

Direct Selection of cDNAs from Filamentous Phage Surface Display Libraries: Potential and Limitations

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Abstract: Over the past decade, powerful technologies devoted to survey large molecular libraries for the presence of specific clones using the discriminative power of affinity selection have been developed. Phage surface display technology is the most established of these methods and has revolutionised our ability to select agonistic and antagonistic peptides, antibodies with desired specificity and other drug targets. Thereby ligands are expressed as fusions to phage coat proteins and their respective genes are packaged within the phage. The basic concept of linking the phenotype, expressed as gene product displayed on the phage coat, to its genetic information integrated into the phage genome, creates fusion proteins covalently associated with the infectious particle itself. Binding of the phage to the target molecule offers a selective system by which rare phage carrying the desired gene product can be selected from large phage populations carrying inappropriate sequences. Phage selected in this fashion can be used for subsequent rounds of selection because they are able to self-replicate in suitable host cells, yielding target-specific phage populations after several consecutive rounds of affinity selection. Over 1500 publications describe the use of phage display technology highlighting its performance. Phage display possesses certain limitations, including its use for selection of genes from cDNA libraries that has lagged behind, despite the many accomplishments of this technology. Here we discuss recent progress in construction and screening of cDNA libraries displayed on phage surface and emerging concepts allowing fast identification of virtually all different clones present in enriched libraries.

INTRODUCTION

Large complex molecular libraries can be broadly divided into two categories. Those that are based on synthetic, low molecular weight compounds generated, for example by combinatorial chemistry [1], and those that are based on biological compounds such as nucleic acids [2, 3], peptides [4-6] or proteins [7, 8]. The unique characteristic of molecular libraries constructed in prokaryotic or eukaryotic vector systems consists in their ability to auto-replicate in a suitable host. Biological libraries consist of large, diverse collections of molecules displayed on the surface of the host. The unifying feature of display systems that distinguishes them from non-display systems is that the cloned gene and its encoded protein are physically linked [9-14]. This linkage allows efficient handling of large libraries based on selective enrichment of clones expressing genes of interest [15]. The library exists as a soluble pool of surface displayed molecules from which individual members with target-binding properties can be efficiently enriched by affinity selection against immobilised target molecules [16]. Because the genetic information is co-selected with the gene product, isolation and amplification of particular clones encoding desired gene products from pools of millions of clones

become feasible. The physical constraint of immobilising entire libraries on a solid support for screening [17] is therefore circumvented allowing exploitation of much larger libraries. Although many surface display systems including baculovirus [18, 19], bacteria [20-22] and yeast [23-25] have been developed, filamentous phage display [26] is the most widely adopted molecular display technique. Thereby, phage populations derived from a selection round can be recovered from the selection surface and used for subsequent rounds of amplification by simply infecting fresh *E. coli* cultures. During repetitive rounds of selection/amplification, potential genes of interest are enriched from very large libraries as a function of the ability of the encoded protein to interact with a given target molecule. Phage display has been successfully applied to the selection of ligands from linear or constraint peptide libraries [27-29] and for the display of functional protein domains such as antibody fragments [30-35], enzymes [36-38], hormones [8, 39] and DNA-binding proteins [40-42]. This wide range of applications clearly demonstrates the high potential of linking genotype and phenotype as a powerful tool for the selection of agonists and antagonists against biological targets and for the development of molecules with new properties [43, 44]. However, most of these systems make use of direct fusion of the encoding DNAs to the N-terminus of the phage coat proteins pIII or pVIII and are, therefore, not suitable for the direct expression of cDNA libraries [10, 11, 43]. Here we summarise available technologies, potential and limitations of phage surface display for construction and screening of cDNA libraries.

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TECHNOLOGY BACKGROUND

The exploitation of the full potential of phage display requires an understanding of the limitations that the phage structure itself, the process of assembly and the process of infection impose to our ability to modify the phage capsid. Filamentous phage (Fig. 1) are relatively simple structures consisting of a fully sequenced single-stranded covalently closed genome spanning about 6400 bp [45-47]. It encodes eleven genes [48] coding for 3 cytoplasmic products involved in phage DNA replication (pII, V and X), 3 gene products required for phage assembly which are not part of the phage particle (pI, IV and XI) and 5 capsid proteins (pIII, VI, VII, VIII and IX). Since replication of DNA or assembly of the phage are not strictly constrained by the size of the DNA, filamentous phages are excellent cloning vectors [49]. By combining the best features of plasmids and filamentous phage, a new generation of cloning vectors called phagemids

has been constructed [50]. They contain the replication origin and packaging signals of a filamentous phage together with the origin of replication, resistance marker and expression system of a chosen plasmid (Fig. 2). Phagemids can maintain themselves as plasmid in the bacterial host; however, infection with a filamentous helper phage activates the phage origin of replication and the resulting single-stranded phagemid DNA is encapsulated into phage-like particles using proteins supplied by helper phage [51]. Phage and phagemid-based vectors have been developed that allow display of foreign peptides and proteins on the surface of biologically functional filamentous phage particles [14, 52]. Repertoires of relatively short peptides of random amino acid sequences [5, 6, 27-29], complex antibody libraries [30-35] or libraries involving more complex scaffolds [53, 54] have been successfully constructed and screened. Most of the work has taken advantage of the ability of phages to assemble in spite of carrying hybrid fusions of the minor

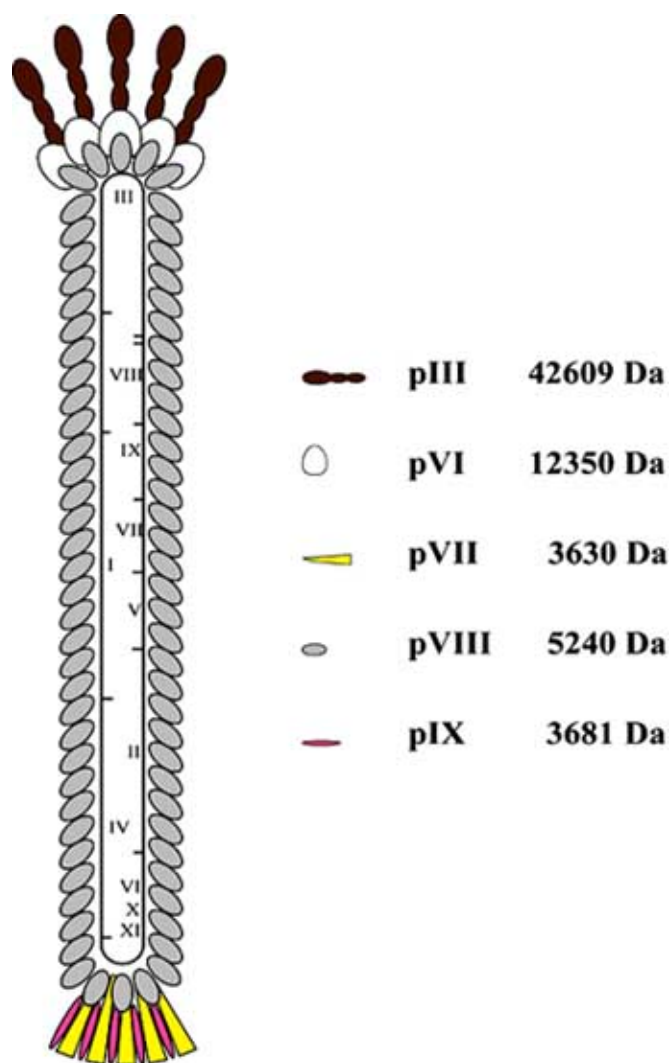


Fig. (1). Schematic representation of a phage particle showing the location of the capsid proteins, the dimensions and the orientation of the DNA.

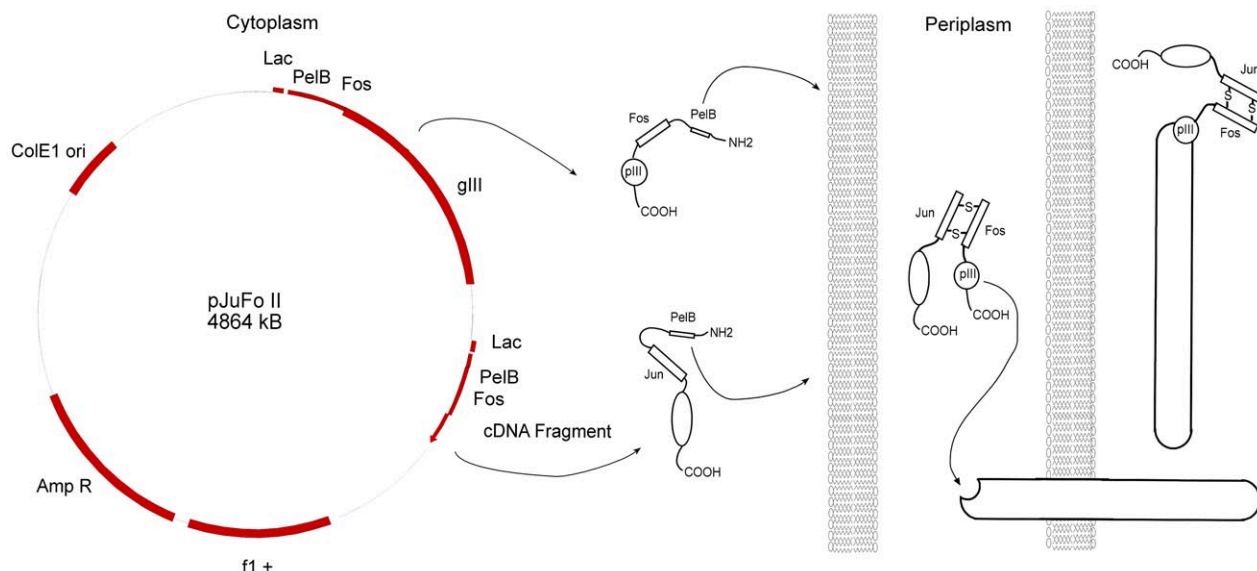


Fig. (2). The phagemid pJuFo and the proposed model for expression and assembly of phage surface-displayed cDNA libraries (Adapted from Ref. [10] and [77]).

coat protein pIII or of the major coat protein pVIII [9, 30, 49, 52]. Both systems can only be used to display foreign polypeptides by fusion to the amino-terminus of the capsid protein since the integrity of the carboxy-terminus of pIII and pVIII is essential for efficient phage assembly [55]. However, also the remaining three capsid proteins pVI [56, 57], pVII [58] and pIX [59] have been used to fuse short peptides or proteins to the filamentous phage surface although wide applications of these approaches have not yet been reported.

Filamentous phage capsids appear as thin and flexible tubular structures of about 900 x 10 nm. They are largely made of the product of gene VIII (Fig. 1) which is synthesised as a 73 aa precursor containing an amino-terminal signal sequence of 23 amino acid residues which is removed after the protein is inserted into the outer membrane of the host for assembly [60]. The amino-terminal portion of the mature 50 amino acids long pVIII protein gets exposed to the outside of the cylindrical phage particle and thus solvent exposed. By insertion of coding regions N-terminal to the capsid protein, chimeric proteins can be produced which can be assembled into phage particles [27, 61, 62], resulting in solvent exposed display of the foreign protein. The same strategy can be used to create N-terminal fusions to the pIII minor coat protein [16, 30]. pIII is synthesised with an amino-terminal signal sequence of 18-residues and the mature 406 amino acids long pIII protein spans the cytoplasmic membrane with the five carboxy-terminal residues in the cytoplasm [63]. Functionally pIII can roughly be subdivided into two domains: the C-terminal domain required for incorporation into the phage coat and the N-terminal domain required for infection [16]. One of the limitations of filamentous phages as display vectors for cDNA libraries is a direct consequence of the bifunctionality

of pIII and pVIII. Since the integrity of the carboxy-terminus of both proteins is essential for phage assembly, insertion of fusion polypeptides can only be tolerated at the amino-terminus [64]. However, cDNA inserts encoding the carboxy-terminus of any protein cannot be assembled into the phage coat as N-terminal fusions since the translational stop codons prevents the synthesis of hybrid coat proteins [16, 65, 66]. In contrast, phage particles containing foreign proteins fused to the carboxy-end of pVI can be produced [56] offering an alternative cloning strategy for cDNAs. Further features related to the life cycle of filamentous phage discussed later might impose serious limitations for cDNA display. However, there is an increasing number of successful applications that will be summarised below.

THE pVI-BASED CLONING SYSTEM

In contrast to fusions to the gIII or gVIII gene products (see below), fusion of foreign inserts at the carboxyl terminus of filamentous phage gene VI is compatible with phage propagation [56]. For the release of assembled filamentous phage from the membrane of infected cells both, pIII and pVI, have to be added to the end of the virion [67]. In the absence of pIII or pVI, phage filaments are not released, but remain associated with the cells [68]. Proteins pIII and pVI appear to specifically recognise each other [69] and the rather hydrophilic carboxyl-terminal region of pVI must be located very close to the surface of the phage particle as deduced from the observation that foreign proteins fused to the carboxyl end of pVI can be displayed as solvent exposed molecules [56]. This experimental evidence is corroborated by structural data suggesting that pVI connects the phage body to the host-binding pIII and might possess a solvent exposed C-terminus [70]. Gene VI-based

cDNA surface libraries have been generated and screened, however, only a few applications of this system have been reported [56, 57, 59].

THE pIII-BASED CLONING SYSTEM

Because display systems with the gene III (and gene VIII) product of filamentous phage employ amino-terminal fusion with the phage coat protein, they are unsuitable for surface expression of cDNAs bearing stop codons [10]. This problem was solved by using the *c-fos* / *c-jun* leucine zipper interaction to provide a covalent linkage between the gIII product and surface-expressed cDNAs [10, 11, 15, 65, 66, 71]. Analogous to the constant domains of antibody heavy and light chain [16], the *jun* and *fos* leucine zipper domains are able to self-associate [72-74] and to form very stable heterodimers with a Kd in the range of 100 nM [75]. To construct the cDNA phage surface display vector pJuFo [10, 66 Fig. 2], the phagemid pComb3 [16] was used as backbone. pComb3 contains two independent *lac*-promoter / operator elements, each followed by the *pelB* signal sequence [76]. One of the promoter elements contains the C-terminal domain (aa 198-406) of pIII cloned in frame with the *pelB* signal sequence, allowing construction of N-terminal pIII-fusions. Obviously this vector is not suitable for cDNA cloning because the stop codons from cDNAs

prevent translational synthesis of pIII fusion proteins [10, 11]. To overcome this limitation, we devised an indirect fusion strategy where cDNA inserts fused to the 3'-end of the Fos leucine zipper are co-expressed as fusion library from one of the promoter elements of pComb3 [10, 65, 66]. These fusions are expressed and directed to the periplasmic space of *E. coli* by the *pelB* leader sequence. The Jun leucine zipper was cloned N-terminally to the truncated pIII gene product to produce Jun-decorated pIII fusions. As these fusions are also directed to the periplasmic space of *E. coli* by the *pelB* leader sequence where phage assembly occurs, and because they conserve an unmodified C-terminus, they can be integrated into the phage coat providing a link for Fos-decorated cDNA fusions. The high affinity interaction between the Jun and Fos leucine zippers effectively links the cDNA products to the phage capsid, allowing solvent exposed display of gene products of large cDNA libraries [66, 77]. The gene products become thereby physically linked to the phage coat through two disulfide bonds provided by cystein residues engineered into both leucine zippers (Fig. 2). An analogous approach was used to generate a Jun / Fos phagemid based on pVIII which has, however, never been used to display cDNA libraries (Cramer and Jaussi, unpublished results). The pJuFo vector has been widely used to clone IgE-binding molecules using serum IgE from allergic patients as ligand (Table 1) and to clone other genes from different sources [15]. To date, more

Table 1. cDNA Libraries Displayed on pJuFo and Screened

Source	Ligand	Sequences*	Ref.
<i>A. alternata</i> cDNA	human serum IgE	2	[15]
<i>A. fumigatus</i> cDNA	human serum IgE	81	[78, 80]
<i>C. comatus</i> cDNA	human serum IgE	37	[102]
<i>C. herbarum</i> cDNA	human serum IgE	28	[78]
<i>C. albicans</i> chromosomal DNA	human serum IgE	4	[15]
<i>M. furfur</i> cDNA	human serum IgE	27	[103]
Wheat germ cDNA	human serum IgE	54	[104]
<i>L. destructor</i> cDNA	human serum IgE	3	[105]
<i>B. burgdorferi</i> chromosomal DNA	human serum IgG	7	[106]
Peanut cDNA	human serum IgE	8	[81]
B-cell cDNA (mouse)	synthetic peptide	2	[107]
Human lung cDNA	human serum IgE	151	[78,108]
HELA cells cDNA	human serum IgG	5	[109]
Chloroplast cDNA	rabbit serum	4	[15]
Celery cDNA	human serum IgE	2	[15]
<i>E. coli</i> chromosomal DNA	polyclonal serum	4	[110]
Mutagenised C5a libraries	U937 cells	6	[111]
B-cell cDNA (human)	Protein G	5	[65, 112]

*Number of clones carrying different sequences identified at DNA-level

than 350 IgE-binding molecules have been isolated from different complex allergenic sources using the pJuFo cloning system [78, 79]. The molecular structures so far cloned using the pJuFo system include secreted and cytoplasmic proteins with or without enzymatic activity [80], storage and structural proteins [81]. Although systematic studies devoted to elucidate the molecular nature of the proteins expressed are lacking, and despite the likelihood of significant under-representation or absence of certain expressed cDNAs from such libraries, the experimental data suggest that they can yield very useful results. An expansion of the utility of phage surface-displayed cDNA libraries will require development of systems allowing introduction of post-translational modification of the displayed gene products prior to screening. Certain target structures within a surface displayed library will require a post-translational modification or binding to a partner molecule to gain the ability to interact with a target ligand. Such modifications can potentially be applied prior to selection as demonstrated by efficient phosphorylation of a target sequence displayed on the lambda tail tube by a protein kinase [82]. However, the potential for *in vitro* activation of display products prior screening remains to be explored.

SELECTION FORMATS

The power of the phage display technology relies on the coupling of the functional display of molecular libraries with the ability of each member in the library to self-replicate and, at the same time, to encode the primary structure of the displayed polypeptide in its genome. Technically it is easy to construct huge cDNA or other molecular libraries and the size of the libraries has grown rapidly over the years reaching, e.g. for combinatorial antibody libraries 10^{10} - 10^{11} individual members [54]. The selection format is usually based on biopanning of the libraries on solid phase surfaces carrying immobilised ligands [11, 16, 83], affinity chromatography [65] or magnetic bead selection [84]. All these methods are based on repeated cycles of selection with target molecules and amplification of binding clones. During selection, those clones able to bind to the target are preferentially enriched from the large number of unspecific clones present in the library. During amplification the relatively small number of selected clones are amplified to form a new population of phage for further rounds of selection. These procedures suffer from several limitations and biases including non-specific binding, washing and elution processes, differences in the ability of the eluted phages to reinfect *E. coli*, a limited number of specific binding clones present in the initial library, a limited number of immobilised target molecules and the small number of clones that can be manually analysed for studying the results [85]. The last point may be the most limiting factor in surveys of cDNA libraries enriched with complex ligands such as serum IgE. It has been shown that complex allergenic sources contain large numbers of IgE binding molecules [78, 79]. Moreover, polyclonal IgE as ligand is highly heterogeneous and contains unknown amounts of molecules raised against different antigens/allergens. Therefore, single clones enriched from cDNA libraries derived from complex allergenic sources using IgE as ligand are expected to be multiple represented in the large

phagemid population obtained after repetitive rounds of selection [66]. The relative enrichment of each single clone will mainly depend on two parameters: the frequency of a specific clone in the original library and the number of clone-specific IgE molecules present in the serum. The first parameter depends on the abundance of the product-specific mRNA species at the time of harvesting for the construction of the cDNA library. The second one depends on the allergenicity of the gene product itself, which dictates the level of allergen-specific IgE present in a given serum used for selection and the individual situation of each serum donor. Both, phage-encoded products derived from abundant mRNA species and from strong immunogens will have a selective advantage over phage derived from rare mRNAs, phages binding to rare IgE species and from phages coding for weak immunogens, respectively. Therefore, the usual format based on biopanning, affinity selection and manual identification of a small number of clones is unlikely to be successful for the isolation of rare clones. Thus, novel formats are needed to characterise all clones present in cDNA libraries enriched with human serum. To create a more robust screening system, efficient parallel handling of large numbers of selected colonies is required [83, 86]. Techniques for high-throughput screening based on robotic picking to generate ordered arrays of selectively enriched libraries and high density spotting of the arrays (Fig. 3) allow fast identification of large numbers of clones [15, 78, 83]. The combination of selective enrichment and automated robot technology for picking and high-density spotting of clones onto filter membranes has proven to be very efficient for the isolation of virtually all allergen-specific cDNA clones present in libraries selected with serum IgE [15, 78, 79]. Selection for IgE binding molecules present in phage surface-displayed cDNA libraries might be regarded as a special case due to the high affinity of IgE for allergens, the high percentage of allergen-specific IgE among total IgE in serum [85, 87], and the ability to generate specific IgE-ligand surfaces through capturing of IgE with anti-human IgE antibodies [11, 66]. Nevertheless, the results obtained clearly show that screening of cDNA products displayed on phage surface is feasible (Table 1).

LIMITATIONS OF PHAGE SURFACE-DISPLAYED cDNA LIBRARIES

All display technologies based on biological systems will suffer from the constraints limiting the system itself. Obvious limitations of filamentous phage display are related to the restricted codon usage of *E. coli* as host, the lack of post-translational modifications [88] and to the potential toxicity of some gene products expressed in heterologous hosts [89]. However, further and more serious limitations of phage display derive from the characteristics of the phage life cycle and from the limited folding capacity of the host. Filamentous phages are released from the host without breaking the integrity of the cell membrane and thus, in contrast to lytic phages, heterologous proteins that should be displayed on the capsid must cross the lipid bilayer of the inner membrane [90]. Any cDNA gene product (fused to a capsid protein) whose properties prevent it from crossing the membrane will not be secreted and, therefore, not be assembled into the phage coat. This is a potential limitation

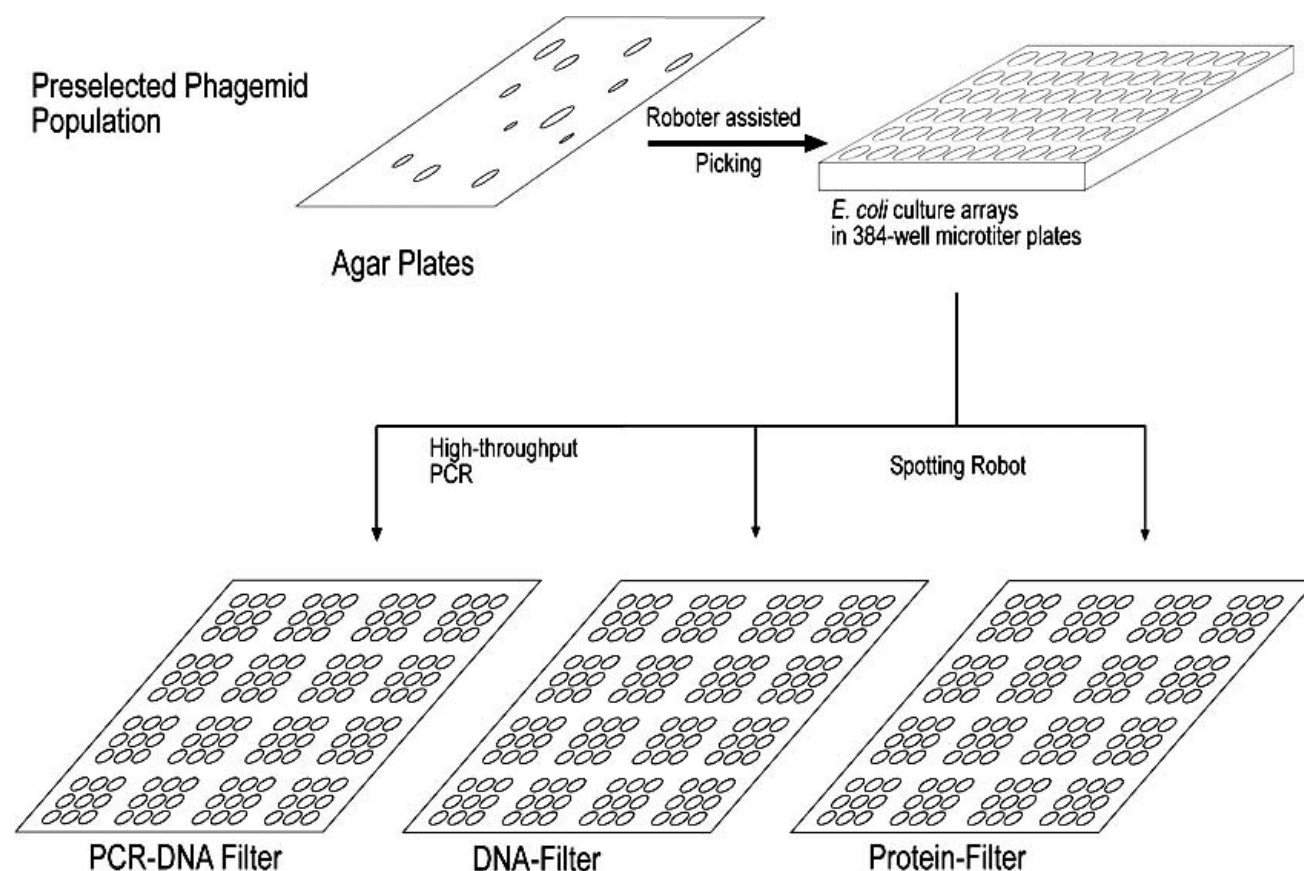


Fig. (3). Robot-assisted parallel handling of large numbers of selected clones for fast identification of desired gene products (Adapted from Ref. [113]).

mainly concerning display of transmembrane proteins such as receptor molecules, cell wall proteins or other structures imbedded into membrane bilayers that have never been reported to be found by screening of phage surface-displayed cDNA libraries. Although an upper limit for a protein to be displayed on phage surface has to be expected from the outer membrane "pore" size that permits the exit of the phage, proteins up to 86 kDa have been shown to be functionally displayed [91]. This fact, together with the demonstration that exclusively intracellular proteins like mitochondrial manganese dependent superoxide dismutase [92] or acidic ribosomal P₂ protein [93] can be selected from phage surface displayed cDNA libraries makes the technology attractive for many applications. However, cDNA libraries displayed on phage surface will be strongly constrained by the limited folding capacity of the *E. coli* machinery [94, 95] and, perhaps, also from the chemical characteristics of the periplasmic environment which may affect folding and stability of some proteins to be displayed. The awareness of these shortcomings prompted the development of alternative display systems that could help to overcome some of these limitations [58, 96]. However, these systems will suffer from other limitations like a limited number of transformants obtained in bacterial [97], yeast [98] and eukaryotic virus surface display systems [99] or instability of the mRNA in both polysome display [100] or RNA-peptide fusion systems [101]. It is unlikely that a single display system will be

optimal for all different fields of application emerging, ranging from screening of cDNA libraries, antibody selection, generation of mutant proteins with tailored activities and drug screening programs. It remains to be seen which of the new display technologies will achieve the widest adoption and, as a consequence thereof, the fastest improvements.

CONCLUSIONS

Molecular display technologies have a great potential in providing target-specific ligands for a fast progress in functional genomics and drug discovery. Selecting complex molecular libraries for specific protein-ligand interaction by functional approaches, combined with rapid parallel handling of large numbers of clones supported by robot-assisted automated technology will speed up the functional identification of gene products. Considering the large number of genomes fully or partly sequenced and the vast amount of genes/ESTs already available, there is a growing interest in functional analysis of gene products. In spite of new emerging technologies based on *in vitro* display systems and intrinsic limitations related to the use of biological systems itself, molecular libraries displayed on filamentous phage surface will still play an important role in gene identification because they can be rapidly and selectively

screened for the presence of specific clones. One of the future challenges for life sciences is the integration of accumulating knowledge into a global understanding of the complex interactional networks governing living systems. It is reasonable to assume that phage display technology, corroborated with rapidly developing technologies based on parallel handling of large numbers of clones will play an essential role in generating information and reagents to facilitate progress in the field of life sciences.

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