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Immune Complex Proteomes: Tools for Vaccine Discovery

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World-wide in tropical and subtropical areas, approximately 200 million cases of malaria are reported annually with about 1 million deaths occurring in children under the age of 5 years. Data from proteomic studies are providing new strategies for identifying *Plasmodium falciparum* proteins with malaria vaccine and therapeutic potential. Purified native and recombinant proteins, and heterogeneous mixtures of proteins have been used traditionally for proteome analysis using 2-D gel electrophoresis (2DE) followed by the identification and excision of differential protein spots stained on sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels and analysis by mass spectrometry. The use of antibodies to add specificity to the identification of proteins analyzed by proteomics provides a practical benefit in small scale studies, in small laboratory settings, to identify changes in protein expression observed in normal and pathogenic states in humans. Antibodies can also be used to identify stage specific and developmental changes occurring in parasitic infections and in identifying expressed proteins of parasite origin present in human serum.

The need for additional levels of specificity in protein identification was recognized in the initial reports of protein identification by sequencing followed by database searches for unambiguous identification of correct proteins [1]. The use of antibodies; either unlabeled or tagged to capture antigens followed by mass spectrometry analysis using single-dimensional chromatography, surface-enhanced laser desorption/ionization time of flight (SELDI-TOF) or multidimensional protein identification technology (MudPIT) have enabled the identification of proteins, reducing the level of ambiguity that can be present in strictly gel-based systems [2-6]. Antibodies prepared as monoclonal or polyclonal antibodies to native proteins, monospecific affinity purified proteins, peptides or recombinant antigens can be employed in immune complex proteomics. Furthermore, the antibodies can be engineered recombinant antibodies identified in gene libraries or fractionated antibodies retaining Fab fragments that can interact with the epitopes of antigens [5]. The ability of the antibodies to be immobilized on sepharose or magnetic bead matrices conjugated to protein A, protein G, species specific antibodies or biotinylated proteins conjugated to avidin, facilitates the formation of immune complexes that can be digested and analyzed by mass spectrometry [3,4,7]. Antibody precipitation of proteins from mammalian cell lines, were analyzed by quantitative mass spectrometry using stable isotope labeling of amino acids in cell culture (SILAC) and used for protein identification [8]. Complementary procedures involving the use of other immunoassays such as western blotting [9] or immunodepletion assays to confirm protein identification before mass spectrometry has been used [10]. The use of patient sera to identify important protein targets with potential as therapeutic and diagnostic biomarkers known as Serological Proteome Analysis (SERPA) is a significant application of antibody based detection of proteins, using western blotting and 2DE combined with proteomics analysis [11]. Immune complex (IC) proteomics is finding increased application in large-scale protein identification for complex proteomes [12]. Antibody-based protein profiling by proteomics has potential uses in diagnostics, vaccine and biomarker discovery.

The use of negative control serum alongside polyclonal, monoclonal and engineered recombinant antibodies in immunoprecipitations or affinity purification protocols is important to enable discrimination of nonspecific contaminating proteins in the assay [6]. The challenge to protein identification, using strictly gel-based systems results in inconsistencies when identifying rare or low abundant proteins. Also, inadequate protein spot excision or elution of proteins from gels, poor protein migration within gels due to protein size or post-translational modification can occur, leading to inadequate or poor recovery of proteins for analysis. The application of antibodies to aid the specificity of protein identification followed by protein elution for mass spectrometry provides an additional critical level of verification for the proteins identified. Hess et al. [4] describe the use of ProACTR, a heat-killed group G Streptoccocal isolate prepared as a 10 % suspension of bacteria, used to immunolocalize antibodies by binding to the Fc region of the antibody molecule. A gene family associated with the Maurer's clefts in *P. falciparum* was identified by immune complex proteomics using monoclonal antibodies [3]. MudPIT was employed to analyze peptides generated from IC formed using monoclonal antibodies (MAbs). Mabs were prepared in Balb/c mice using 1% SDS extracts of mature schizont pellets [13], and the antibodies were immobilized on goat antimouse sepharose beads followed by antigen capture from schizont extracts to form IC [3]. Following database matches of IC peptides to *P. falciparum*, human, mouse and rat protein sequences, the genes encoding the proteins complexed with Mabs were identified [3]. Proteome based profiling represents a powerful way to identify functional domains, regions, or motifs, within the protein sequence with the use of gene-mining techniques. While proteome analysis identifies proteins, defining the function for the proteins, knowing the protein distribution within the cell and identifying functional multi-protein complexes remains challenging. Expression of proteins such as biomarkers, in human infections by pathogens, changes in cellular protein expression in pathogenic states in response to infection, changes in cytokine protein expression and changes in autoimmune disease and other chronic disease states can be identified using antibodies [14]. Moreover, if functional proteins occur in multi-protein complexes or if new protein interactions develop, co-immunoprecipitation using specific antibodies will enable identification of proteins. IC proteomics using Mabs bound to their cognate antigen as the starting sample for MudPIT analysis enabled the identification of genes encoding the

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Maurer's cleft gene family *PfMC-2TM*, with gene paralogs identified among *P. falciparum* strains [3].

IC proteomics can be used to query protein-protein interactions in cell signaling pathways and receptor-ligand interactions in host cell-pathogen associations. Functionally interacting proteins complexed together can be precipitated with antibodies and analyzed by proteomics resulting in gene identification. The use of IC to analyze the proteome extends the use of antibodies beyond merely tools to detect proteins and allows for genes and gene families to be identified. The ability to identify genes encoding proteins using specific antibodies makes this technique highly versatile. Antibodies are employed for screening gene libraries to detect proteins, followed by gene cloning for gene identification. Use of IC to characterize proteomes provides two important advantages; 1) the antibody concentrates the specific protein (s) as immune complexes are formed and 2) if the specific (cognate) protein is also bound to other proteins in a multi-protein complex, the antibody will co-precipitate the complex and all the proteins can be analyzed by mass spectrometry techniques such as MudPIT in shotgun proteomics in one tube. The genes encoding the proteins can then be identified from the peptides generated. The technique saves time and can be scaled up to identify multiple genes in a microarray format. The use of specific antibodies will help clarify cases of ambiguous protein and gene identifications because the peptides used for identification are generated from proteins specifically bound to antibodies. Background contaminating proteins can also be identified. The technique can be applied to protein profiling when the proteome cannot be analyzed using conventional gene cloning, sequencing techniques or gel-based techniques. The technique can be optimized by evaluating differences in the types of matrices (beads) used for "pulling down" the IC following antibody precipitation with protein and types of buffers used for protein elution. Additionally, negative controls provide a means of delineating specific proteins bound to antibodies from background contaminating proteins. IC proteome analysis is highly feasible and a faster alternative to standard gene identification methods, when specific antibodies are available. IC proteome analysis also provides more detailed information about the genes once they are identified. Antibodies highly specific for disease antigens, can confirm gene identifications. Post-translationally modified proteins, rare proteins, intermittently expressed proteins and proteins expressed in response to external perturbations, in stage specific or developmental stage analysis can be identified using the IC technique. Agarose and sepharose conjugated species specific antibodies (anti mouse, anti-rabbit or antihuman antibodies) can be optimized for the "pull down" of IC formed using mouse, rabbit or human antibodies for the identification of individual and multi-subunit proteins or protein complexes.

In the design of experiments for IC proteomics, the experimental objective (s) or hypothesis being tested is crucial in determining the type of antibody to be employed for immunoprecipitation as well as the type of solid matrix to be used. The specificity of antibodies should be validated by immunoassays, in particular, by western blotting and immunoprecipitation to confirm that the IC can be formed following incubation of the antibody with protein extracts or antigen sources. The enrichment of the proteins bound to specific antibodies as the proteins are depleted from extracts is a crucial step that will decrease the detection of contaminant and non-specific proteins from the sample of the IC analyzed. This will ensure that the peptides generated are from the most abundant proteins; those proteins specifically bound to the antibodies as well as bona fide co-precipitated proteins. Additionally, protein contaminants should be minimized by judiciously determining if additional proteins such as bovine serum albumin, gelatin or milk

proteins are necessary in wash buffers. Stringency in wash steps should be maintained in cases where individual specific proteins are to be analyzed. In contrast, when cross-reactive antibodies are used, stringency may be relaxed with the caveat that background protein contamination may be increased in the IC to be digested for proteome analysis. The reproducibility of polyclonal antibody batches collected following immunization-boost protocols should be verified and the protocol for protein extractions should be standardized to ensure reproducible amounts of antigen for immunoprecipitations. A lack of specificity and sensitivity of antibody in immunoassays will be compounded by poor reproducibility of the proteome analysis. This can affect the individual protein or heterogeneous protein mixtures subjected to proteome analysis following IC formation [2,6].

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