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How to Nail Down Trace Proteins in any Sample

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Abstract

Proteins present in most biological mixtures are expressed over a vast concentration range (up to 10–12 orders of magnitude in human sera), the most abundant ones complicating the detection of low-abundance species or trace components, Classical approaches, such as pre-fractionation and immuno-depletion methodologies are frequently used to remove the most abundant species. Unfortunately, these methods not only are unsuccessful in concentrating trace components, which could remain below the detection limits of analytical approaches, but also may cause a non-specifically depletion of other components (including low abundance ones). In case of immuno-depletion, the situation is less than brilliant: untargeted proteomic analyses using current LC–MS/MS platforms with immuno-depletion cannot be expected to efficiently discover low-abundance, disease-specific biomarkers in plasma, since the increment in detection of these trace components after such a treatment, results in a meagre 25% increase, accounting for only 5–6% of total protein identifications in depleted plasma. The characterization of minor components in complex protein systems, has been revolutionized by the introduction of the combinatorial peptide ligand libraries technology. This more recent methodology is based on the use of hexa-peptide baits to capture and normalize the relative concentrations of the components of any proteome under investigation. The major advantage of this technique, in comparison with other pre-fractionation methods, is that it not only diminishes the concentration of the more abundant proteins, but also concentrates low-abundance and even trace components, thus providing access to the “invisible” proteome. In addition, the loss of low-abundance species that may be accidentally eliminated by co-depletion using immuno-subtraction methods, is avoided.

Keywords: Glycoproteins; Phosphoproteins; Biological Proteins

The Problems with Current Depletion Methods

In a number of cases the removal of high-abundance proteins in view to evidence possible biomarkers is operated by immune-depletion using specific solid-phase antibodies against the proteins to be suppressed. The method is quite effective; however, it suffers from a vicious circle which starts from the use of small volumes of expensive immunosorbents which accept only small samples to deplete. In small samples the amount of targeted low-abundance protein is very low while the process itself contributes to their dilution, thus rendering the detection even more challenging without any post concentration. Naturally concentration is possible but it contributes to protein losses. Immunosorbents are also limited for sample treatment due to their species specificity; moreover, immunosorbents available for proteomics depletion are restricted to the treatment of only human blood plasma. Conversely the use of enrichment methods based on solid-phase adsorption of targeted group species (e.g., glycoproteins, phosphoproteins and other classes) or the use of solid-phase combinatorial affinity ligands are by far more effective since they allow much larger initial biological samples and hence larger quantities of targeted low-abundance proteins. The Combinatorial Peptide Ligand Library (CPLL) is a technology for sample treatment that repeatedly demonstrated its capability to allow detecting proteins that are most of the time ignored because well below the level of sensitivity of proteomics equipment and methods. It is additionally of general use for various biological material and various sources (animal, plant, cell extracts). This original procedure, that takes its origin from affinity chromatography mechanisms, when used under overloading conditions, contributes not only to improve the knowledge in proteomics but, more importantly, to detect dilute proteins that are expressed at the early-stage of metabolic diseases. It is after years of applications in various conditions and various sample situations that low-abundance protein detection by CPLL in early-stages of diseases is gaining momentum as a potential discovery of new protein markers allowing the design of diagnostic tools. Explanation of the mechanism of action is given in the following sections as well as examples of detection of panels of exclusive low abundance proteins present in various diseases as well as contaminating host cell proteins from purified recombinant biopharmaceuticals.

This peculiar procedure when used under large overloading conditions, contributes not only to improve the knowledge in proteomics but, more importantly, to detect dilute protein from any biological extract.

Proteins and their variants are produced in a very large number and their individual concentration difference is extremely large, ranging throughout at least a dozen of orders of magnitude if not more. This situation renders the detectability of low- and very low-abundance species very challenging or clearly impossible in practice. Without any kind of sample treatment, the large majority of proteins cannot be detected because their concentration is either below the detectability levels or because their signal is suppressed by the presence of most abundant proteins. The use of enrichment methods based on solid-phase adsorption of targeted species or groups (e.g., glycoproteins, phosphoproteins and other classes) or the use of solid-phase combinatorial affinity ligands are very effective since they allow much larger initial biological samples and hence larger quantities of targeted low-abundance proteins. They have demonstrated their capability to enlarge significantly the knowledge of protein diversity content from samples of animal origin (blood, urine, milk, tears, saliva, tissue extracts), of plant origin (leaves, roots, fruits, seeds, exudates) and of microorganism origin) attested by thousands of published scientific reports.

Condensed Explanations of CPLL Mechanism of Action

A combinatorial peptide ligand library (CPLL) is a quite recent technology now extensively described for successful applications in animal and plant proteomics investigations. Its utilization has been reported with a major interest in the discovery of low- and very low-abundance proteins that are undetectable even after the use of immuno-depletion of major species. In practice the CPLL procedure allows compressing the dynamic concentration range of protein components by simultaneously decreasing the concentration of high-abundance species and enriching for low- and very low-abundance ones (for reviews see references). This concept has been coined several years ago and since then had not reduced its interest for many applications including the discovery of markers of diagnostic and prognostic interest. The library is composed of millions of spherical gel porous beads each of them covalently carrying many copies of a single hexapeptide structure. The library is made via a combinatorial synthesis process that uses natural amino acids grafted the one after the other (split-and-pool procedure). Each bead can be considered as an affinity chromatography sorbent addressing a single or a group of proteins from a crude biological sample with a common affinity for the same peptide structure. Considering that the mixture of beads carries millions of different affinity beads with millions of diverse hexapeptides, most, if not all, proteins are adsorbed. Under large overloading sample conditions, concentrated proteins (high abundance species) saturate rapidly the corresponding affinity

beads while the excess remains free in solution. Conversely very dilute proteins (very low-abundance species) converge towards their specific beads and are thus concentrated. Upon completion of the binding process, dominated by not only adsorption, but also by quite intensive displacement effects, the beads are washed and all proteins in solution, mainly the excess of large abundance proteins, are eliminated. The adsorbed proteins are then desorbed using dissociation compounds such as those adopted in affinity chromatography; the collected sample thus comprises all captured proteins where their respective dynamic concentration range is much reduced. In this sample low-abundance proteins are detectable because first they are concentrated by the affinity process and also because their signal is not any longer obscured by the high-abundance species that are now largely diluted. The intense competition effect among proteins during the adsorption phase is the result of numerous molecular interactions singularly or collectively present generated by the mixed-mode affinity ligand library (the peptide). Among them are hydrophobic associations, electrostatic interactions and hydrogen bonding. The interaction forces are governed by the mass action law for systems that associate together by molecular affinity; the association and dissociation of partners depend on environmental conditions such as the pH, the ionic strength of the buffer, the temperature, the presence of competitors, their concentration and the extent of overloading. All these physicochemical parameters need to be considered with care in order to get the maximum reproducibility between samples. The nature of the molecular interaction allows also to design the best desorption buffer for protein harvesting. The two major success factors of the process are (i) the enrichment of low abundance species, which is dependent on the availability of biological sample (the larger the sample, the higher level of enrichment) and (ii) the ability to desorb all proteins captured by the beads. In comparison to the so-called "depletion" or "immune-depletion" technologies, CPLLs show large distinctive characteristics. While depletion does not concentrate the low-abundance proteins, the CPLL main property is to concentrate most of very dilute species to bring them to the level of detectability by current analytical methods. High abundance proteins are not eliminated as this is the case with depletion methods, but rather maintained at a certain level of concentration conserving thus the property to carry other interacting polypeptides that are consequently not co-depleted as it can be the case with immunosorbents. To reduce the risk of protein losses due to non-specific binding on solid supports, adsorbed proteins need a complete elution using various appropriate dissociation methods harvesting thus all adsorbed proteins. Alternatively, after extensive washings the beads loaded with proteins can be directly trypsin zed in order to produce peptides that are collected and streamlined within a LC-MS/MS equipment for protein identification. This second approach is very convenient for identification of all protein components from the sample, but less applicable in differential expression analysis when attempting to detect protein markers.

Detection of Low-Abundance Proteins from Early-Stage Gene Expression

It is after years of applications in various conditions and various sample situations that low-abundance protein detection by CPLL in early-stages of diseases is gaining momentum as a potential discovery allowing the design of diagnostic tools. A large number of pathologies including cancers, are associated with a mis-regulation or the expression of modified polypeptides. These changes can be taken as signatures for diagnostic confirmation. Certain proteins are up-regulated and others are down-regulated creating situations where panels of proteins can be used all together as a marker of a pathological situation. Mis-regulation of protein expression is an evolutive process that depends on the size of a cancer (or the number of cancer cells). In other worlds, the extent of expression is progressively more pronounced as the pathology makes progress. At the beginning this phenomenon is characterized by an extremely small modification of the expression and novel proteins are considered as early-stage markers. They are by definition of low abundance compared to all the bulk of other current proteins and are extremely difficult to detect if not specifically targeted or treated by an appropriate enrichment process.

In a large number of situations, the disease-related protein markers are expressed at a too low rate to be detected by current ELISA-based assays or even by other proteomics methodologies. The massive presence of other bulk proteins masks the signal of very dilute novel proteins precluding any possibility to make a proper comparison with a healthy biological sample. In all cases the benefit of an enrichment process based on CPLL makes a full sense.

A large scientific literature survey tends to demonstrate that the approach is rich in discoveries. Numerous published reports discuss the discovery of proteins panels after CPLL treatment of biological samples from specific pathologies. Cancer taken as a generic word is probably the most representative example with multiple reports for the characterization of hepatocellular carcinoma, breast cancer, pancreatic cancer, ovarian cancer, cervical carcinoma, colorectal cancer and lung cancer, just to mention a few.

Beyond cancer, the use of combinatorial peptide ligand library has been described as very effective to evidence biomarkers of other pathologies such as atherosclerosis, rheumatoid arthritis,

Duchenne muscular dystrophy and many others. In summary a number of recent studies suggest that low-abundance multi-marker discovery pave the way to design new diagnostic tools and to implement predictive medicine for the coming years. It may in addition develop approaches that would be more patient-centered therapies.

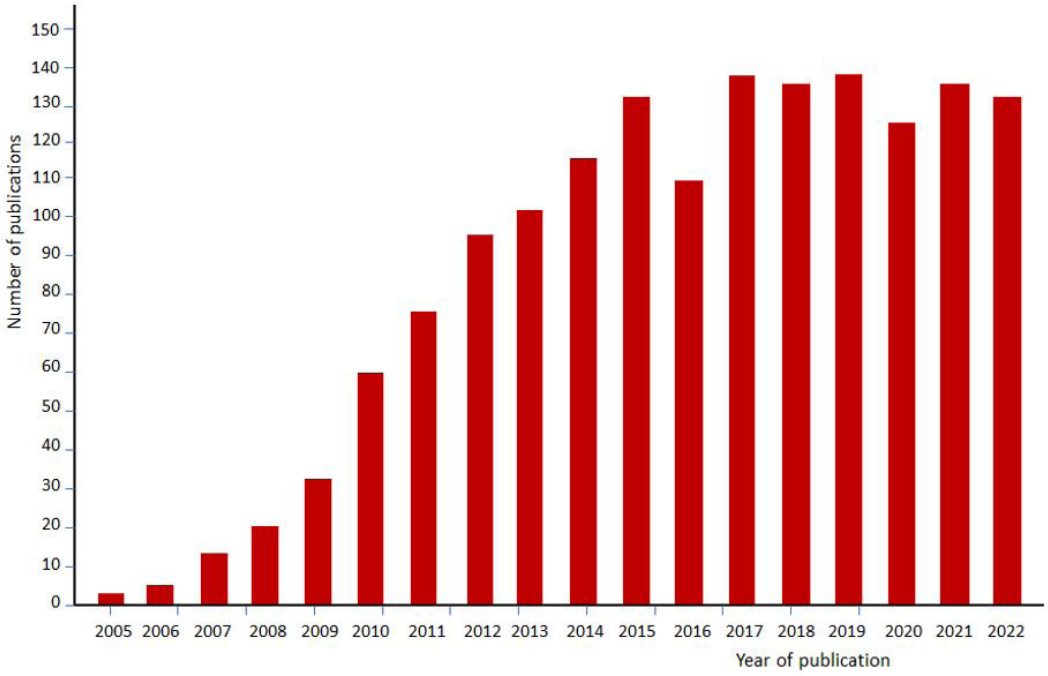
Statistical calculations demonstrate that CPLL technology is more and more applied to this end. Fifteen years ago, about 12% of published reports using CPLL were dedicated to the pathological marker discovery, while at present this goal represents almost 60% of total scientific paper describing the use of CPLL.

Detection of Host Cell Proteins (HCP) from Biopharmaceuticals

Host cell proteins are polypeptides that are commonly found in biopharmaceuticals as by-products of the entire production by recombinant technologies such as cultures of living cells. They are currently eliminated during the overall downstream processing because they are considered as possible components that may compromise the quality and safety of the final biopharmaceuticals if still present even in trace amount. They are thus naturally considered as low-abundance proteins. To validate the efficacy of their elimination, specific and sensitive analytical assays are devised. ELISA-based assays are commonly adopted to check that HCP are completed absent from the final purified bioproduct; however, these analytical determinations are able to detect only proteins corresponding to the antibodies for which the assay is designed creating a biais. Thus, any other possible host cell protein present escape the detection. Alternative methods are proposed such as LC-MS/MS. They provide a bulk HCP identification while giving a picture of their relative abundance. Nevertheless, this approach has the limitation described above due to the very low amount of HCP in the final purified bioproduct. To implement an effective detection of HCP by LC-MS/MS, enrichment strategies are proposed. As repeatedly described, the integration of combinatorial peptide library enrichment mass spectrometry methods allows detecting low-abundance HCP impurities in the presence of highly concentrated recombinant proteins even within an extremely large dynamic concentration range. In the recent years this relatively old approach described for the first time a dozen of years ago arouses a large scientific interest as witnessed by a growing number of published reports. The enrichment of HCP present in the final biopharmaceutical is most generally followed by a direct shotgun MS proteomic analysis. This is easy to apply all along the purification process including the final purity check; it can be effectively used in association to ELISA based assays using an appropriate pool of antibodies.

Expected Upcoming Developments with CPLL

Recent technological developments about identifying early-stage modifications of protein expression for various critical pathologies are a promise for a great future. Statistical observations of low-abundance proteins expressed during the development of some cancers will improve the reliability of selection of marker candidates (Figure 1).



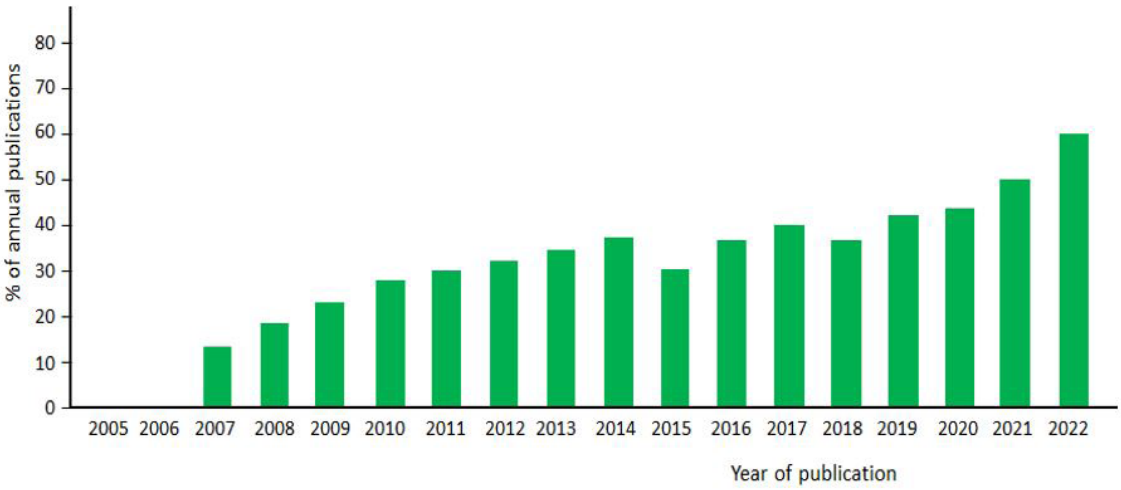


Figure 1: Progression of the number of publications over years mentioning the use of CPLL. Upper panel: representation of the number of published papers from 2005 to 2022. Lower panel: progression of published reports on CPLL evidencing or mentioning their use within the domain of biomarker discovery. Each bar is expressed in % of the total number of published papers shown in the panel above

Post-translational modifications such as truncations, mis-glycosylations, mistaken phosphorylations and others, that are also tracked as potential biomarkers, could eventually be circumvented if the enzymes that are at the origin of such modifications are identified as very low-abundance proteins that are dependent on a bad or modified regulation of the expression system. Although the performance of CPLLs has largely contributed to the progress of novel discoveries, other complementary approaches associated to modern and more sensitive equipment, will increase the probability of novel reliable and affordable findings (Figure 2).

On CPLL technology itself possible developments are envisioned that could be advantageously associated to specific enrichment technologies. Thus, the general enrichment governed by this multi-affinity principle could be enhanced by adding to the libraries various accurately selected adsorbents in order to either increase the low-abundance species or to further enrich a special group of proteins. Main principles for this approach were already suggested in 2015. The mode of use of CPLLs could also be progressively normalized as a function of the type of biological samples. For the discovery phase of novel biomarkers, two main general routes are presently used: (i) the direct comparative expression difference of previously fluorescently labelled samples by 2D-DIGE separation analysis and (ii) the indirect comparative tryptic digests of enriched samples, classified as bottom-up approach.

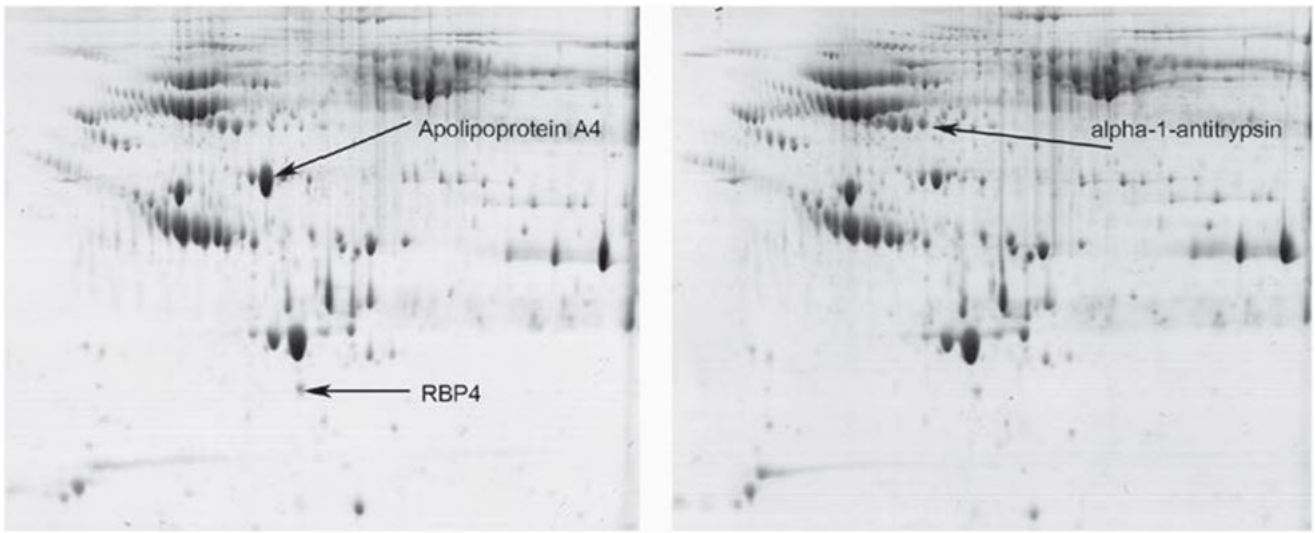


Figure 2: Two-dimensional polyacrylamide gel electrophoresis of serum from healthy women (left panel) and from epithelial ovarian cancer (right panel). The first-dimension separation was performed by using a relatively narrow pH gradient from 4 to 7. Both serum samples were treated by CPLL. Three spots of significantly different density between the groups were found (see arrowed indications) and then identified by MALDI mass spectrometry

Both are more rapid than other intricate protein capture methods and multiple sequential elutions from beads followed by technological clean-up and/or fractionation methods with the additional risk of protein losses. Although to date the return from massive efforts in proteomics is quite scarce in terms of diagnostic tests, the search for early-stage protein expression modifications continues. The acceleration of exploitable results in view of bringing findings to clinical practice is contingent upon deep collaborations between laboratories having complementary skills and also complementary interests including industrial organizations as well as bio-banks and clinicians. In this endeavour it is believed that CPLLs as are described or enriched by additional features may contribute to novel discoveries for early-stage potential protein biomarkers allowing differentiation of patient's subgroups to fit with the current trends in personalized medicine.

Conclusion

In the early years of CPLL applications, we felt the growth of the methodology was in a stage of "Andante moderato", like in the second theme of the third movement of the famous Symphony No. 9 by Ludwig van Beethoven (LvB). Yet, as the years went by, and as witnessed by the graph in Figure 1, it would appear that now CPLLs have reached the stage of "Andante maestoso", as in the fourth movement of the Symphony, which ends with the Hymn to Joy from Friedrich Schiller (FS). It is hoped that more and more scientists will pick up the technique, given its high performance and maturity.

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