

The Powerful Combination of Phage Surface Display of cDNA Libraries and High Throughput Screening

Reto Cramer^{1*} and Rimantas Kodzius^{1,2}

¹ Swiss Institute of Allergy and Asthma Research (SIAF), Obere Strasse 22 CH-7270 Davos, Switzerland

² Max Planck Institute of Molecular Genetics, Ihnestrass 73, D-14195 Berlin, Germany

Abstract: Phage surface display of cDNA libraries facilitates cloning, expression and rapid selection of functional gene products physically linked to their genetic information through gene product-ligand interactions. Efficient screening technologies based on selective enrichment of clones expressing desired gene products allows, within a short time, the isolation of all ligand-specific clones that are present in a library. Manual identification of clones by restriction analysis and random sequencing is unlikely to be successful for the isolation of gene products derived from rare mRNA species resulting from selection of the libraries using polyvalent ligands like serum from patients. Here we describe rapid handling of large numbers of individual clones selected from molecular libraries displayed on phage surface using the power of robotics-based high throughput screening. The potential of the combination of cDNA-phage surface display, with selection for specific interactions by functional screening and robotic technology is illustrated by the isolation of more sequences potentially encoding IgE-binding proteins than postulated from Western blot analyses using extracts derived from raw material of complex allergenic sources. The subsequent application of functional enrichment and robotics-based screening will facilitate the rapid generation of information about the repertoire of protein structures involved in allergic diseases.

INTRODUCTION

Expression of cDNA libraries using various systems including bacterial viruses such as λ -phage derivatives has been used as a tool to clone genes through protein-protein interactions since the first days of genetic engineering [1, 2]. The general ability of λ lysogens to express gene products from cloned inserts has been widely exploited to enhance the sensitivity and efficiency of antigen screening [3]. Historically, the first combinatorial antibody libraries, later constructed and displayed almost exclusively on filamentous phage surfaces [4-6], were based on λ phage vectors [8, 9]. Therefore, molecular cDNA libraries as bodies of molecules designed to deal simultaneously with large numbers of single clones are per se not novel. The construction of a cDNA library in lysogen λ phage derivatives permits the

growth, induction, and lysis of antigen-producing cells directly on nitrocellulose filters [2].

Thereafter, the expression libraries can be screened for appropriate translation products by using either functional [10, 11] or immunological assays [12, 13]. Although classical λ vector-based cloning approaches have been extremely successful in cloning cDNAs from different origins, the technology suffers from practical limitations [14]. The strongest one results from the lack of a physical linkage between phenotype and genotype. In λ -based libraries, the cloned inserts, integrated into the phage genome, and the translated gene products, expressed exploiting the machinery of the host cell during the lytic process, represent functionally independent units. As a consequence, this kind of library requires immobilization to solid phase supports for screening [1, 3] and, obviously, immobilization of a library hampers selective enrichment of clones by specific gene-product/ligand interaction [15, 16]. Approaches devoted to solve these problems are reviewed elsewhere in this issue [17]. In

*Address correspondence to this author at the Swiss Institute of Allergy and Asthma Research (SIAF), Obere Strasse 22, CH-7270 Davos, Switzerland, Telephone +41 81 420 07 31, FAX +41 81 410 08 40, e-mail crameri@siaf.unizh.ch

contrast to classical cloning systems, the pivotal work of Smith [18] showed that peptides can be displayed on the surface of filamentous phage capsids by genetic fusion of the encoding sequences to capsid genes. Thus, cloning systems based on filamentous phage fulfill the basic requirement of linking genotype as genetic information integrated into the phage genome to the phenotype as gene product displayed on the phage surface [19-21]. Since peptides or proteins displayed on phage surface are exposed to solvent, phage carrying fusion proteins can be separated by affinity purification from a large excess of uncognate phage. This is achieved by incubating the phage mixture in the presence of a ligand immobilized to a solid phase [22, 23]. The basic concept and different possibilities for linking genotype and phenotype by phage surface display technology are depicted in Fig. 1. This field of molecular display technology has developed rapidly from display of random peptide libraries used to select agonists or antagonists of natural ligand-receptor interactions [24-26] to more complicated applications [27, 28]. To date, combinatorial antibody and peptide libraries represent the most important applications of phage surface display technology [26, 29-32].

Spectacular advances in increasing the size of phage antibody libraries, which reach now a diversity of $>10^{11}$ different molecules [7], allow the isolation of high-affinity antibodies to virtually any antigen [33]. However, other important applications have clearly demonstrated the general applicability of linking phenotype and genotype as a rational approach to screen molecular libraries. Enzymes [34-37], enzyme inhibitors [38-40], receptors [41], hormones [42, 43], lymphokines [44] and DNA binding proteins [45-47] have been displayed on filamentous phage. Therefore, phage surface display technology can be considered as one of the most important developments in modern molecular biology. The selection format based on biopanning has proven to be very efficient for isolation of phage displaying proteins with affinity for discrete ligands. However, if complex ligands are used to screen complex libraries biopanning, affinity selection and identification of clones by restriction analysis and random sequencing represent limiting steps [48]. To enable the characterization of all clones present in an enriched library in a fast and effective way, high-throughput screening technology is required (see below).

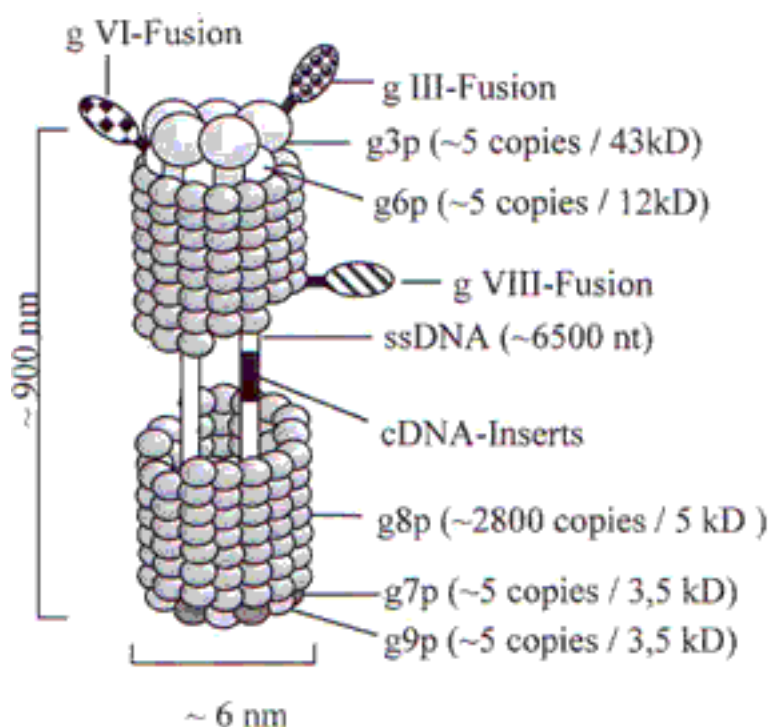


Fig. (1) Linkage of genotype and phenotype by phage surface display technology. Genomic or cDNA libraries can be constructed by fusion to the gVI gene of filamentous phage. However, fusions to gIII and gVIII are not directly possible and require linkage through protein-protein interaction (modified from ref. [104]).

DISPLAY OF cDNA LIBRARIES ON PHAGE SURFACE

As demonstrated for random peptide libraries [22, 23] and combinatorial antibody libraries [7, 19, 49], phage surface display can be used to generate large libraries containing a high diversity of different molecules. The diversity of these libraries is based on randomization of the codons determining the amino acid sequences of short peptides [50], PCR amplification of natural structures like VH and VL antibody fragments [6], or combinations thereof [31, 51]. In the most general term, the total set of phage-displayed molecules can include the diversity of structures present within any expressed cDNA library. Randomly fragmented prokaryotic genomic libraries lacking introns [52] behave functionally similar to cDNA libraries when expressed on phage surface and are discussed elsewhere in this issue [53]. Because the most common approaches used to link phenotype and genotype employ amino-terminal fusion to the gene III or VIII products of filamentous phage, they are unsuitable for surface expression of cDNAs bearing stop codons [14, 54]. However, this problem has been solved using different strategies. Fusion of cDNAs to the C-terminus of the phage VI gene (Fig. 1) is compatible with phage propagation and packaging [55] and a cDNA library has been generated and screened using this approach [56]. More sophisticated approaches are based on the ability to separate the gene III product of filamentous phage into two functional domains [Fig. 2]: 1) The N-terminal domain binding to the F' pili which is responsible for the infectivity of the phage, and 2) the C-terminal domain morphogenically involved in capping the trailing end of the filament according to the vectorial polymerization model [57, 59]. Although cleavage of the gene III product into

these separate functional entities is incompatible with phage propagation, infectivity can be restored by joining the segments through noncovalent protein-protein interactions [60-61]. The basic concept of this technology termed SIP for selectively infective phage [62] shows great potential for future developments. As demonstrated in a few cases, SIP can be efficiently used to screen cDNA libraries for selection of proteins within a library that interact with a target molecule [63, 64]. However, the most widely used vector for direct selection of gene products linked to their genetic information from phage surface displayed cDNA libraries is based on the strong interaction between the *c-fos/c-jun* leucine zippers [14, 48, 54]. Although most of the libraries constructed and screened using the pJuFo vector (Table 1) were devoted to the isolation of IgE-binding molecules from complex allergenic sources, many other applications have been reported. For example, mapping of protein-ligand interaction using whole genome phage display libraries [65], construction of vectors for stable immobilization of multimeric recombinant proteins [66] and detailed analysis of the C5a anaphylatoxin effector domain [67-70]. Furthermore, selection of a C5a receptor antagonist attenuating the inflammatory response in immune complex disease has been successfully performed [71], demonstrating the versatile applicability of the cloning system. The methodology used to construct the pJuFo vector has been reported elsewhere [14, 48, 51, 72] and does not need to be discussed in detail here. Briefly, the Jun leucine zipper [73] was fused to the N-terminus of the pIII coat protein of filamentous phage, thus anchoring Jun outside of the phage coat through the free N-terminal end of a truncated pIII product [14]. cDNAs were decorated with the Fos leucine zipper [73] by fusion to their N-terminal ends [14]. Both

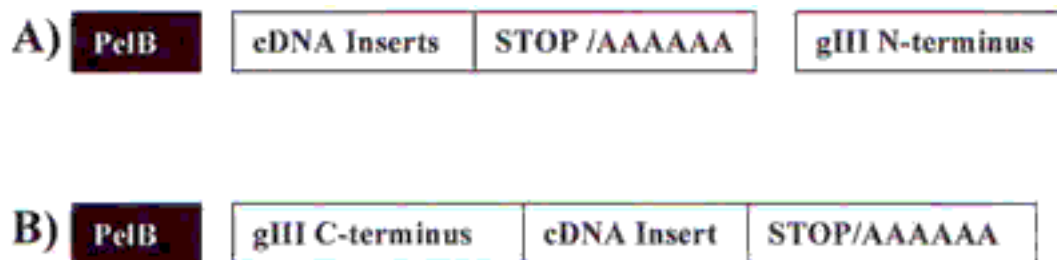


Fig. (2) Schematic structure of the gIII gene of filamentous phage. A) Fusions N-terminal to the gIII gene product are not possible due to the presence of translational stop codons. B) C-terminal fusions to gIII are possible but block the incorporation of the fusion into the phage coat.

Table 1. cDNA Libraries Displayed on pJuFo and Screened with Different Ligands

cDNA source	Ligand	References
<i>Alternaria alternata</i>	human serum IgE	[48]
<i>Aspergillus fumigatus</i>	human serum IgE	[15, 82, 90]
<i>Candida albicans</i>	human serum IgE	[105]
<i>Cladosporium herbarum</i>	human serum IgE	[48, 77]
<i>Coprinus comatus</i>	human serum IgE	[77, 83]
<i>Malassezia furfur</i>	human serum IgE	[77, 78]
Peanut	human serum IgE	[77, 79]
Storage mites	human serum IgE	[106]
Wheat germ	human serum IgE	[107]
Human lung tissue	human serum IgE	[77, 108]
Human peripheral blood lymphocytes	Staphylococcal protein A	[48, 72]
	HIV reverse transcriptase	[64, 72]
HeLa cells	human serum IgE	[109]
<i>E. coli</i> chromosomal DNA	polyclonal mouse serum	[65]
Celery	human serum IgE	[48]
Chloroplasts	polyclonal rabbit serum	[48]

functional entities, Jun/pIII and Fos/cDNA, were placed under control of separate lac promoter/operator elements of phagemid pComb3 [5] and directed to the periplasmic space of *Escherichia coli* by *pelB* signal sequences [14]. During helper phage superinfection, the modified Jun/pIII product becomes structurally incorporated into phage particles, providing a covalent link for Fos-decorated cDNAs. Therefore, complex cDNA libraries can be displayed on phage surface and screened using the discriminative power of affinity purification commonly used in screening of molecular libraries displayed on phage surface.

ENRICHMENT OF PHAGE SURFACE-DISPLAYED cDNA LIBRARIES

Filamentous phage cDNA surface display libraries will be subject to the same constraints limiting the transport and secretion of proteins in such phage systems. All virion proteins in filamentous phage must undergo transport into the *Escherichia coli* host cell periplasm prior to assembly and extrusion from the host as assembled viral particle [74]. This may actually be beneficial

for promoting the correct folding of certain foreign proteins, deleterious, however for some others. Part of this problem might be solved using display systems in bacteriophage λ , which has a lytic or lysogenic life cycle (reviewed elsewhere in this issue [17]). In turn, incorporation of large proteins into λ capsids seems to be limited [75, 76], and it is unlikely that a single phage surface display system will be compatible with all proteins that could theoretically be displayed. However, enrichment of libraries constructed in pJuFo using cDNA from different complex allergenic sources with serum IgE from allergic individuals has proved to be extremely successful for the isolation of IgE-binding molecules (Table 1). The repertoire of structures detected so far covers secreted, cytoplasmic and surface proteins with or without enzymatic activity as well as structural and storage proteins [77]. These results indicate that phage surface display allows expression of a wide range of proteins with different biochemical properties. Selection is accomplished by interaction between the displayed gene product and solid phase-immobilized serum IgE, and the selected phage are amplified by infection of *E. coli* cells which, after helper rescue, produce large numbers of new phage [5]. Usually, after five rounds of selection and

amplification, 100% of the phage tested display IgE-binding proteins [78, 79] and, as a consequence of the physical linkage between gene product and genetic information, carry the encoding cDNAs. Obviously, sequencing the cDNA of the integrated section of the phage genome can readily elucidate the amino acid sequence of the displayed gene product allowing fast identification of the allergens present in enriched libraries. However, single clones enriched from complex allergenic systems using a polyclonal ligand like serum IgE are expected to be multiply represented in the large phagemid population selected [54]. The absolute number of clones displaying the same gene product will depend on both the number of clones displaying the product present in the original library and the number of allergen-specific IgE molecules present in the serum pool used for screening. Therefore, identification of clones encoding allergens derived from rare mRNA species or from allergens eliciting weak IgE responses and thus resulting in low amounts of allergen-specific IgE in serum among billions of phage obtained after several rounds of selection and amplification remains a major problem. To enable characterization of all clones of an enriched cDNA library in a fast and cost-effective way, high-throughput screening technology is required (see below). However, the success in screening of cDNA libraries displayed on phage surface will depend from several parameters influencing the selection of targets with affinities to given ligands immobilized on solid phase [80, 81]. In general, the known or expected characteristics of the ligand will dictate the screening procedure used. The most important sources of error and bias related to screening of phage surface displayed libraries have been discussed considering detailed probabilistic descriptions of every laboratory step used in phage selection [81]. According to these calculations the affinity between IgE and allergen is high enough to allow efficient enrichment of allergen displaying phage, assuming that the amount of allergen-specific IgE present in serum is not a limiting factor. However, serum IgE represents a heterogeneous ligand with affinity to whole arrays of allergens [48, 82, 83], where the molar ratio of ligand raised against a given allergen is unknown [72]. Therefore, if the concentration of allergen-specific IgE against a single allergen in the serum used for screening is high, the allergen will

be selectively enriched. On the other hand, allergens for which the ligand (i. e. the number of IgE molecules in serum) is limited will not be enriched from the library and either be lost during biopanning or be present in the enriched phage population at very low frequency. Obviously, clones present in an enriched library at low frequency can only be detected if the sample size analyzed is large enough.

ROBOTIC-BASED SCREENING OF ENRICHED cDNA LIBRARIES

Selective enrichment and manual picking of clones from cDNA libraries displayed using the pJuFo cloning system allowed fast identification of IgE-binding molecules from different allergenic sources [Table 1]. However, the potential of these molecular libraries could not be fully exploited. The remarkably polyclonal immune responses to multiple allergens present in complex allergenic sources [79, 82, 83] and the serum-dependent enrichment of IgE binding phage during each round of selection [45, 77] generate large populations of phage. These enriched libraries contain a wide spectrum of multiply represented clones. The frequency of each clone in the enriched libraries depends on its frequency in the original library and on the amount of clone-specific IgE in the serum used for screening. Therefore, manual identification of clones is strongly biased towards clones derived from abundant mRNA species and from clones able to elicit strong IgE responses. As a consequence thereof, only the most prevalent clones will be found in manually processed small sample sizes. Clones expressing products derived from rare mRNA species or from allergens inducing weak IgE immune responses will hardly be found with a reasonable effort. In contrast, robotics-based automatic devices for picking and high density spotting of clones onto filter membranes allow fast survey of representative samples of clones [48, 84-87]. We have adapted this technology, originally developed for automated library handling in genomics [84-87], to screen cDNA libraries displayed on phage surface. A schematic representation of the steps required for picking and high throughput screening of cDNA libraries is presented in Fig. 3. In a first step, *E. coli* cells are infected with pJuFo phagemids and plated onto square 23 x 23 cm agar

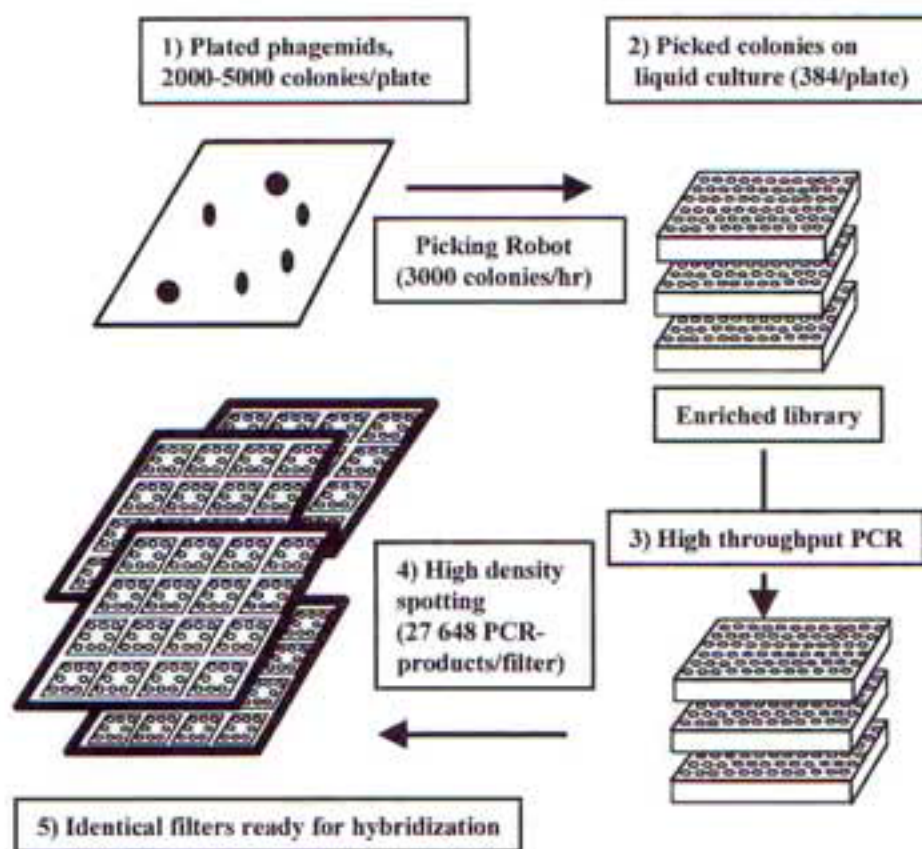
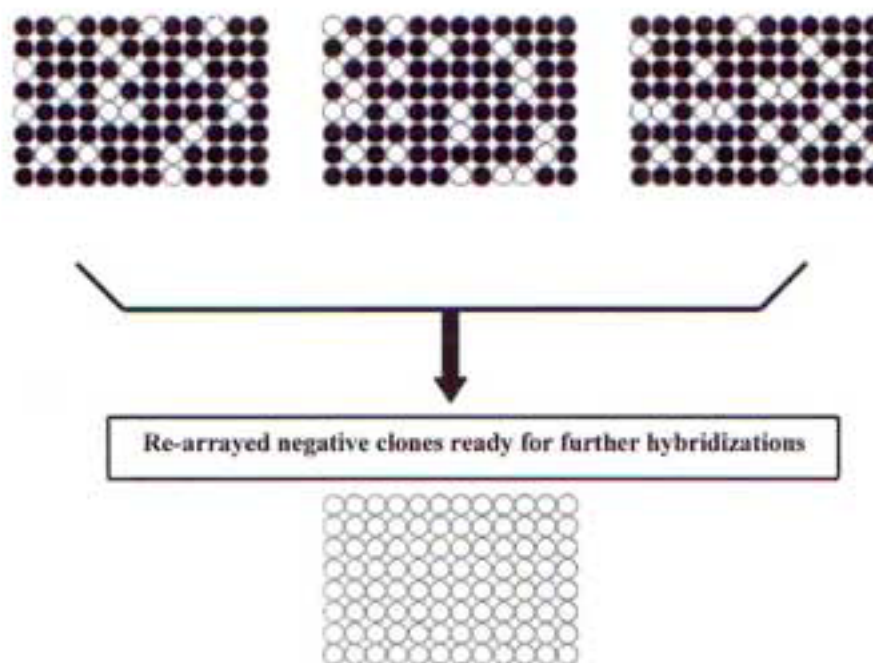
A) Picking, PCR amplification and gridding of PCR products**B) Re-arranging of hybridization negative PCR products**

Fig. (3). Schematic representation of the steps required for selection and high throughput screening of cDNA libraries displayed on phage surface.

plates at a density of 3000-5000 colonies per plate. The low colony density is required to allow picking of well separated colonies identified by a CCD camera mounted onto the picking robot. Single colonies are individually picked with a 96-pin picking device and collectively transferred into bar-coded 384-well microtiter plates filled with 2 x YT medium supplemented with freezing mix. After growth overnight at 37 °C, picked clones are replicated into new microtiter plates using 384-pin replicating tools to produce working copies. Master plates, as well as working copies of the picked libraries, are stored at -80 °C. At this stage colonies can be directly gridded onto Hybond N⁺-Nylon filter membranes at high density (147'456 clones per filter) and filters placed onto square (23 x 23 cm) agar plates for growing overnight at 37 °C [84]. After *in situ* lysis, filters can be screened by hybridization with labeled cDNA probes derived from the insert of interest to identify all clones representing a specific gene as described [48, 85]. We have extended the automated technology for picking representative samples of clones to high-throughput PCR amplification of all inserts and high-density gridding of the PCR products onto filter membranes (Fig. 3). This simple technology allows the amplification of all cDNA inserts in 384-well format, starting from the picked bacterial colonies as DNA templates and two pJuFo-specific primers in 30 µl reaction volumes [77]. PCR products can be gridded at high density onto Nylon filter membranes using a 384-pin gridding device to produce hybridization filters. Due to the small volume of PCR product needed for spotting (150 nl), large numbers of identical filters can be produced using the product of a single PCR reaction. Thereafter, each filter can be hybridized with a different cDNA probe. Starting from ten filters and ten randomly chosen inserts as hybridization probes, a first round of hybridization allows identification of all corresponding clones present in the enriched library. Repeated automated re-arraying of PCR products from negative clones onto new filters followed by hybridization with different cDNA probes allows identification of all different sequences present in an enriched library in a short time. We have used this approach to characterize enriched phage surface displayed cDNA libraries derived from different complex allergenic sources (Table 2). Although the IgE-binding capacity of each single gene product needs to be confirmed by subcloning, production of recombinant protein and

in vitro and *in vivo* analysis of its allergenicity [88, 89], very promising results have been obtained so far. All previously described allergens from *A. fumigatus* [82, 90], *C. comatus* [83], *C. herbarum* [91], *M. furfur* [78] and peanut [79] could be isolated by high-throughput screening technology of enriched cDNA libraries displayed on phage surface (Table 1). Moreover, subcloning of five randomly chosen new inserts derived from the *A. fumigatus* library, production of recombinant proteins and serologic investigations demonstrated that all five gene products were able to bind serum IgE in allergen-specific ELISA experiments performed as described [88, 92, 93]. This indicates that the combination of selective enrichment of cDNA libraries and robotic-based high throughput screening is perfectly suitable for the rapid characterization of complete allergen repertoires contained in complex allergenic sources. Cloning, characterization and production of allergenic molecules have already contributed to improve the diagnosis of allergy [94-98]. There is no doubt that recombinant allergens show a high potential for the immunotherapy of allergic diseases [99-101] which have become an important socio-economic problem causing an increasing health care burden to the society in industrialized countries [102, 103].

OUTLOOK

The work summarized suggests that phage surface display combined with robotic-based high throughput screening technology have the potential for rapid handling of large numbers of individual proteins/ligands required to identify gene products of interest present in molecular libraries. Selecting for specific interaction by functional screening of cDNA libraries displayed on phage surfaces and automated screening of the enriched clones enables, within a short time, the characterization of all desired gene products in a library. However, the key to success will depend from the successful enrichment of specific clones from a given library. Selective enrichment of filamentous phage depends on efficient display of proteins and will only be successful for proteins exported through the bacterial membrane and folded in a (native-like) form allowing interaction with the ligand used for screening. In spite of these limitations, application of functional enrichment of cDNA libraries displayed on phage surface and robotic-based screening will contribute to the rapid

Table 2 High Throughput Screening of Different cDNA Libraries Displayed on Phage Surface Enriched for IgE-Binding Clones with Serum of Sensitized Patients

Library	Clones spotted negative ^{a)}	% Clones hybridiz. found	% Clones analyzed	Different sequences ^{b)}	Described sequences ^{c)}	[Ref.]
<i>A. fumigatus</i>	13440	0.6	100	81	14	[90]
<i>C. herbarum</i>	5376	3.3	>80	28	4	[91]
<i>C. comatus</i>	2688	31.5	>70	37	5	[83]
<i>M. furfur</i>	5376	4.2	>90	27	9	[78]
Peanut	2688	0.0	4	9	7	[79]
Human lung	8064	1.8	100	151	8	[77]

a) Picked after five rounds of affinity selection against serum IgE from sensitized individuals

b) Absolute number of different sequences detected after affinity selection and hybridization

c) Number of sequences already described as IgE-binding molecules and detected by the screening

generation of information about the biochemical nature of proteins easily accessible with phage surface display technology. The basic knowledge gathered from arrays of successful and unsuccessful applications of cDNA display will serve as a basis to further develop the technology in order to overcome the biological limitations of the current systems.

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