ELSEVIER

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Journal of Chromatography B, 815 (2005) 65–75

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Review

Immunoproteomics

Jennifer L. Hess[a], Levi Blazer[a], Terence Romer[b], Lee Faber[c], R. Mark Buller[d], Michael D.P. Boyle[a,*]

[a] Department of Biology, Juniata College, 1700 Moore Street, Huntingdon, PA 16652-2196, USA
[b] BioCheck Laboratories, Toledo, OH 43614-2779, USA
[c] Department of Physiology and Molecular Medicine, Medical College of Ohio, Toledo, OH 43614-5804, USA
[d] Department of Molecular Microbiology and Immunology, College of Medicine, St. Louis University, St. Louis, MO 63103, USA

Received 2 July 2004; accepted 20 July 2004
Available online 23 September 2004

Abstract

A novel immunoproteomic assay, combining specificity of antibody with precision of mass spectral analysis is described, and a number of practical applications are presented. The assay is carried out in three steps. The first step of the assay involves antibody immobilization, using a bacterial Fc binding support. The second step is antigen capture and washing to remove non-specific binding. The third step involves analysis of the captured antigens by SELDI-TOF. The assay has many advantages in sensitivity, speed, and economy of reagents in detection of specific antigens or antibodies. In addition, under appropriate experimental conditions, semi-quantitative data may be obtained. By combining the increasing range of selective specific antibody reagents available, in part due to advances in antibody engineering technology, and the resolving power available, using mass spectrometry, immunoproteomics is a valuable technique in proteomic analysis. A number of examples of the application of this technique to analysis of biological systems are presented.
© 2004 Elsevier B.V. All rights reserved.

Keyword: Immunoproteomics

Contents

1. Introduction 66
2. Immunoproteomics 66
3. Analysis of phosphorylation of a model signaling peptide 68
4. Immunological signaling molecules 69
5. Monitoring antibody responses 71
6. Quantification of antigens using immunoproteomics 72
7. Albumin detection and depletion 73
8. Summary and future prospects 74
Acknowledgements 74
References 74

* Corresponding author. Tel.: +1 814 641 3553; fax: +1 814 641 3685.
E-mail address: boyle@juniata.edu (M.D.P. Boyle).

1570-0232/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2004.07.047

1. Introduction

It is generally recognized that analysis of the proteome is going to be much more technically challenging than analysis of the transcriptome [1–3]. Not only are there potentially multiple forms of each translated transcript but the analysis of those products is not amenable to the amplification procedures and the rapid sequencing techniques that can be applied to different nucleic acid sequences. The importance of post-translational modifications, such as phosphorylation and glycosylation are becoming critical, as attempts to understand such key features of cellular behavior as molecular localization, protein–protein interaction, and gene regulation [4]. Consequently, the study and analysis of the variety of distinct products that can be generated following translation of a single cDNA becomes essential if the connection between the transcriptome and the physiome in normal and abnormal cells is to be understood.

Analysis of virulence factors and coordinately regulated gene expression in bacteria, using 2D gel electrophoresis represents the foundation of the current concept of proteomics [3–6]. These methods have recently been complemented with developments in protein chip mass spectrometry [7–10]. Protein chips offer the possibility of performing a simple and rapid analysis of the proteome. Although, this system lacks the resolving power of 2D gels, it provides a quick and sensitive system to monitor a single protein with known characteristics or compare major differences in paired samples. These studies are frequently complicated by a large molar excess of one or small number of proteins that dominate the sample.

One of the most successful approaches to rapid analysis of proteins in complex mixtures, has been the use of antibody-based assays. With the availability of in vitro selection techniques, such as phage or ribosome display, antibody probes to peptides synthesized from expressed sequences tags (EST) are being used to screen tissue sections [11–14]. In addition, plastibodies and nucleic acid antibody mimics have been developed that may have advantages for certain applications [11,15].

A number of methods have been described that seek to combine the specificity of antibody capture with the resolving power of mass spectrometry. The initial antibody-based mass spectral methods involved the direct immobilization of antibody on a chip surface that can be used to capture antigen for subsequent mass spectral analysis [16–18]. These approaches are technically limited by the concentration of antibody that can be immobilized and the volume of antigen-containing sample that can be analyzed. Furthermore, attempts to analyze multiple samples on a single chip results in significant cross-contamination between sample spots, even if a bioprocessing device is used.

2. Immunoproteomics

Our laboratories have previously described the concept of "immunoproteomics" (Fig. 1). Immunoproteomics employs a single reagent (proACTR) for immobilization of antibody, capture of antigen, and transfer into the mass spectrometer for analysis [19–23]. ProACTR is a group G streptococcal isolate that has been selected for very high surface expression of the immunoglobulin binding protein, protein G. This reagent is prepared as a heat killed, 10% wet weight/volume, suspension of the bacteria. ProACTR can immobilize the antibody through the antibody's Fc region, orienting the Fab_2 regions for efficient, selective antigen capture. This approach to antibody immobilization allows for higher capture capacity than approaches, using antibody-coated beads [23]. Captured antigens can then be directly transferred to a platform for mass spectral analysis (Fig. 1).

Key requirements for this procedure include antibodies with high selectivity and appropriate buffer systems for sample extraction, preparation, antigen capture, and washing. The goal of the immunoproteomic protocol is to capture and quantify antigens in crude samples.

There are a number of significant features of the immunoproteomic protocol outlined in Fig. 1. In the first stage of this procedure, antibody from an appropriate antiserum is immobilized. The antibody is selectively captured from crude antiserum through a high affinity Fc binding interaction [24]. Unbound serum proteins are removed by washing. This step represents an antibody purification step. Furthermore, the mode of antibody binding promotes orientation of the antigen combining Fab_2 regions away from the supporting bacterial structure to favor subsequent interaction with its cognate antigen.

The second step is antigen capture. The sample-containing antigen is mixed with the immobilized antibody in 1.5 ml Eppendorf tubes and incubated for 30 min to allow antigen–antibody interaction (step 2, Fig. 1). The bacterial antibody support is inherently stable and remains so during centrifugation. By performing the capture reaction in larger volumes and subsequently resuspending the bacterial pellet in a smaller volume there is potential for the concentration of antigen (step 2 of the protocol outlined in Fig. 1). This overcomes some of the technical difficulties of sample volume that were noted in studies, using antibody directly immobilized on a protein chip spot.

In step 3, the immobilized antigen–antibody complex is applied to the protein chip and analyzed directly by SELDI-TOF mass spectrometry (Fig. 1). This approach eliminates some of the limitations associated with using small volume samples and allows the absolute concentration of captured antibody to be measured and allows different antigen–antibody ratios to be compared for efficiency of capture from solutions, containing a low concentration of the targeted antigen [21,23]. Critical to the success of this approach is that the antigen can be selectively dissociated from the antibody and that the signal is not masked by noise from the antibody coated proACTR.

Preliminary studies demonstrated that at laser energies required to fly peptides and proteins of less than 80,000 amu (atomic mass units), there was no significant signal observed

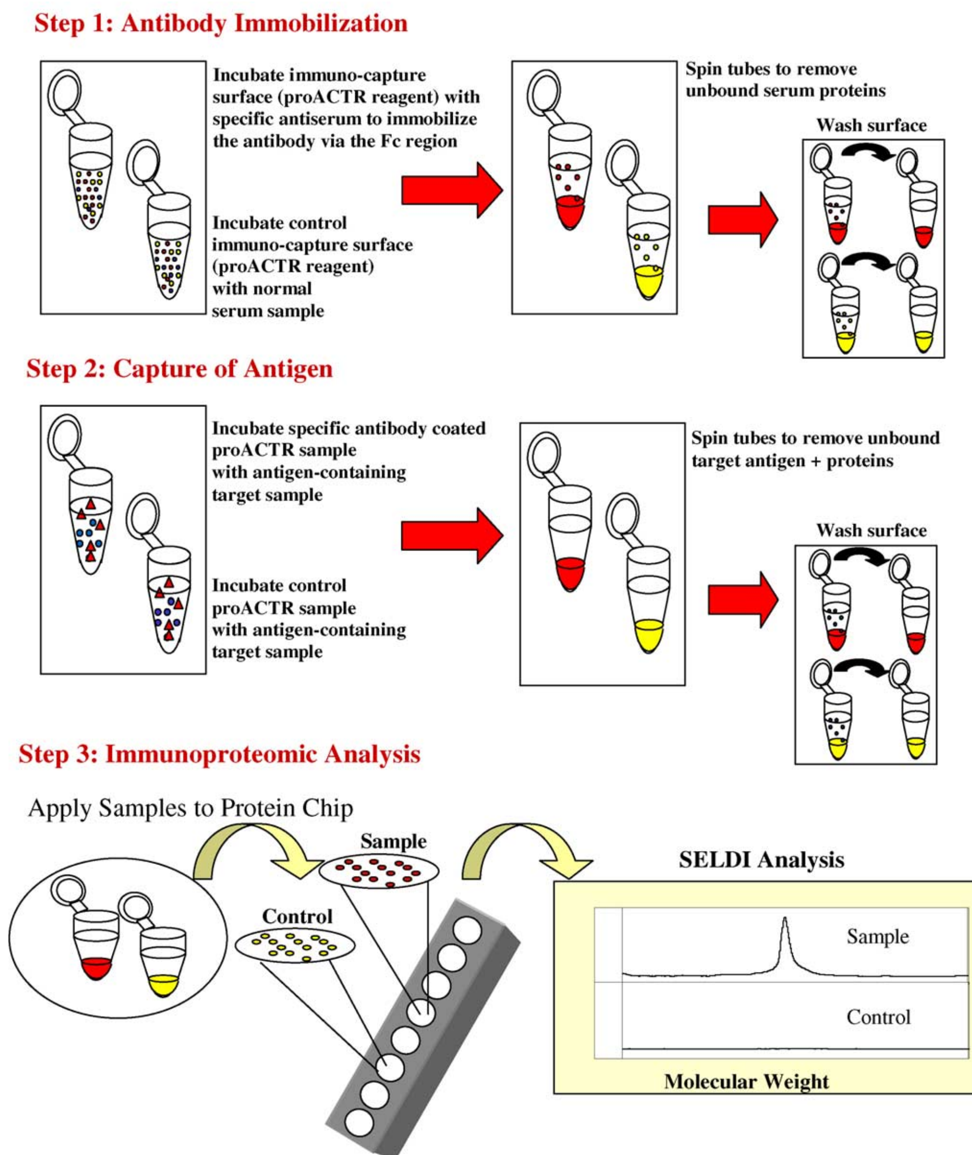


Fig. 1. Schematic representation of immunoproteomic assay to detect antigens. Step 1 involves antibody immobilization; step 2 involves antigen capture; and step 3 is the analytic phase in which the immobilized antigen–antibody complexes are transferred to a protein chip and then analyzed by SELDI-TOF mass spectrometry.

from either proACTR alone or antibody coated proACTR. However, laser energies sufficient to both dissociate antigens from antigen–antibody complexes and have them fly in the mass spectrometer could be achieved. The differential energy required to dissociate antigen from antibody is consistent with differences in the respective affinity of the interactions. Antigen–antibody interactions are of the order of 10^{-7} to 10^{-8} M, while the binding protein–Fc interaction is one to two-logs greater. Analysis of the mass spectrum in the 140,000–160,000 amu range also failed to demonstrate any protein peaks indicating that the antibody was not dissociated under the experimental conditions tested (data not shown). At higher laser energy levels, an antibody peak could be detected from either the specific or non-specific immobilized antibody support.

In this paper, we review extension of the immunoproteomics methodology to:

1. Analyze phosphorylation of a model signaling peptide,
2. Detect cytokines, and
3. Monitor production of antibodies.

The quantitative nature of the assays and the limitations of the system to analyze low abundance samples in the presence of a large molar excess of other proteins, e.g. serum albumin, are also addressed. The goal of our studies has been to evaluate whether immunoproteomics is likely to provide significant advantages over existing 2D gel systems, coupled with a western blotting step or traditional immunoprecipitation assays for proteomic analysis.

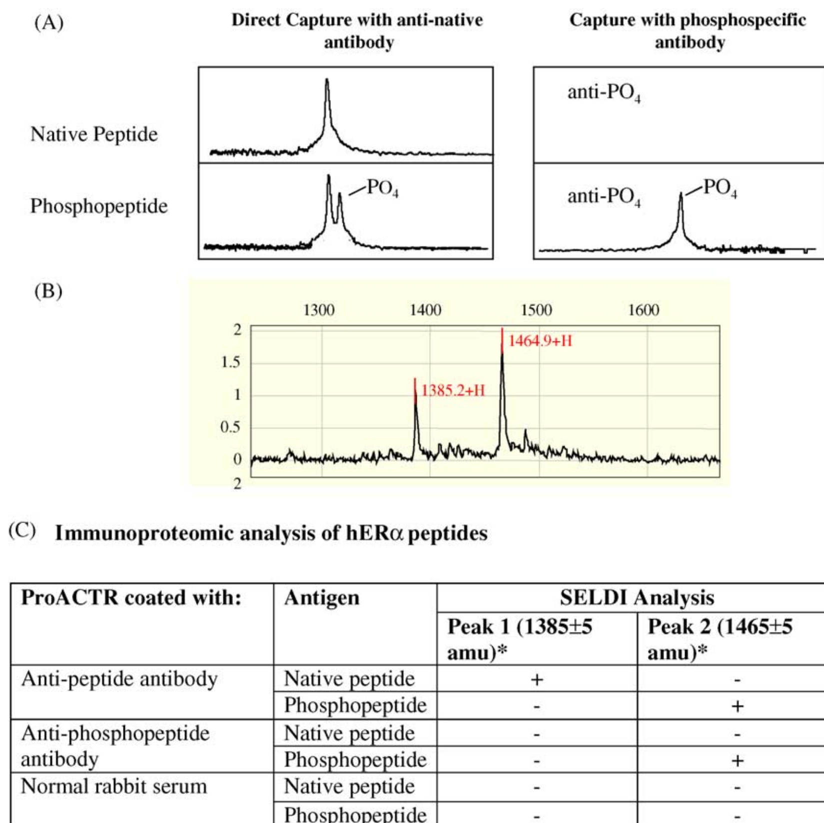
3. Analysis of phosphorylation of a model signaling peptide

Our earlier studies of streptococcal virulence factors suggested that the resolving power of SELDI-TOF mass spectrometry coupled with specific antigen capture might allow for analysis of other post-translational modification reactions [19–23]. The logical extension of these studies would be directed towards analysis of common post-translational modification events, such as phosphorylation. The availability of specific antibodies to signaling peptides as well as to phosphoserine residues makes this strategy particularly attractive. The theoretical approach to these studies is summarized in Fig. 2A.

To test the practicality of this model we have evaluated a model system, containing phosphorylated and non-phosphorylated peptides derived from human estrogen receptor alpha (hERα). Commercially available peptides corresponding to amino acids 160–171 of hERα and their cognate antibodies were purchased from AnaSpec (San Jose, CA). Sequences of both native (unphosphorylated) and phosphorylated peptides differ only in the presence of a phosphate on serine 167. The molecular mass difference between the two forms of the peptide is consequently ∼90 amu.

In the initial studies, conditions to detect the two forms of hERα, using SELDI-TOF mass spectrometry were established [25]. The native peptide could be resolved as a single peak with a molecular mass of 1385 ± 5 amu, while the phosphorylated sample contained a major peak at 1465 ± 5 amu. When mixed in equimolar proportions the two peptides could be readily distinguished (Fig. 2B).

Having demonstrated that SELDI-TOF could resolve phosphorylated and non-phosphorylated peptides, we next set out to establish the utility of the proACTR system in this model. Fifty microliters of washed proACTR was added to a 1 ml of a 2 μg/ml solution of an IgG preparation of monospecific rabbit anti-hERα or an IgG fraction of a rabbit anti-phosphopeptide specific antiserum (Anaspec). As a control for immunological specificity, proACTR coated with 2 μg of rabbit IgG of irrelevant specificity were also prepared. The Ab–proACTR mixtures were allowed to stand at room temperature for 60 min, and then washed three times with 1 ml of Tris buffer (100 mM, pH 7.4) to remove unbound antibody and unrelated plasma proteins. The specific



(C) **Immunoproteomic analysis of hERα peptides**

ProACTR coated with:	Antigen	SELDI Analysis	
		Peak 1 (1385±5 amu)*	Peak 2 (1465±5 amu)*
Anti-peptide antibody	Native peptide	+	-
	Phosphopeptide	-	+
Anti-phosphopeptide antibody	Native peptide	-	-
	Phosphopeptide	-	+
Normal rabbit serum	Native peptide	-	-
	Phosphopeptide	-	-

* The native peptide had a molecular mass of 1385±5 amu (peak 1) and the phosphorylated variant had a molecular mass of 1465±5 amu (peak 2).

Fig. 2. Immunoproteomic analysis of hERα peptides. (A) Theoretical spectrum of a native and a phosphorylated peptide analyzed by SELDI-TOF following immunoproteomic capture with selective anti-native or anti-phosphopeptide antibody. (B) SELDI-TOF resolution of a human estrogen receptor α peptide (hERα) and a phosphorylated derivative. The hERα peptide consisted of amino acids 166–171 (1385 ± 5 amu), and the phosphorylated peptide differed only by the presence of a phosphorylation site on serine 167 (1465 ± 5 amu). (C) Summary of proACTR capture of native and phosphorylated hERα peptide. For precise experimental details, see the text.

immobilized antibody and control antibody reagent were then added to a solution, containing equimolar concentrations of the phosphorylated and non-phosphorylated peptides (250 μl of a 500 ng/ml mixture of each peptide). The reagents were incubated with the Ab–proACTR for 60 min at ambient temperature in the presence of protease inhibitors. The Ab–proACTR–peptide complex was then washed three times with 1 ml of Tris buffer and resuspended in 20 μl 10 mM Tris. Subsequently, 3 μl of Ab–proACTR suspension was applied to a hydrophobic Ciphergen™ protein chip (H50) and subjected to SELDI-TOF mass spectrometry. After drying, each spot was coated with an energy-absorbing matrix consisting of saturated cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.5% trifluoroacetic acid. A high voltage detector sensitivity of 5 and a laser intensity setting of 165 was empirically determined to be optimal for efficient mass spectral analysis. All data were generated by averaging 65 laser shots on different positions of each protein chip spot.

The results summarized in Fig. 2C demonstrate that both antigenic forms of peptide were detected when captured with the antibody that recognized the native peptide. If the capture reagent utilized antibody specific for only the phosphorylated peptide, then only one peak corresponded in molecular mass to the phosphorylated peptide (1465 ± 5 amu) was be observed in the spectrum (Fig. 2C). The capture was immunologically specific, since when the capture reagent was prepared, using normal rabbit serum no peaks were observed.

The capture experiments, summarized in Fig. 2C, represent the proof-of-concept of a novel bioassay for a single-site phosphorylation. Preliminary data also suggest that the incubation time for antigen capture can be shortened to less than 30 min without loss of sensitivity and that the Ab–proACTR reagent can be stored for up to 3 days without loss of capture capacity (data not shown). Furthermore, preliminary analysis suggests that by measuring the areas under the peaks in the spectra, a semi-quantitative analysis of the relative concentrations of two peptides can be performed. These variations in the assay design may, in turn, facilitate more detailed kinetic studies of phosphorylation reactions in cells. By using a panel of capture antibodies to different native and phosphorylated signaling kinases, detailed phosphoprofiling of a number of different signal transduction pathways in a cell or tissue sample could potentially be achieved.

4. Immunological signaling molecules

The immune system is highly sophisticated and involves interactions of many different cell types, intracellular signaling pathways, and cell to cell communication. Critical to defining and supporting an immune response are the secreted signaling molecules known collectively as cytokines [26,27]. The nature of the cytokine response (T_h1 versus T_h2) can determine whether a cell-mediated or an antibody response results [28]. Furthermore, if the production of cytokines is not tightly regulated, there is the potential for an uncontrolled response that can lead to fatal septic shock [29–31]. Because of these key properties of cytokines, a rapid detection system would be advantageous.

In the initial series of immunoproteomic studies of cytokines, the key T_h1 cytokine interferon-γ was targeted. A commercial source of human interferon-γ (Pepro Tech, Rocky Hill, NJ) and a monoclonal mouse anti-human interferon-γ (IgG_1) were obtained from Pharmingen (San Diego, CA). Preliminary studies demonstrated that the interferon-γ preparation contained major peak at 16,800 amu when analyzed by SELDI-TOF mass spectrometry (data not shown).

For the immunoproteomic analysis the capture reagent was prepared by mixing 50 μl of the proACTR cells with 1 ml of a 1:500 dilution of the mouse interferon-γ monoclonal antibody (1 mg/ml) in 0.01% CHAPS/0.01M EDTA in phosphate buffered saline (CE buffer). The reaction was incubated at ambient temperature for 15 min and was then washed once with CE buffer. The antibody dilution was determined empirically in a series of preliminary studies and was found to be optimal for capturing the cytokine from solution. A control reagent generated in an identical manner, using a mouse IgG_1 monoclonal antibody of irrelevant specificity was prepared and used as a control for immunological specificity.

Using these experimental conditions, the ability to capture different concentrations of human interferon-γ from CE buffer in an immunologically specific manner was tested. Varying amounts of human interferon-γ were then mixed with the proACTR–specific antibody complex in a final reaction volume of 1 ml in CE buffer. Control reactions, containing interferon-γ and proACTR–irrelevant antibody complexes or proACTR alone were also performed. After adding interferon-γ, the reactions were incubated at 4 °C for 20 min and were then washed once with Tris-buffered saline (TBS). After washing, the pelleted complexes were resuspended in 10 μl TBS. Subsequently, 1 μl of Ab–proACTR suspension was applied to a hydrophobic Ciphergen™ protein chip (H50) and subjected to SELDI-TOF mass spectrometry. After drying, each spot was coated with an energy-absorbing matrix consisting of saturated cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.5% trifluoroacetic acid. A high voltage detector sensitivity of 5 and a laser intensity setting of 165 was empirically determined to be optimal for efficient mass spectral analysis. All data were generated by averaging 65 laser shots on different positions of each protein chip spot.

The results of a representative experiment are shown in Fig. 3. Using these experimental conditions, interferon-γ amounts of 20 ng per spot could be detected. The area under the peak increased as the cytokine level in the sample increased. The entire assay for interferon-γ could be completed in less than 45 min, which is considerably faster than alternative methods, using ELISA or antibody-bead fluorescence technology [32]. While these studies suggest that an immunoproteomic approach to cytokine detection and potential quantification is practical, it was of major concern as

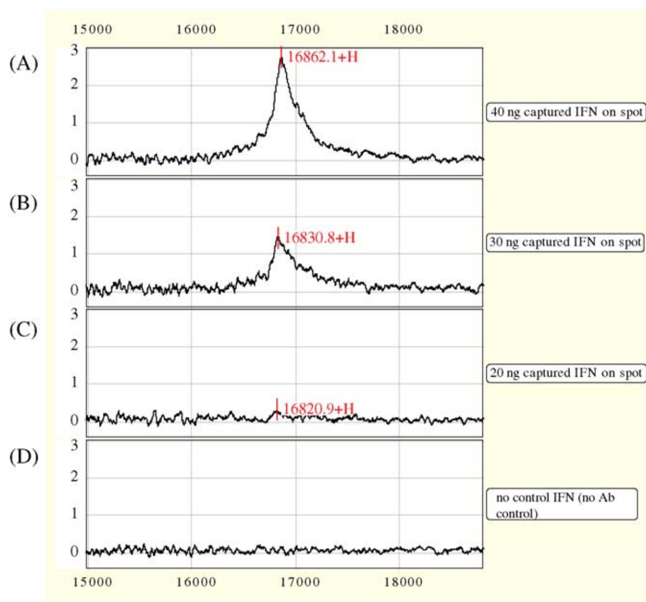


Fig. 3. Immunoproteomic analysis of IFN-γ. Differing concentrations of human IFN-γ, diluted in PBS, containing 0.01% CHAPS and 0.01M EDTA (CE buffer) were incubated with proACTR coated with a specific anti- IFN-γ antibody. Following a series of washing steps, the sample was transferred to an H50 protein chip and was analyzed by SELDI-TOF mass spectrometry. (A) Sixteen thousand eight hundred atomic mass unit peak corresponding to IFN-γ was observed only in samples, containing the proACTR coated with anti- IFN-γ antibody. (A–C) The area under the peak decreases in a dose-dependent manner as the amount of IFN-γ in the reaction decreases. (D) ProACTR coated with an antibody of irrelevant specificity failed to capture any detectable quantity of the cytokine. For precise experimental details, see text.

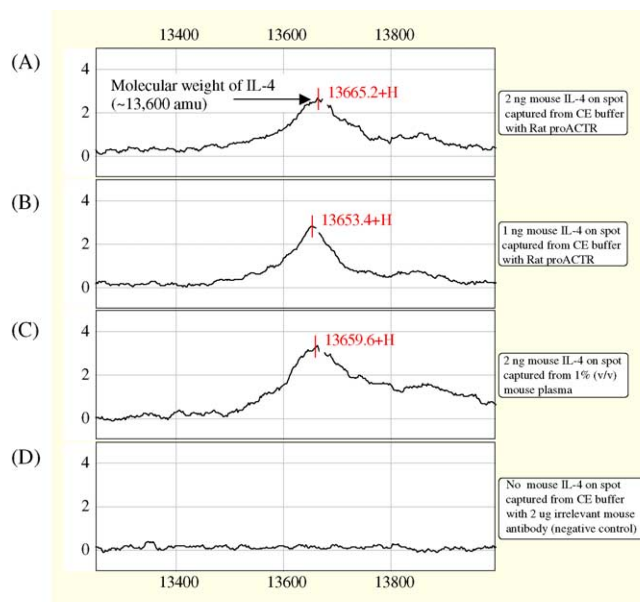


Fig. 4. Immunoproteomic analysis of IL-4, using a rat monoclonal anti-IL4 antibody. Differing concentrations of a recombinant preparation of mouse IL4, diluted in PBS, containing 0.01% CHAPS and 0.01M EDTA (CE buffer) were incubated with a rat selective proACTR coated with a rat monoclonal anti-IL4 antibody. Following a series of washing steps, the sample was transferred to an H50 chip and was analyzed by SELDI-TOF mass spectrometry. (A and B) A broad peak at ∼13,700 amu, corresponding to IL-4 was observed only in samples, containing the proACTR coated with the rat anti-IL-4 antibody which decreased in a dose-dependent manner as the amount of IL-4 in the reaction decreased. (C) Rat proACTR coated with a rat monoclonal antibody specific for mouse IL-4 could capture mouse IL-4 from 1% mouse plasma. (D) The capture was immunological specific since no IL-4 was captured when a proACTR coated with an irrelevant rat monoclonal antibody was used. For precise experimental details, see text.

to whether this approach could be used with serum samples that contain a large molar excess of other proteins.

To address this question, interferon-γ was seeded into samples, containing 1% (v/v) human plasma in CE buffer and analyzed as described as above (data not shown). In the 16,000–17,000 amu molecular weight range, interferon-γ could be detected in an immunologically specific manner. Interferon-γ amounts of 20 ng per spot could be detected. However, the spectra also show a major peak for albumin at approximately 66,800 amu and a double charged peak at 33,400 amu. Thus, if the targeted antigen had a molecular mass close to albumin or one of its multiple charged peaks this assay would be difficult to interpret. The challenge of analyzing trace molecules in complex biological samples (e.g. plasma) is discussed further below.

In addition to analysis of cytokines in human samples, murine models, using inbred strains and transgenic models have been of great interest immunologists [33–35]. As a consequence many antibodies to murine cytokines have been developed as monoclonal reagents in the rat. The use of rat antibodies with the protein G-based proACTR reagent is not practical, since protein G demonstrates minimal affinity for rat IgG [24]. Previous studies from other laboratories have, however identified a Type VI Fc binding protein that demonstrates good reactivity with wild type IgG monoclonal and polyclonal antibodies [36]. In addition, the Type VI expressing strain has been subjected to extensive selection for a variant that displays high levels of surface binding protein [36].

In the next group of experiments, the highly expressed Type VI binding protein positive strain was tested to determine if it could function as a proACTR in the immunoproteomic assay, using a rat anti-mouse IL-4 monoclonal antibody to detect this prototypic T_h2 cytokine.

In preliminary experiments, antibody coating and capture conditions were optimized, using the rat proACTR and then tested for their ability to capture mouse IL-4 from solution, using similar experimental conditions to those described for the interferon-γ assay. The results presented in Fig. 4 demonstrate that the immunoproteomic system can be adapted for use with rat antibody reagents. In this experiment, IL-4 concentrations of greater than 25 pg/spot could be detected. The ability to extend the technique for use with rat monoclonal antibodies expands the platform to a wider range of specific antibody reagents and should prove beneficial for studying target antigens, particularly in murine models of disease for which many rat monoclonals currently exist.

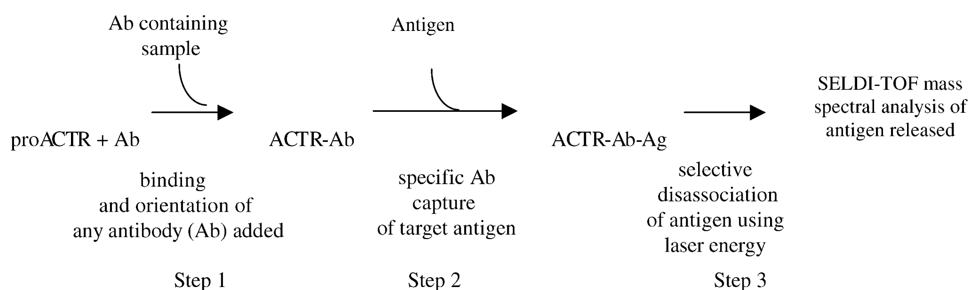


Fig. 5. Schematic representation of a modified immunoproteomic assay to measure antibodies. In this assay, the presence of antibody to a pre-determined antigen is measured. In step 1, any antibody in the sample is immobilized by proACTR. In step 2, the targeted antigen is added and, following a series of washing steps, bound antigen is detected by SELDI-TOF analysis (step 3). Detection of the target antigen indicates the presence of specific antibody in the sample. Controls, using a source of antiserum against an unrelated antigen are included to account for any non-specific binding of antigen.

The studies described in this section, suggest that the immunoproteomic approach can be used to measure cytokines in plasma and other biological samples. This approach has also been shown to work for IL-6 using a goat anti-IL-6 antibody and for RANTES and other key cytokines using appropriate rabbit or mouse antibodies (data not shown).

The availability of a proACTR compatible with rat IgG should enable this strategy to be extended to a wide range of cytokine specific antibodies and enable cytokine profiles to be monitored under different experimental conditions. The speed of the assay has a number of advantages and future studies will be focused on increasing the sensitivity of the assay, optimizing conditions to measure multiple cytokines in a single sample, and adapting the precise molecular weight read out to enable cytokines bound to regulatory proteins to also be monitored.

5. Monitoring antibody responses

All of the applications outlined thus far, have been designed to use specific antibodies to capture their cognate antigens. Like all immunoassay formats, it is also possible to use the same basic strategy to monitor the presence of a specific antibody to a predetermined antigen (Fig. 5). For example, determining whether an immunized animal is responding to a vaccination protocol or whether a well, containing a single hybridoma is secreting the desired monoclonal antibody. To address the practicality of using the immunoproteomic assay to follow antibody production we monitored several bleeds from a rabbit that had been immunized with a specific peptide coupled to keyhole limpet hemocyanin (KLH) as a carrier. This peptide conjugated to the carrier protein KLH was used to immunize the rabbit used in this study had an expected mass of 2595 amu (Fig. 6).

In the initial screen a 1:500 dilution of rabbit serum was mixed with 75 μl of the proACTR reagent for 1 h at ambient temperature in 100 mM Tris buffer. Unbound IgG and uncaptured serum proteins were removed by washing and then mixed with 1 ml of 10 ng of the immunizing peptide for 60 min at ambient temperature. The mixture was washed three times in Tris to remove unbound antigen. One microliter of sample was applied to a normal phase Ciphergen^{TM} protein chip (NP20). After drying, each spot was coated with an energy-absorbing matrix consisting of saturated cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.5% trifluoroacetic acid. The optimum laser intensity setting was empirically determined and the data were generated by averaging 65 laser shots on different positions of each protein chip spot.

Control samples of proACTR alone and proACTR coated with normal rabbit serum (pre-bleed) were included as controls. The presence of antibody could be detected by the appearance of a peptide peak (in this case a 24 amino acid peptide of 2595 amu). Purified peptide could be resolved as a single peak by SELDI-TOF at this molecular mass (data not shown). The results of the initial screen of timed samples

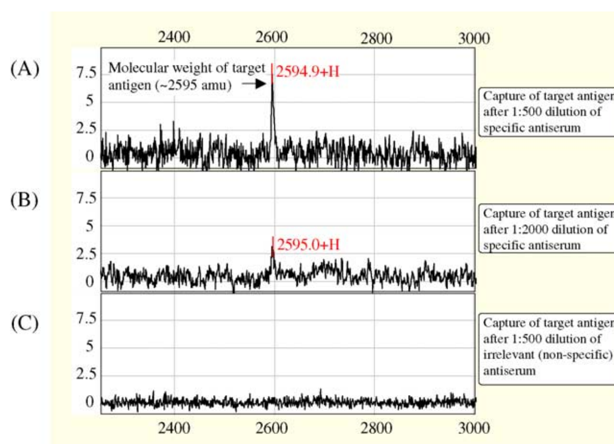


Fig. 6. Detection of specific antibody by immunoproteomics. Serum samples from a rabbit immunized with a 24 amino acid (2595 amu) peptide conjugated to KLH were tested following the protocol outlined in Fig. 5. The presence of a 2595 amu peak was only observed in samples in which a dilution of serum from an immunized rabbit was present. (A–C) The area under the peptide peak decreased as the concentration of serum added in step 1 of the assay decreased. (D) No peptide peak was observed when an irrelevant antiserum was added in step 1 of the assay. For precise experimental details, see text.

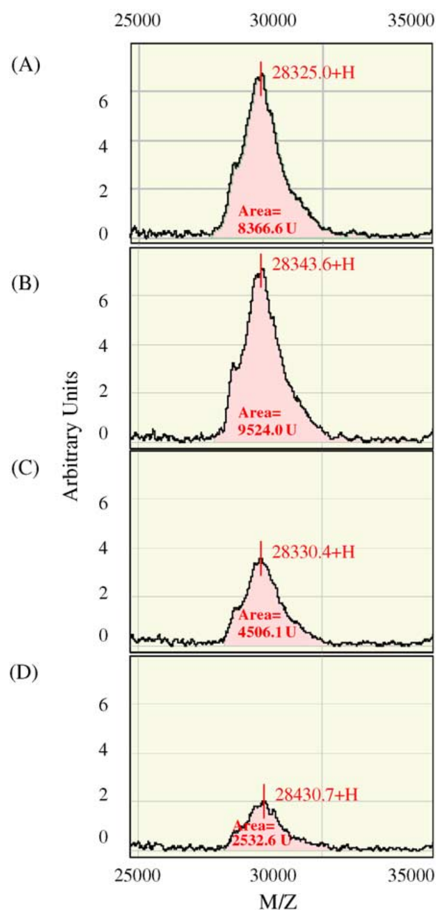


Fig. 7. Application of immunoproteomics to detection and quantitation of the streptococcal cysteine protease, SpeB. Capture of different concentrations of purified SpeB in Todd Hewitt broth, containing 0.1% yeast (THY) by proACTR coated with a specific anti-SpeB antibody was performed as described in the text. The area under the SpeB peak (at ∼28,400 amu) was analyzed, using the Ciphergen™ PBS II software package. (A–D) Over a limited dilution range the area under the peak could be related to the concentration of SpeB in the initial sample. The capture of SpeB was immunologically specific since no peak was observed for the same samples analyzed, following incubation with proACTR coated with normal rabbit serum (data not shown).

from the immunized rabbit demonstrated the absence of any antibody in the pre-immune or early bleeds; however, selective capture of the peptide antigen was observed in the final bleed.

In subsequent experiments, the positive serum sample from the final bleed was serially diluted and evaluated for selective binding of the peptide and demonstrated a dose-dependent capture of antigen (Fig. 7). The results presented in Fig. 7 were consistent with the antibody levels determined for the same samples by ELISA (data not shown).

The rapid immunoproteomic assay for antibody should enable antibody responses in vaccinated or infected animals to be monitored efficiently. The use of the protein G-based proACTR system, as well as the rat proACTR system, should allow this approach to be used to monitor humoral immune responses in a wide range of mammalian hosts used for the production of monoclonal and polyclonal antibodies. The ability to capture antibody via the Fc region and potentially concentrate the antibody during the first step of the protocol (Fig. 1), may be of particular value for screening hybridoma supernatants to identify positive clones quickly and efficiently.

6. Quantification of antigens using immunoproteomics

The ability to quantify antigen in any immunoassay is complicated by difficulties in determining on rates, off rates, and the equilibrium that occurs during washing steps in a given procedure. Since the pioneering work of Yalow, that led to the development of radio-immunoassays, and subsequently many different types of quantitative immunoassays, the necessity for inclusion of a standard curve in each assay has been appreciated [37–39]. Given the parallels between the immunoproteomic assay and other immunoassays, it should be possible to relate antigen concentration to some measure of area under the peak corresponding to the molecular mass of a targeted specific antigen.

In a series of preliminary experiments the area under the peak for a variety of different peptide and protein antigens was shown to be proportional to the concentration of antigen spotted on the chip [23]. As expected, it was possible to saturate the capacity of the chip surface, so the linearity of the dose response curve was only observed over a narrow concentration range.

A similar series of studies were carried out, using different dilutions of a preparation of the active streptococcal cysteine protease SpeB added to a crude bacterial culture broth, Todd Hewitt broth, containing 0.1% yeast extract, (THY) [22]. The SpeB antigen was captured from THY, using proACTR coated with a monospecific rabbit anti-SpeB antibody (Toxin Technologies, Sarasota, FL.) 50 μl of washed proACTR was added to a series of 1 ml aliquots of a 1:2000 dilution of rabbit anti SpeB anti-serum. A control tube, containing 1 ml of a 1:2000 dilution of non-immune rabbit serum was included as a control for immunological specificity. The Ab–proACTR mixtures were incubated at ambient temperature for 60 min. Each tube was washed three times with 1 ml Tris buffer to remove unbound antibody and serum related proteins. Serial two-fold dilutions of purified SpeB ranging from 1:2000 to 1:16,000 were made in THY. One milliliters of each dilution was added to an aliquot of the Ab–proACTR complex and allowed to incubate at ambient temperature for 60 min. The tube, containing non-immune rabbit control–proACTR complex was incubated with a 1:2000 dilution of THY/SpeB mix. The Ab–proACTR–SpeB complexes were washed three times with 1 ml of Tris buffer to remove unbound SpeB and media related proteins. The Ab–proACTR–SpeB complexes were resuspended in 20 μl Tris buffer. 3 μl of Ab–proACTR–SpeB suspension was spotted on a Ciphergen™ hydrophobic (H4)

protein chip. After drying, each spot was coated with 1 μl of an energy absorbing matrix consisting of saturated SPA in 50% acetonitrile and 0.5% trifluoroacetic acid. The chip was subsequently read in the SELDI protein-chip reader. A high voltage detector sensitivity of 10 and a laser intensity setting of 285 was empirically determined to be optimal for efficient mass spectral analysis.

Data were generating by averaging 65 laser shots on different positions of each protein chip spot. Peak area was calculated by the SELDI software. No significant binding was detected in the non-immune control sample (data not shown). Following this protocol, a peak corresponding to the expected molecular mass of active SpeB was observed (Fig. 7). The area under the peaks was integrated, using the Ciphergen™ PBS II software package. At the highest two concentrations of SpeB tested, there was no significant change in the area under the peak suggesting that the system was saturated, most probably due to limiting capture antibody. However, as the concentration of the SpeB in the THY broth was diluted further, there was a proportionality between the area under the antigen peak on the mass spectra and the quantity of antigen in the system (Fig. 7). These findings suggest that the immunoproteomic assay may be amenable to providing at least semi-quantitative data. By inclusion of a standard curve in each assay and a demonstration of the reproducibility of results among replicate samples within a single assay, it may ultimately be possible to determine absolute levels of a targeted antigen.

The immunoproteomic assay is likely to be most useful for rapid distinction between different molecular forms of a single antigen. This may be valuable in identifying potential disease markers and post-translational modification events. It is likely that the value of this technique will be in rapid detection of qualitative rather than quantitative differences. Based on our studies, this technique will be able to distinguish two-fold concentrations differences but it is unlikely that the methodology, as currently envisioned, would have the quantitative precision of ELISA or radioimmunoassay. It is possible that by including an antigenically identical competitor antigen that could be differentiated by size on the mass spectral readout, a more quantitative assay could be achieved.

Recently, Lill reviewed technical methods for the quantification of proteins and peptides by mass spectrometry [40]. The use of internal standards and metabolic labeling to incorporate isotopic labels seem to offer an enhanced potential for quantification of the targeted molecule. If suitable antibody is available these approaches should also be applicable to the immunoproteomic format.

7. Albumin detection and depletion

One of the major problems with any proteomic assay, using a relevant biological sample is the ability to detect the targeted molecule in the presence of a large molar excess of other proteins. This is particularly true if the starting material is a plasma sample. In this case, over 80% of the total protein mass consists of just six proteins. These include: albumin, IgG α-1 antitrypsin, IgA, transferrin, and haptoglobin. As noted above, when measuring interferon-γ captured from human plasma, the presence of human serum albumin in the spectra was readily detected at 66,800 amu or as the double charged peak at 33,400 amu. This contamination, even after an antibody capture step, is not surprising when you consider the normal serum albumin levels of 35–50 mg/ml in healthy adults.

A number of procedures have been proposed for removing albumin as part of a sample pre-treatment protocol. These include use of the affinity dye ligand, Cibacron blue, binding to immobilized lectin, or absorbing the serum sample with immobilized antibody specific for human albumin [41–44]. None of these procedures have proved very efficient and all are expensive. In addition, Cibacron blue is not specific for albumin, and thus, removes other proteins and enzymes from the sample, including a variety of enzymes, containing a NAD binding site. Similarly, lectins bind to sugar moieties and can remove many glycosylated proteins. Immunoaffinity resins require concentrations of specific antibody in the 35–45 mg/ml range and immobilization is both time-consuming and expensive. This approach, however, is highly versatile since additional antibody specificities can be added to the immobilized resin to remove multiple different targeted plasma proteins.

A low-cost high affinity, selective binding protein for human serum albumin, and other abundant plasma proteins, would be of value for use in pre-treating samples prior to proteomic profiling. For over 25 years, it has been known that certain bacteria and other pathogens can express surface structures that can interact with human plasma proteins [45]. Streptococcal protein G, for instance, binds human albumin as well as IgG, while other bacterial isolates appear to express surface binding proteins that are more selective for albumin [46,47]. Other bacteria receptors have been described that can bind transferrin, protease inhibitors, like α-2 macroglobulin, as well as IgA, fibrinogen, plasminogen and other abundant plasma proteins [47–54]. Some of these binding activities have been mapped to unique domains of the protein and all appear to have high affinity binding characteristics [45].

The use of selective bacterial binding proteins in immunochemistry is well established. For example, intact bacteria expressing IgG-binding proteins has been widely used for immunoprecipitation studies and for depletion of IgG from clinical samples to facilitate detection of IgM selective responses [55,56]. In a series of preliminary studies, we have found that incubation of diluted serum samples with a heat-killed preparation of bacteria expressing albumin selective binding proteins can result in 80–90% depletion of albumin from 1 to 5% samples of human serum in approximately 15–20 min (data not shown). This approach may merit further study as a rapid, economic approach to pre-treat plasma or serum samples prior to proteomic analysis.

8. Summary and future prospects

For a complete understanding of dynamic biological processes, proteins must be analyzed carefully because gene sequences alone do not predict post-transcriptional or post-translational modifications. Post-transcriptional modifications, such as alternative transcript splicing and RNA editing result in variation or modification of the primary amino acid sequence. In addition, many post-translational modifications (e.g. proteolytic processing, phosphorylation, glycosylation, etc.) can change the function and sub-cellular localization of proteins and their interaction with other proteins. Therefore, it is essential to measure these changes at the phenotypic level in order to understand the dynamics of biological reactions.

In this review, we describe a novel immunoproteomic assay that combines specificity of antibody capture with precision of mass spectral analysis. The assay provides a new level of sensitivity, speed and sophistication compared to other conventional methods of immunoprecipitation and 2D-gel analysis. This immunoproteomic approach has many potential advantages in speed, sensitivity and economy of reagents. The method allows a protocol to be developed that enables analysis and potentially quantitation of specific antigens in complex mixtures. In addition, the ability to monitor post-translational modifications in complex samples will be practical. This type of analysis will be of great value for the ultimate goal of understanding the dynamics of complex biological processes.

The next generation of immunoproteomic applications is likely to incorporate advances in antibody engineering, development of different capture reagents to allow use of a wider range of antibody species and other specific binding receptor molecules as well as improved quantitation techniques. The use of antibody-based proteomic systems offers the best opportunity to move from analysis of the proteome to an understanding of the physiome.

Acknowledgements

The authors would like to thank Emily Coyle, Christine Dorman, and Abby White for expert their technical assistance. This work was supported in part by Grants AI 50713, AI 53718, AI 53346, and HD 643171 from the National Institutes of Health.

References

[1] P.M. Harrison, A. Kumar, N. Lang, M. Snyder, M. Gerstein, Nucleic Acids Res. 30 (5) (2002) 1083.
[2] N. Anderson, N. Anderson, Electrophoresis 19 (1998) 1853.
[3] N. Anderson, A. Matheson, S. Steiner, Curr. Opin. Biotechnol. 11 (2000) 408.
[4] R. VanBogelen, K. Greis, R. Blumenthal, T. Tani, R. Matthews, Trends Microbiol. 7 (1999) 320.
[5] M.J. Dutt, K.H. Lee, Curr. Opin. Biotechnol. 11 (2) (2000) 176.
[6] M.P. Washburn, J.R. Yates III, Curr. Opin. Microbiol. 3 (3) (2000) 292.
[7] H. Zhu, M. Bilgin, M. Snyder, Annu. Rev. Biochem. 72 (2003) 783.
[8] E.F. Petricoin, L.A. Liotta, J. Nutr. 133 (Suppl 7) (2003) 2476S.
[9] C.J. Campbell, P. Ghazal, J. Appl. Microbiol. 96 (1) (2004) 18.
[10] S. Weinberger, T. Morris, M. Pawlak, Pharmacogenetics 1 (2000) 395.
[11] D. Drolet, R. Jenison, D. Smith, D. Pratt, B. Hicke, Comb. Chem. High Throughput Screen 2 (1999) 271.
[12] H.R. Hoogenboom, A.P. de Bruine, S.E. Hufton, R.M. Hoet, J.W. Arends, R.C. Roovers, Immunotechnology 4 (1) (1998) 1.
[13] C. Schaffitzel, J. Hanes, L. Jermutus, A. Pluckthun, J. Immunol. Methods 231 (1–2) (1999) 119.
[14] G. Winter, A.D. Griffiths, R.E. Hawkins, H.R. Hoogenboom, Annu. Rev. Immunol. 12 (1994) 433.
[15] K. Haupt, K. Mosbach, Trends Biotechnol. 16 (11) (1998) 468.
[16] H. Zhu, M. Snyder, Curr. Opin. Chem. Biol. 7 (1) (2003) 55.
[17] N. Tang, P. Tornatore, S.R. Weinberger, Mass Spectrom. Rev. 23 (1) (2004) 34.
[18] D.J. Cahill, E. Nordhoff, Adv. Biochem. Eng. Biotechnol. 83 (2003) 177.
[19] S.S. D'Costa, T.G. Romer, M.D. Boyle, Biochem. Biophys. Res. Commun. 278 (3) (2000) 826.
[20] T.G. Romer, M.D. Boyle, Proteomics 3 (1) (2003) 29.
[21] M. Saouda, T. Romer, M.D. Boyle, Biotechniques 32 (4) (2002) 916.
[22] M.D. Boyle, T.G. Romer, A.K. Meeker, D.D. Sledjeski, J. Microbiol. Methods 46 (2) (2001) 87.
[23] T. Romer, M. Rezcallah, M. Boyle, Recent Res. Dev. Mol. Microbiol. 1 (2002) 1.
[24] M. Boyle, E. Faulmann, D. Metzger, Applications of Bacterial Immunoglobulin-Binding Proteins to the Purification of Immunoglobulins, Plenum Press, New York, 1993.
[25] L. Faber, T. Romer, M. Boyle, Proceedings of the Jensen Symposium, Cincinnati, OH, 5–7 December, 2003.
[26] H. Holtmann, K. Resch, Naturwissenschaften 82 (4) (1995) 178.
[27] J.J. Oppenheim, Int. J. Hematol. 74 (1) (2001) 3.
[28] S.L. Reiner, R.M. Locksley, Annu. Rev. Immunol. 13 (1995) 151.
[29] G.E. Griffin, J. Antimicrob. Chemother. 41 (Suppl A) (1998) 25.
[30] S. Zanotti, A. Kumar, Expert Opin. Investig. Drugs 11 (8) (2002).
[31] A. Beishuizen, I. Vermes, C. Haanen, Adv. Clin. Chem. 33 (1998) 55.
[32] E. Morgan, R. Varro, H. Sepulveda, J.A. Ember, J. Apgar, J. Wilson, L. Lowe, R. Chen, L. Shivraj, A. Agadir, R. Campos, D. Ernst, A. Gaur, Clin. Immunol. 110 (3) (2004) 252.
[33] J. Buer, R. Balling, Nat. Rev. Genet. 4 (3) (2003) 195.
[34] S.H. Kaufmann, C.H. Ladel, Trends Microbiol. 2 (7) (1994) 235.
[35] N. Osterrieder, E. Wolf, Rev. Sci. Tech. 17 (1) (1998) 351.
[36] K. Reis, M. Boyle, in: M. Boyle (Ed.), Bacterial Immunoglobulin Binding Proteins, Academic Press, San Diego, CA, 1990, p. 165.
[37] R.S. Yalow, Endocrinology 129 (4) (1991) 1694.
[38] A. Ronald, W.H. Stimson, Parasitology 117 (Suppl) (1998) 13.
[39] J.P. Gosling, Clin. Chem. 36 (8 Pt. 1) (1990) 1408.
[40] J. Lill, Mass Spectrom. Rev. 22 (3) (2003) 182.
[41] H. Brzeski, R.A. Katenhusen, A.G. Sullivan, S. Russell, A. George, R.I. Somiari, C. Shriver, Biotechniques 35 (6) (2003) 1128.
[42] M. Kocakulak, A. Denizli, A.Y. Rad, E. Piskin, J Chromatogr. B. Biomed. Sci. Appl. 693 (2) (1997) 271.
[43] A. Denizli, M. Kocakulak, E. Piskin, J. Chromatogr. B. Biomed. Sci. Appl. 707 (1–2) (1998) 25.
[44] H.A. Chase, N.M. Draeger, J. Chromatogr. 597 (1–2) (1992) 129.
[45] M. Boyle, in: M. Cunningham (Ed.), Effects of Microbes on the Immune System, Williams and Wilkins, Lippincott, 2000, p. 195.
[46] R. Raeder, R.A. Otten, M.D. Boyle, Infect. Immun. 59 (2) (1991) 609.
[47] U. Sjobring, L. Bjorck, W. Kastern, Mol. Microbiol. 3 (3) (1989) 319.
[48] M. Tsivitse, M.D. Boyle, Can. J. Microbiol. 42 (11) (1996) 1172.

[49] J.M. Taylor, D.E. Heinrichs, Mol. Microbiol. 43 (6) (2002) 1603.
[50] M. Rasmussen, H.P. Muller, L. Bjorck, J. Biol. Chem. 274 (22) (1999) 15336.
[51] I. Ramshaw, A. Ramsay, G. Karupiah, M.S. Rolph, S. Mahalingam, R. Jc, Immunol. Rev. 159 (1997) 119.
[52] B. Modun, P. Williams, Infect. Immun. 67 (1999) 1086.
[53] S. Mahalingam, P.S. Foster, M. Lobigs, J.M. Farber, G. Karupiah, Immunol. Rev. 177 (2000) 127.
[54] M. de Chateau, L. Bjorck, J. Biol. Chem. 269 (16) (1994) 12147.
[55] M. Boyle, E.L. Faulmann, Application of Bacterial Expressing Immunoglobulin Binding Proteins to Immunoprecipitation Reactions, Academic Press, 1990.
[56] L. Rasmussen, D. Kelsall, R. Nelson, W. Carney, M. Hirsch, D. Winston, J. Preiksaitis, T.C. Merigan, J. Infect. Dis. 145 (2) (1982) 191.