Molecular Diversity, **5:** 7–12, 2000.
KLUWER/ESCOM

Heteroduplex mobility assay (HMA) pre-screening: An improved strategy for the rapid identification of inserts selected from phage-displayed peptide libraries

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Received 15 February 2000; Accepted 22 May 2000

Key words: DNA conformation, heteroduplex mobility assay (HMA), mismatch, mutation detection, phage display

Summary

Phage-displayed peptide libraries represent an efficient tool to isolate peptides that bind a given target molecule. After several selection rounds, generally a large pool of target binding phages is obtained. Conventional analysis of the selected phage population involves extensive sequencing of many clones, most of which can be identical. We have adapted the Heteroduplex Mobility Assay (HMA) for pre-screening of phage inserts that were amplified by direct colony PCR of ELISA-positive clones. This strategy allowed for the rapid and reproducible assignment of insert sequences to different 'heteroduplex migration groups'. Sequence analysis of only one representative of each HMA migration group then completes the characterisation of the binding phage population. In our model experiments, only 16% of HMA pre-screened clones required further sequence analysis.

Introduction

Phage-displayed random peptide libraries [1–3] are highly efficient and increasingly popular tools to define specific ligands of receptors or enzymes, to map linear B cell epitopes or to identify peptide mimics of conformational epitopes (mimotopes). The pioneering work of G.P. Smith [4] provided the phage vectors for the insertion of foreign DNA fragments into genes encoding capsid proteins of the filamentous fd phage, thus yielding phage particles which display a unique peptide sequence on each phage. The majority of the available peptide libraries use an N-terminal display on the pIII protein for the presentation of up to five identical peptides per phage particle.

Random libraries that contain billions of different peptides are available for different insert sizes, with or without conformational constraints such as disulphide bonds. The identification of specific binders somewhat resembles the process of 'finding a needle in a haystack' and requires several selection and amplification rounds during which the phage population is gradually enriched for high affinity binders. This is achieved by biopanning, i.e. incubating the ligand under investigation with the library, then washing away non-binding phages, eluting and subsequently amplifying the selected phages in bacteria. Such amplified phages are then used in the next selection round. After each selection round individual clones are tested to identify specific ligand-binding phages using for example a phage-ELISA (Figure 1, top part).

At the end of the selection procedure a pool of binding phages is generated with the insert diversity largely depending on the ligand used for biopanning. In some cases these phages may all be derived from a single clone bearing the same insert sequence; however, most targets select a pool of phages with several distinct inserts since different peptides may specifically interact with one ligand. Therefore, a large number of individual clones need to be analysed to fully appreciate the frequency and diversity of the interacting peptide sequences. This is usually achieved by sequencing the insert region of several dozens or more phages, which requires the expansion of phage producing bacteria, the purification of the amplified phage particles, DNA extraction and purification for

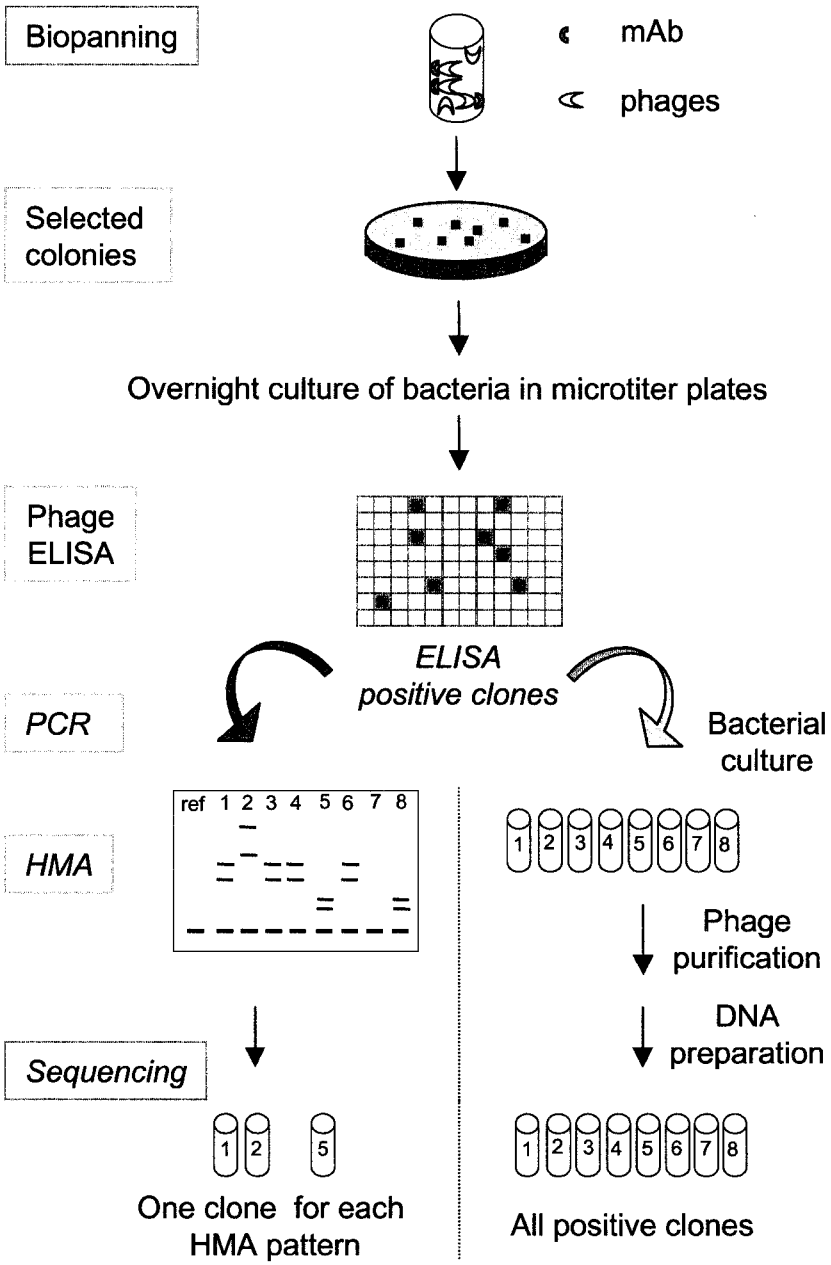

Figure 1. Overview of the conventional procedure for analysis of phage inserts (right) and the HMA pre-screening (left).

sequencing (Figure 1, right). Hence, the analysis of a representative number of peptide inserts may become a very time consuming, cumbersome and expensive process. The aim of this study was to find an alternative strategy for analysis of positive phages which would circumvent repetitive sequencing of identical clones.

Here we describe a simple and rapid method for the characterisation of positive clones using direct PCR amplification of the peptide insert region of binding phages followed by Heteroduplex Mobility Assay [5,6] (HMA) pre-screening (Figure 1, left). This way, the phage DNA preparation and purification after selection and above all the sequence analysis of each

positive clone becomes obsolete, limiting sequencing to a non-redundant set of phages.

Materials and Methods

Phage display

Phage display technology was used to identify mimotopes of neutralising and protective epitopes of the measles virus hemagglutinin protein (MVH). An in-house monoclonal antibody (mAb) BH15, which recognises a discontinuous B cell epitope of the Edmonston wild-type MVH, was used to screen four different type III libraries: constrained random 7-mer [7] and semi-constrained 9-mer [8] libraries (inserts CX_7C and CX_9, respectively; kindly provided by R. Pasqualini) as well as linear 6-mer and 15-mer libraries (kindly provided by G.P. Smith) following a modified protocol of Parmley and Smith [9]. Briefly, 2×10^{11} infective phages of a given library were incubated with the immobilised mAb and unbound phages were washed off seven times before binding phages were eluted with an acidic buffer and amplified in *E. coli* TG1 cells. After each selection round, clones were grown in 96-well plates and binding phages were determined using a standard phage-ELISA [10].

PCR

PCR of ELISA-positive clones was performed either directly on bacterial colonies introduced with a tooth pick into the PCR mixture or with a 10 μl fraction (1:2 diluted in H_2O) of a 200 μl microwell liquid culture which was boiled for 5 min and then centrifuged for 5 min at 13 000 rpm to pellet bacterial debris. Ten μl of supernatant (diluted 1:5 in H_2O) served as a template for PCR. The insert of ligand binding phages was amplified using primers F1 (sense primer, 5′ TCGAAAGCAAGCTGATAAACCGATACA 3′) and F2 (anti-sense primer, 5′ AGCATTCCACAGACAGCCCTCATAGTT 3′), which generated a 299 bp fragment containing two flanking regions of 122 and 129 bp length, respectively and the peptide-encoding region of the fUSE5 vector [10] in a central position. The PCR mix contained 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.4 μM of each primer, and 1U of Platinum Taq Polymerase (Gibco BRL Life Technologies, U.S.A.). Cycling conditions were as follows: 1× (95 °C–3 min), 10× (95 °C–15 s, 63 °C–40 s, 72 °C–45 s), 15× (95 °C–20 s, 64 °C minus 0.4 °C in each cycle – 25 s, 72 °C–45 s), 1× (72 °C–8 min).

HMA

HMA analysis was performed as described previously [5]. Briefly, the PCR product of an arbitrarily selected clone served as a reference fragment without prior knowledge of its sequence. For the homoduplex control this fragment was mixed with H_2O. For heteroduplex reactions, the reference fragment was consecutively added to each of the PCR fragments derived from the remaining ELISA positive cultures. The mixtures, containing equal quantities of two different PCR fragments, were then denatured for 5 min at 95 °C and re-annealed by slow cooling to room temperature in a water bath. Upon annealing of the single DNA strands of the reference PCR fragment with the complementary strands of a clone-derived PCR product, two species of heteroduplexes are normally generated. Sequence changes in the insert region (relative to the reference fragment) form mismatches, which lead to bulges in the dsDNA. Such bulges confer a particular conformation [11] to the heteroduplexes, which is detected by a slower electrophoretic migration compared to the perfectly paired dsDNA fragments (homoduplex). Homo- and heteroduplex fragments were resolved by non-denaturing gel electrophoresis on a 1× mutation detection enhancement (MDE) gel (FMC Bio Products, U.S.A.) containing 5% glycerol. Gels were stained with GelStar Nucleic Acid gel stain (FMC Bio Products) which is approximately 10 times more sensitive than conventional ethidium bromide, for 1 h. The MDE gel matrix yielded better resolution of heteroduplexes when compared to a standard 5% polyacrylamide gel (data not shown).

Results and Discussion

Phages binding to BH15 were selected by biopanning of a CX_9 library. Inserts of antibody-binding phage clones were amplified directly from bacterial cultures using the PCR protocol described above. PCR products of 51 different ELISA-positive clones were then pre-screened by HMA. Representative results for 17 PCR products are shown in Figure 2. Lane 1 represents the homoduplex of the reference fragment alone. Lanes 2 to 17 show banding patterns for different phage clones. Eight different migration patterns became evident (Figure 2A, patterns A to H). The majority (24 of 51) of analysed phage inserts did not form heteroduplexes (pattern A), indicating that these inserts shared the same sequence with the refer-

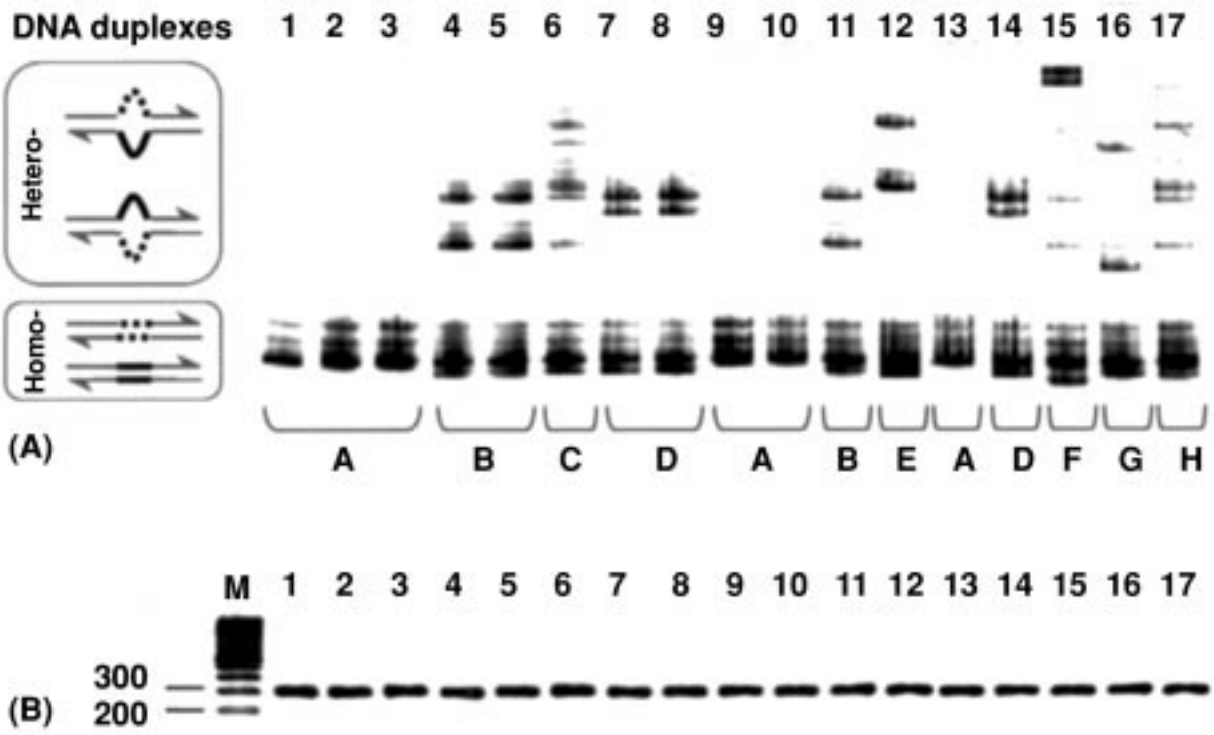

Figure 2. (A) HMA analysis of clones selected from a CX_9 peptide library using the mAb BH15 that recognises a discontinuous epitope on measles virus H protein. The MDE gel shows the homoduplex control in lane 1 (pattern A) and distinct HMA patterns (B–H) for the different clones (lanes 2–17). The cartoon on the left illustrates the principle of HMA analysis with the central insert regions of the reference and test fragments shown as dotted or bold lines, respectively. (B) PCR products of 17 clones selected for HMA analysis. Five μl of PCR reactions (lanes 1–17) were separated on a 2% agarose gel and stained with ethidium bromide. PCR products migrated as single bands of similar intensity at their sequence deduced size.

ence fragment. Patterns B to H displayed heteroduplex bands with a characteristically reduced electrophoretic mobility. Moreover, several clones unambiguously revealed the same HMA pattern (Figure 2A, lanes 4, 5, 11 and lanes 7, 8, 14) and were thus expected to carry the same insert sequence. For this pilot study, all clones were subsequently sequenced on an ABI 377 automated sequencer (Perkin Elmer) confirming HMA results in all cases (Table 1).

These findings illustrate that the electrophoretic mobility of the heteroduplexes is insert sequence dependent and yields specific and reproducible HMA patterns, which allow for the identification of subpopulations with distinct inserts. The efficiency of the HMA was independent of the sequence of the randomly chosen reference fragment and although migration patterns may change with different references, the screening results remain identical. PCR fragments of 299 bp with flanking vector sequences of approximately 120 bp length produced optimal resolution of heteroduplexes. All PCR products showed one distinct band and equal concentrations when analysed on a 2% Agarose gel (Figure 2B).

HMA pre-screening revealed that only 16% of the selected clones were unique and required sequencing. Furthermore, the sequences of several GC-rich fragments that repeatedly showed ambiguous sequences (mimotope 17, Table 1) could be resolved by HMA.

The bands that migrated close to the homoduplex originated from the PCR amplification. These fragments were not visible on a standard agarose gel and did not interfere with the separation of heteroduplexes. The presence of more than two expected heteroduplex bands (Figure 2A, lanes 6, 15, 17) was probably due to different conformations and/or different annealing possibilities of the various ssDNA fragments [6].

HMA pre-screening also proved to be successful for identification of distinct phage clones selected from CX_7C, CX_9, X_{15} and X_6 libraries with several mAbs directed against proteins of different pathogens

Table 1. Insert-specific HMA patterns of CX_9 phages selected with mAb BH15

Mimotope no.	Nucleotide sequence $5' \rightarrow 3'$[a]	Amino acid sequence[a]	HMA pattern	Frequency of clones[b]
1, 2, 3, 9, 10, 13	TGTTATAGTATGATGATTTCGGAAGATTGT	C Y S M M I S E D C	A	24
7, 8, 14	. . . G T . T C . T G . . . T G A G . . T . . G	. V . W I E	D	11
12	. . . A T . . T G C G . C C T T A . G . . . C G T T G . . .	. I M R P Y A A L .	E	7
4, 5, 11	. . . G T G T C G T . T A T T C . . A A T . . G	. V . F I L N . . .	B	5
6	. . . A T G T T G C G . C C T T A . G . . . C G . . G . . .	. M L R P Y A A E .	C	1
15	. . . . G G . . . T A T . C . C . . A G T . . G	. W . Y T L	F	1
16	. . . . T G . C . . G . C C . T A . G . T T C T T T G . . .	. L T R P Y A S L .	G	1
17	. . . A T . T T G C G . C C T C . . N . . . . G T . . . T .	. I L R P L ? E Y F	H	1

[a] Only nucleotides and amino acids differing from those of pattern A are shown.
[b] Frequencies of clones after five selection rounds. Mimotope 1 served as the reference clone.

(data not shown).

The data presented here illustrate that the method allows for the pre-screening of pIII displayed random or gene-fragment peptide libraries [12,13] constructed in fd phages. In principle, HMA screening applies to any peptide display system provided that the random insert can be amplified specifically as the central part of a PCR fragment flanked by conserved regions.

The technique can further be adapted to pIII displayed phagemid libraries such as semisynthetic scFv libraries, where one CDR [14] is randomised, by designing an appropriate vector-specific forward primer (F1) for PCR amplification or by using primers that bind in the conserved regions of the scFv backbone, respectively. HMA pre-screening is a DNA-based method for differentiation of short peptide inserts selected by protein–peptide interactions and thus differs from the approach described by Bartoli et al. [15] where nucleic acid hybridisation was used to enrich or deplete phage populations as part of the selection procedure.

In recent years, random libraries have been used to identify ligands that recognise increasingly complex target structures in vivo [16] as well as in vitro (e.g. polyclonal serum [17], whole cells [18]). Moreover, affinity selections under different stringencies augment the variety of binders, thus generating a broader set of selected sequences [19] for a better understanding of the target–ligand interaction and the identification of consensus structures. Such developments lead to binding phage populations of growing complexity and emphasise the need for an efficient pre-screening method.

Acknowledgements

This work was funded by the EU Biotechnology Project BIO4 CT980242 and by the Centre de Recherche Public-Santé, Luxembourg. S.D. was supported by a ‘Bourse Formation Recherche’ of the Ministère de l’Education Nationale, Luxembourg.

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