mouse memory effector cells isolated during the recall of protective immunity to tuberculosis. Interspecies analysis by immunoblotting and PCR demonstrated that CFP29 is widely distributed in mycobacterial species.

Pollock JM, Andersen P
Predominant recognition of the ESAT-6 protein in the first phase of interferon with *Mycobacterium bovis* in cattle
Infect Immun 65, 2587–2592 (1997)

Tuberculosis continues to be a worldwide health problem for both humans and animals. The development of improved vaccines and diagnostic tests requires detailed understanding of the immune responses generated and the antigens recognized during the disease. This study examined the T-cell response which develops in cattle experimentally infected with *Mycobacterium bovis.* The first significant T-cell response was found 3 weeks after the onset of infection and was characterized by a pronounced gamma interferon (IFN-gamma) response from peripheral blood mononuclear cells directed to antigens in culture filtrates. Short-term culture filtrate (ST-CF) was separated into molecular mass fractions and screened for recognition by T cells from experimentally infected and field cases of bovine tuberculosis. Cattle in the early stages of experimental infection were characterized by strong IFN-gamma responses directed predominantly toward the lowest-mass (<10-kDa) fraction of ST-CF, but cattle in later stages of experimental infection (16 weeks postinfection) exhibited a broader recognition of antigens of various molecular masses. Field cases of bovine tuberculosis, in comparison, preferentially recognized low-mass antigens, characteristic of animals in the early stages of infection. The major T-cell target for this dominant IFN-gamma response was found to be the secreted antigen ESAT-6. This antigen was recognized strongly by the majority of field cases of bovine tuberculosis tested. As ESAT-6 is unique to pathogenic mycobacterial species, our study suggests that ESAT-

6 is an antigen with major potential for vaccination against and specific diagnosis of bovine tuberculosis.

Ravn P, Boesen H, Pedersen BK, Andersen P
Human T cell responses induced by vaccination with *Mycobacterium bovis* bacillus Calmette-Guerin
J Immunol 158, 1949–1955 (1997)

Many aspects of the widely used bacillus Calmette-Guerin (BCG) vaccine against tuberculosis are still the subject of controversy. There is a huge variation in efficacy from one clinical trial to another and no relationship between vaccine-induced skin test conversion and subsequent protection. We have studied in vitro cell-mediated immune responses primed by BCG vaccination in 22 healthy Danish donors with different levels of in vitro purified protein derivative (PPD) reactivity before vaccination. The study demonstrated a markedly different development of reactivity to mycobacterial Ags depending on the prevaccination sensitivity to PPD. Previously sensitized donors mounted a potent and highly accelerated recall response within the first week of BCG vaccination. Nonsensitized donors, in contrast, exhibited a gradually increasing responsiveness to mycobacterial Ags, reaching maximal levels between day 56 and 365 postvaccination. The recognition of different classes of Ags were induced in a stepwise manner: culture filtrate Ags were recognized 1 wk postvaccination followed by cell wall, membrane, and the cytosolic Ag fraction. The T cell response primed by BCG vaccination was characterized as a CD4 response with a Th1-like cytokine pattern and substantial levels of Ag-specific cytotoxicity. The specificity of the T cell response generated was broad and directed to a range of culture filtrate Ag fractions. The study shows that BCG vaccination of previously nonsensitized donors can provide important data on potentially protective immune responses in humans and suggest a careful evaluation of prevaccination sensitivity when investigating vaccine-induced immunity.

Brandt L, Oettinger T, Holm A, Andersen AB, Andersen P
Key epitopes on the ESAT-6 antigen recognized in mice during the recall of protective immunity to *Mycobacterium tuberculosis*
J Immunol 157, 3527–3533 (1996)

The recall of long-lived immunity in a mouse model of tuberculosis (TB) is defined as an accelerated accumulation of reactive T cells in the target organs. We have recently identified Ag 85B and a 6-kilodalton early secretory antigenic target, designated ESAT-6, as key antigenic targets recognized by these cells. In the present study, preferential recognition of the ESAT-6 Ag during the recall of immunity was found to be shared by five of six genetically different strains of mice. Overlapping peptides spanning the sequence of ESAT-6 were used to map two T cell epitopes on this molecule. One epitope recognized in the context of H-2b,d was located in the N-terminal part of the molecule, whereas an epitope recognized in the context of H-2a,k covered amino acids 51 to 60. Shorter versions of the N-terminal epitope allowed the precise definition of a 13-amino acid core sequence recognized in the context of H-2b. The peptide covering the N-terminal epitope was immunogenic, and a T cell response with the same fine specificity as that induced during TB infection was generated by immunization with the peptide in IFA. In the C57BL/6j strain, this single epitope was recognized by an exceedingly high frequency of splenic T cells (approximately 1:1000), representing 25 to 35% of the total culture filtrate-reactive T cells recruited to the site of infection during the first phase of the recall response. These findings emphasize the relevance of this Ag in the immune response to TB and suggest that immunologic recognition in the first phase of infection is a highly restricted event dominated by a limited number of T cell clones.

Boesen H, Jensen BN, Wilcke T, Andersen P
Human T-Cell responses to secreted antigen fractions of *Mycobacterium tuberculosis*
Infect Immun 63, 1491–1497 (1995)

The T-cell response of human donors to secreted antigen fractions of *Mycobacterium tuberculosis* was investigated. The donors were divided into five groups: active pulmonary tuberculosis (TB) patients with minimal and with advanced disease, *Mycobacterium bovis* BCG-vaccinated donors with and without contact with TB patients, and nonvaccinated individuals. We found that patients with active minimal TB responded powerfully to secreted antigens contained in a short-term culture filtrate. The response to secreted antigens was mediated by CD4+ Th-1-like lymphocytes, and the gamma interferon release by these cells was markedly higher in patients with active minimal TB than in healthy BCG-vaccinated donors. Patients with active advanced disease exhibited depressed responses to all preparations tested. The specificity of the

response to secreted antigens was investigated by stimulating lymphocytes with narrow-molecular-mass fractions of short-term culture filtrate obtained by the multielution technique. Considerable heterogeneity was found within the donor groups. Patients with active minimal TB recognized multiple secreted targets, but interestingly, six of eight patients demonstrated a predominant recognition of a low-mass (< 10-kDa) protein fraction which induced high levels of gamma interferon release in vitro. Only a few of 12 previously characterized secreted antigens were recognized by T cells isolated from TB patients, suggesting the existence of a number of as yet undefined antigenic targets among secreted antigens.

Andersen P, Heron I
Simultaneous electroelution of whole SDS-polyacrylamide gels for the direct cellular analysis of complex protein mixtures
J Immunol Methods 161, 29–39 (1993)

A novel procedure which allows the rapid screening of complex protein mixtures in cellular assays is described. A device has been developed which allows a convenient, simultaneous electroelution of separated proteins from whole SDS polyacrylamide gels into narrow chambers each containing single or a few protein bands. We have optimized the conditions of the procedure and have obtained an efficient removal of SDS, leading to non-toxic protein fractions in a physiological buffer suited for direct testing in cell cultures. The responses generated by stimulating lymphocytes with the purified products have been compared to the native protein and a corresponding preparation of protein transferred to nitrocellulose particles. The method was used to investigate murine T cell responses to secreted mycobacterial antigens during infection with *M. tuberculosis.* An immunodominant secreted protein fraction was purified in a semipreparative scale by the procedure and used to immunize mice. The specificity of and lymphokine production by T cells generated in these animals were investigated. The device developed has various applications and provides a tool for the possible identification of new T cell antigens of importance for protective immunity.

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