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Array-in-well binding assay for multiparameter screening of phage displayed antibodies

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ABSTRACT

Phage display is a well-established and powerful tool for the development of recombinant antibodies. In a standard phage display selection process using a high quality antibody phage library, a large number of unique antibody clones can be generated in short time. However, the pace of the antibody discovery project eventually depends on the methodologies used in the next screening phase to identify the clones with the most promising binding characteristics e.g., in terms of specificity, affinity and epitope. Here, we report an array-in-well binding assay, a miniaturized and multiplexed immunoassay that integrates the epitope mapping to the evaluation of the binding activity of phage displayed antibody fragments in a single well. The array-in-well assay design used here incorporates a set of partially overlapping 15-mer peptides covering the complete primary sequence of the target antigen, the intact antigen itself and appropriate controls printed as an array with 10 × 10 layout at the bottom of a well of a 96-well microtiter plate. The streptavidin-coated surface of the well facilitates the immobilization of the biotinylated analytes as well-confined spots. Phage displayed antibody fragments bound to the analyte spots are traced using anti-phage antibody labelled with horseradish peroxidase for tyramide signal amplification based highly sensitive detection. In this study, we generated scFv antibodies against HIV-1 p24 protein using a synthetic antibody phage library, evaluated the binders with array-in-well binding assay and further classified them into epitopic families based on their capacity to recognize linear epitopes. The array-in-well assay enables the integration of epitope mapping to the screening assay for early classification of antibodies with simplicity and speed of a standard ELISA procedure to advance the antibody development projects.

1. Introduction

Recombinant antibodies based drugs have become an elementary part of the pharmaceutical toolbox to combat various diseases including cancers and autoimmune disorders. Also, the use of recombinant antibodies, in diagnostic assays and as research tool, is rapidly increasing. Recombinant antibodies can be obtained by cloning the rearranged variable domain genes from hybridoma cell line or B-cells for expression in a recombinant host cell [1]. Another widely used approach utilizes an *in vitro* platform involving large man-made repertoires of antibody molecules and use of a display technique for enrichment of the clones with desired properties [2,3]. Phage display is the longest-standing and still the most widely used display technique. Subjecting a suitable high quality phage display antibody library to a few iterative rounds of selection, generally results in a strong enrichment of binders against the selection target. In this technique, the target can be freely chosen including self-antigens and toxic molecules [3,4]. A single phage display selection experiment against the target of interest typically yields a strongly enriched antibody phage pool, in many cases, containing a large number of different target specific antibodies.

After the rapid phage display based enrichment phase, the individual target specific binders are identified in a screening process which typically involves picking the individual colonies, expressing antibody, and revealing the positive clones by binding assays such as conventional ELISA [5]. The number of colonies taken to the screening often ranges from hundreds to thousands, and for practical reasons, the screening is typically performed in two stages: potentially interesting clones are first identified by a simple and coarser primary screening assay and the binding activity of the

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shortlisted clones is then verified in a more diligent secondary screening assay. The unique antibody clones can then be identified by sequencing and the promising ones directed to more detailed analysis pertaining to e.g. the strength and specificity of the target recognition. After the screening, there can be tens or even hundreds of active binders deserving further characterization, and compared to the fairly straightforward and rapid phage display selection phase, the screening and especially the following closer characterization is often considerably slower and labor-intensive process, constituting a major bottleneck in the antibody development process.

Information on the epitope, antibody's binding site in the target protein, is useful for many applications. Such information has several important uses, such as advancing the understanding of the possible mechanism of action, identifying functional antibody pairs for immunoassay development and securing intellectual property rights [6]. Moreover, understanding the nature of the epitope, whether linear or conformational, is valuable for many purposes. The most accurate approach to map an epitope is the crystallographic analysis of the antigen-antibody complex. However, this method is time-consuming and technically challenging [7]. Other methodologies for epitope mapping include hydrogen-deuterium exchange coupled to mass spectrometry [8], site directed mutagenesis [9] and mimotope analysis with phage display peptide library [10]. Each of these having their own technical limitations, making them too laborious and slow for routine analysis of a large number of antibody clones. The most widely used and simple epitope mapping approaches use consecutive, overlapping synthetic peptides that cover the complete primary sequence of the protein antigen. Each peptide can be immobilized on ELISA wells [11,12] or the peptides can be printed or directly synthesized as an array on cellulose membranes [13,14], slides [15], glass chips [16], polyethylene pins [17] or single-walled carbon nanotube field-effect transistors [18]. The peptide-based approach is primarily suited for identification of linear epitopes, although the identification of conformational epitopes can be promoted by constrained, cyclized, peptides [19]. The advantages of overlapping synthetic peptides based assays include that the interpretation of results is straightforward and performing the assay itself is typically technically less demanding compared to other epitope mapping techniques. Owing to the advances in peptide synthesis process, the reagents for such assays are today reasonably affordable.

Here, we present an array-in-well concept [20] based method for simultaneous assessment of binding activity and epitope specificity of phage displayed antibodies. This method allows reliable analysis of an antibody clone against a number of targets such as various proteins and a series of antigen-derived peptides in a single microtiter plate well. The assay can help to develop more efficient and economic screening processes with high information content.

2. Material and methods

2.1. Phage antibody production

Phage-displayed single-chain variable fragment (scFv) antibodies were isolated from the synthetic antibody phage library scFvP [21] by selections against HIV-1 p24 protein antigen (NEXT BIOMED Technologies, Finland). The first round of phage display selection was performed with 250 μg of streptavidin magnetic beads (Dynabeads ®MyOne™streptavidin T1, Invitrogen, Norway) saturated with the biotinylated p24 antigen and 5.0×10^{12} colony forming units (cfu) of phage from the library. The phage pool was first incubated with the beads without the antigen for 1 h to eliminate undesired antibodies against streptavidin, and the unbound phage were then let to react with the antigen containing beads for 30 min on rotatory mixer at room temperature. After washing, the bound phage were eluted from the beads by adding 50 μL of 50 μg/ml trypsin (Sigma Aldrich, USA) and incubating 10 min at 37 °C with shaking. Trypsin activity was blocked by adding 12.5 μg trypsin inhibitor (Sigma Aldrich) and incubating for 15 min. The scFv-genes were rescued from the eluted phage by PCR-based amplifications. The PCR mixture included 0.5 μM primers JLe01_s (5′CGGCAGCCGCTGGATTGTTATTAC) and JLe01_as (5′-ACCAGAACCGCCACGACCTTC), 200 μM dNTP's, 25 U/mL Pfu DNA polymerase, 1× Pfu buffer with $MgSO_4$ and eluted phage (heat-treated at 95 °C for 5 min prior the PCR amplification) in the total reaction volume of 360 μL. The PCR consisted of 2 min initial denaturation at 95 °C, followed 30 cycles of 30 s at 95 °C, 30 s at 60 °C, and 2 min at 72 °C, the final extension 10 min at 72 °C. The PCR products were purified with geneJET™ purification kit (Fermentas, Lithuania) according to manufacturer's instruction. The products were digested with 20 U SfiI enzyme in 40 μL. The reactions were incubated overnight at 50 °C. The SfiI digestion product of 768 bp was isolated from 0.8% agarose gel, and ligated to 100 ng of PEB32x vector (molar ratio 1:3) overnight at 16 °C. After inactivation of ligase at 65 °C for 10 min, 2 μl of ligation mixture was used to transform to electrocompetent cells XL1-blue *E. coli* [*rec*A1 *end*A1 *gyr*A96 thi-1 *hsd*R17 *sup*E44 *rel*A1 *lac* [F' *pro*AB l*acI*^{q}ZΔM15 Tn10 (Tet^r)]] (Stratagene, USA). The electroporation was conducted with Bio-Rad Genepulsed (Biorad, USA) with settings 200 Ω, 1.25 kV, 25 μF. After recovery in 1 mL SOC at 37 °C at 100 rpm for 1 h, samples were plated on LA (25 μg/mL cm, 10 μg/ml tet and 1% glc) and incubated overnight at 37 °C. The cells were used as an inoculum for 20 mL phage production as described by Brockmann et al. [2]. The second round of phage display selection was performed with 50 μg of magnetic beads saturated with the biotinylated p24 antigen and 3×10^{11} cfu of phage from the first round. After the two selection rounds, 960 individual bacterial colonies were randomly selected from a culture plate to produce individual phage antibody cultures in a 96-plate format as described by Brockmann et al. [2]. Monovalent phage antibodies were obtained using VCS M13 helper phage (Agilent Technologies, USA) and multivalent phage antibodies with a genetically pIII-deficient helper phage, known as hyperphage M13 K07ΔpIII (Progen Biotechnik, Germany) [22].

2.2. Time resolved fluorescence (TRF) based immunoassay

The binding activity of the enriched phage pools and individual phage antibody clones towards the antigen was measured by TRF immunoassay. Streptavidin-coated microtiter plates, assay buffer and wash solution used in TRF immunoassay were procured from Kaivogen Oy (Finland). The total volume of the immunoassay was 100 μL and all incubations were performed at room temperature (RT) in shaking, 900 rpm. First, 30 ng of biotinylated p24 antigen or assay buffer for blank were added to the streptavidin wells and incubated for 1 h. The wells were washed four times, followed by the addition of 100 μL of diluted phage pools (1×10^9 cfu) for the evaluation of the phage pools or 100 μL of 1:10 dilution of culture supernatant for the evaluation of individual clones. After 1 h incubation, the wells were washed four times and 25 ng of Eu-N1-labelled anti-M13 monoclonal antibody 9E11 (Turku University) was added to trace the bound phage. After 1 h incubation, the wells were again washed four times and 200 μL of DELFIA enhancement solution (Perkin Elmer, Finland) was added for signal development. Time-resolved fluorescence signal was measured after 15 min with Victor 1420 multilabel counter (Perkin Elmer), using Europium program (λ excitation = 615 nm, λ emission = 340 nm, delay time 400 μs and window time 400 μs)