COMBINATION OF RIBOSOME AND PHAGE DISPLAY FOR FAST SELECTION OF HIGH AFFINITY VHH ANTIBODY FRAGMENTS

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Selection of antibodies using phage display involves the preliminary cloning of the repertoire of sequences encoding antigen-binding domains into phagemid, which is considered the bottleneck of the method, limiting the resulting diversity of libraries and leading to the loss of poorly represented variants before the start of the selection procedure. Selection in cell-free conditions using a ribosomal display is devoid from this drawback, however is highly sensitive to PCR artifacts and the RNase contamination. The aim of the study was to test the efficiency of a combination of both methods, including pre-selection in a cell-free system to enrich the source library, followed by cloning and final selection using phage display. This approach may eliminate the shortcomings of each method and increase the efficiency of selection. For selection, alpaca VHH antibody sequences suitable for building an immune library were used due to the lack of VL domains. Analysis of immune libraries from the genes of the VH3, VHH3 and VH4 families showed that the VHH antibodies share in the VH3 and VH4 gene groups is insignificant, and selection from the combined library is less effective than from the VHH3 family of sequences. We found that the combination of ribosomal and phage displays leads to a higher enrichment of high-affinity fragments and avoids the loss of the original diversity during cloning. The combined method allowed us to obtain a greater number of different high-affinity sequences, and all the tested VHH fragments were able to specifically recognize the target, including the total protein extracts of cell cultures.

Keywords: nanobodies, VHH antibodies, ribosome display, phage display, biopanning, PDLIM4

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ИСПОЛЬЗОВАНИЕ КОМБИНАЦИИ РИБОСОМНОГО И ФАГОВОГО ДИСПЛЕЕВ ДЛЯ БЫСТРОГО ОТБОРА ВЫСОКОАФФИННЫХ VHH-ФРАГМЕНТОВ АНТИТЕЛ АЛЬПАК

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Селекция антител с помощью фагового дисплея предполагает предварительное клонирование репертуара последовательностей, кодирующих антигенсвязывающие участки, в фагмиду, что считается «бутылочным горлышком» метода, ограничивающим итоговое разнообразие библиотек и ведущим к потере слабо представленных вариантов еще до начала процедуры селекции. Отбор в бесклеточных условиях при помощи рибосомного дисплея лишен этого недостатка, однако отличается высокой чувствительностью к артефактам ПЦР и присутствию РНКаз. Целью работы было исследование эффективности сочетания двух методов: проведения предварительной селекции в бесклеточной системе для обогащения исходной библиотеки с последующим клонированием и заключительной селекцией при помощи фагового дисплея. Предполагалось, что такой режим селекции позволит устранить недостатки каждого из методов и повысить эффективность отбора. Для селекции использовали последовательности VHH-антител альпаки, удобные для построения иммунной библиотеки из-за отсутствия VL-доменов. Анализ иммунных библиотек из генов семейств VH3, VHH3 и VH4 показал, что в группах генов VH3 и VH4 доля VHH-антител незначительна, и селекция из комбинированной библиотеки менее эффективна, чем из библиотеки последовательностей семейства VHH3. Мы установили, что комбинация рибосомного и фагового дисплеев приводит к более высокому обогащению высокоаффинными фрагментами и позволяет избежать потери исходного разнообразия при клонировании. Комбинированный метод позволил получить большее количество различных высокоаффинных последовательностей, а все протестированные VHH-фрагменты оказались способными специфично распознавать мишень, в том числе в тотальных белковых экстрактах клеточных культур.

Ключевые слова: наноантитела, VHH-антитела, рибосомный дисплей, фаговый дисплей, биопаннинг, PDLIM4

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Соблюдение этических стандартов: работу с животными проводили в соответствии с принципами и требованиями Международной лаборатории по уходу за животными и Директивой совета европейских сообществ (86/609/EEC) от 24 ноября 1986 г.

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Monoclonal antibodies, as well as their antigen-binding fragments, are one of the most important tools in biological research, diagnostic applications, and disease therapy. The traditional method of antibody identification by testing of individual clones of hybridomas is laborous and inefficient. More advanced approaches, including *in vitro* selection from libraries of antigen-binding fragments of antibodies, simplify the production of antibodies, and allow to select antibodies that have certain properties, for example, the ability to block specific interactions between a receptor and its ligand. Among the methods of selection *in vitro*, phage display technology has become the most widely used, as it allows to create libraries of antigen-binding fragments relatively quickly and with high reliability. This method is based on production of genetically modified phage particles exposing antigen-binding sites of antibodies on their surface. Selection involves incubating a mixture of phage particles with an immobilized antigen. After washing and elution, bound variants of phage particles are propagated in permissive bacteria, followed by the selection of individual variants that are most abundant in the mixture [1]. One of the drawbacks of the method is the requirement to clone the repertoire of fragments encoding the antigen-binding fragments into a special phagemid and then to obtain a pool of transformed cells to produce libraries of phage particles. This stage is considered to be the bottleneck of the method, limiting the diversity of the resulting libraries. As a result, rare variants of antibody fragments may be lost before the beginning of the selection process [2]. Another disadvantage follows from the very structure of traditional antibodies, in which the binding of the antigen is carried out by the interaction of antigen-binding sites located on two separate polypeptide chains. When creating clonal libraries, the probability that the antigen-binding regions of the heavy and light chains of a certain immunoglobulin will be joined in one single-chain scFv antigen-binding fragment is extremely low due to the randomness of the combinatorial process. As a result, the final mixture contains mostly inactive combinations of sections of heavy and light chains, which significantly complicates the selection of scFvs with the desired properties.

To overcome the drawback associated with the need to transform bacteria, there have been developed several modifications of the method, that are based on selection in cell-free conditions, in particular, a ribosome display. This method is based on a coupled cell-free transcription and translation system, where complexes consisting of transcripts (mRNA), ribosomes, and growing protein chains corresponding to the repertoire of antigen-binding regions of antibodies are subjected to affinity selection on the immobilized antigen. Bound variants are amplified by RT-PCR, resulting in an enrichment of the desired variants [3]. Although the initial diversity of the repertoire when using this method is much higher, selection is often biased towards variants of antigen-binding sites that are most easily amplified in PCR [4]. It can be assumed that by combining the methods of ribosome and phage display it will be possible to overcome the weak spots of both methods and to increase the overall efficiency of selection. In this case, it would be possible to preliminarily select variants in a cell-free system, achieving enrichment with high-affinity variants, and then clone the resulting repertoire of fragments into a phagemid and carry out the final selection using phage display.

Animals of the camelid family possess, in addition to antibodies of the traditional structure, antibodies consisting only of heavy chains of immunoglobulins (variable heavy-heavy, VHH antibodies) [5]. This type of antibodies was formed as a result of a mutation in the hinge region of the heavy chain, which resulted in the deletion of the heavy and light chain binding site. To compensate for the absence of the antigen-recognizing region of the light chain, the VH domains of such antibodies have longer CDR3 regions, which ensures high affinity and specificity for the recognition of antigens. Antibodies of this structure make it possible to obtain compact antigen-recognition sites. The process of *in vitro* selection of VHH antibodies is more efficient due to the lacking stage of random joining of two polypeptide chains, as the resulting libraries are devoid of inactive combinations. VHH antibodies are already widely used for *in vitro* selection by phage display, and several products were already created on their basis for diagnostic applications and disease treatment [6].

The aim of this work was to select for VHH antibodies to the tumor marker PDLIM4 from the immune library of alpaca antibodies with different selection methods. We wanted to test whether the combination of ribosomal and phage displays allows for more efficient selection of sequences of antigen-binding fragments with high affinity and specificity.

METHODS

Animal immunization

The source of biological material (blood) was the alpaca (*Vicugna pacos*) of the camelid family. 700 μg of purified PDLIM4 protein was dissolved in complete (for primary immunization) or incomplete (for repeated immunizations) Freund's adjuvant (Pierce; USA) according to the manufacturer's protocol. Immunization of alpaca was carried out in three stages (primary and two boosts, with an interval of 3 weeks) by intramuscular injection into the thigh of the hind leg of the animal. 4 weeks after the last boost, 100 ml of heparinized blood was collected. Peripheral blood mononuclear cells (PBMC) were isolated by centrifuging with a 1.077 Ficoll solution (PanEco; Russia) according to a standard protocol.

RNA isolation, cDNA synthesis and PCR

Total RNA was exctracted from PBMC cells using ExtractRNA reagent (Evrogen; Russia)

in accordance with the manufacturer's recommendations. The mRNA molecules encoding VHH fragments were used to prepare the cDNA with the *CH2-IgG–sp rev* primer (Table) using the ProtoScript cDNA kit (NEB; USA). Further amplification was carried out using high-precision polymerase Tersus (Evrogen; Russia) and primers AlpVHH3 uni fwd, AlpVH4 uni fwd, AlpVH3 uni fwd, AlpVHH-R1 and AlpVHH-R2 for creating libraries containing collections genes VH3, VHH3 and VH4, or with primers AlpVHH3 uni fwd, AlpVHH-R1 and AlpVHH-R2 to create a library containing only genes from the VHH3 family (Table).

Construction of expression cassetes for selection by ribosome display

Linear expression cassetes were prepared by performing bridge-PCR with Ck gene fragment, which was amplified with primers Flag-Ck (CGGATCCGGATTACAAGGAC GACGACGATAAGACTGTGGCTGCACC) and Ck/for 4 (AACACTCTCCCCTGTTGAAGCT); and a set of VHH fragment sequences. Bridge-PCR was performed with primers RD1x (GGATCCTAATACGACTCACTATAGGGAACAGACCACCATGT CTAG) and Ck/for 4. Resulting DNA fragments were isolated from agarose gel using the Cleanup standard (Evrogen; Russia) reagent kit and used for ribosome display selection.

Preparation of phagemid libraries for phage display selection

Phagemid libraries were prepared by cloning the amplified VHH sequences into a modified pHEN2-XB phagemid containing the restriction sites XbaI and BamHI between the sequences of the periplasmic localization signal PelB and c-Myc epitope at the indicated restriction sites, using XbaI and BamHI-HF restriciton endonucleases (NEB; USA) и T4 DNA Ligase (NEB, USA). Phagemid libraries weretransduced into TG-1 electrocompetent cells using the Genepulser (Bio-Rad; USA) electroporator.

Selection with ribosome display

Selection was carried out according to the method described elsewere [7]. Briefly, the TNT T7 Quick for PCR (Promega; USA) *in vitro* transcription-translation kit was used, 20 µl of the mixture and 100 ng of expresson constructs were taken for the reaction. The target (PDLIM4 fragment, amino acids 111–224, corresponding to the linker region between the LIM and PDZ domains) was immobilized by the C-terminal biotin on hydrophilic streptavidin magnetic microspheres conjugated to streptavidin (NEB; USA) in an amount of 100 ng per 5 µl of microspheres; microspheres treated with biotin were used for counter-selection. Restoration of a full-size cassetes after each selection round was performed with OneTaq Onestep RT-PCR kit (NEB; USA) and HSTaq polymerase (Evrogen; Russia) using primers RD1x, RT1 (ACTTCGCAGGCGTAGAC) and Kc/for 9 (AACACTCTCCCCTGTTGAAGCTCTTTGTGAC GGGCGAGCTCAGGCCCTGATGGGTGACTTCGCAGGCG TAGACTTTG). Selected constructs were cloned directly after reverse transcription step using XbaI and BamHI-HF restriciton endonucleases (NEB; USA).

Phage display selection

Selection was carried out according to the published protocol [8] using an antigen immobilized in accordance with the description given above, without the counter-selection step.

Production of soluble forms of VHH fragments

Individual clones of TG-1 cells identified after selection were used to obtain bacteriophage preparations, which were then transduced to HB-2151 cells cultured in M9 medium. Transduced cells were cultured on solid medium in the presence of ampicillin, after which individual colonies were used to obtain soluble protein in a liquid medium in accordance with the protocol [9]. Purification was performed using Ni-NTA magbeads (Cube Biotech; Germany) as recommended by the manufacturer.

Evaluation of library enrichment during ribosome and phage displays

For phage display, the assessment was carried out in accordance with the published protocol [8]. Wells of an immunological plate with sorbed PDLIM4 (111–224 aa) or

Table 1. Primer sequences used for amplification of VHH fragments

AlpVHH3 uni fwd	GAACAGACCACCATGTCTAGASAGKTGCAGSTSGTRGAGTCTGKGGGAGG
AlpVH4 uni fwd	GAACAGACCACCATGTCTAGASAGGTGCAGSKGCAGGAGTCGGGCCCAGGC
AlpVH3 uni fwd	GAACAGACCACCATGTCTAGASARKTGCRRSTSGTRGAGWCYGKGGGRGG
AlpVHH-R2	CCTTGTAATCCGGATCCGGTTGTGGTTTTGGTGTCTTGGG
AlpVHH-R1	CCTTGTAATCCGGATCCGGGGGGTCTTCGCTGTGGTGCG
CH2-IgG–sp rev	GGTACGTGCTGTTGAACTGTTCC

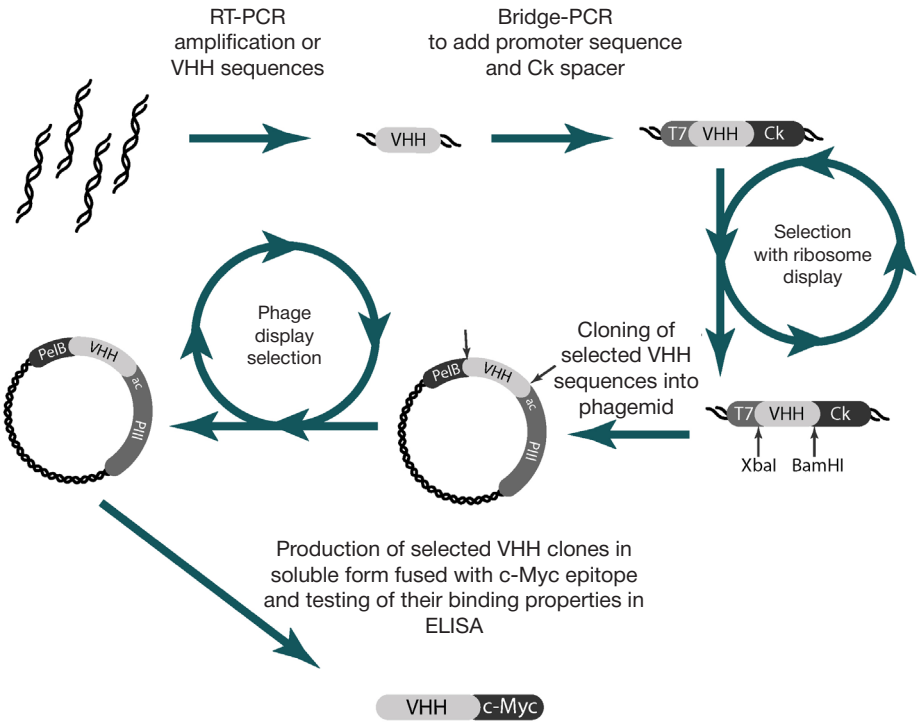

Fig. 1. Schematic representation of selection process combining ribosome and phage display

bovine serum albumin (control) were treated with preparations of phage particles displaying VHH fragments, obtained after each round of selection. Detection was performed using horseradish peroxidase-labeled antibodies to phage M13 (Sino Biological; China; cat. 11973-MM05T-H). For selection using the ribosome display, instead of phage particles, mixtures of mRNA–ribosome–protein complexes were obtained using *in vitro* transcription-translation kit TNT T7 Quick for PCR (Promega; USA). Detection was performed using antibodies to the Flag epitope labeled with horseradish peroxidase (Proteintech; USA; cat. HRP-66008).

Evaluation of the binding capability of individual variants of VHH fragments

The wells of immunological plate were coated either with PDLIM4, or with total protein extracts obtained from MBA-MB-231 and T47D cell lines in accordance with standard protocols, in an amount of 10 μg of cell extract per well. Next, wells were treated with preparations of purified VHH fragments, obtained according to the procedure described above; detection was performed using biotinylated antibodies to the Myc epitope (SciStoreLab; Russia; cat. PSM003BN-100) and streptavidin conjugated with horseradish peroxidase (R&D Systems; USA; cat. DY998). To obtain statistically reliable results, each reaction was reproduced in three independent replications.

RESULTS

Many studies on the selection of VHH fragments for various targets are based on the use of a single primer set developed over 15 years ago for amplifying VHH sequences [10]. Since then, the alpaca and llama VHH3 genome locus has been studied using deep sequencing and the sequences of all of its genes have been determined [11]. In addition, it has been reported that some genes of the VH4 and VH3 loci can also participate in formation of mature VHH antibodies. We collected all known sequences of the VH3, VHH3 and VH4 genes, and made a set of primers for amplifying all members of each of the gene families (Table). For reverse primers, we used well-proven sequences corresponding to unique hinge regions of VHH antibodies. Each primer variant was tested in PCR on alpaca cDNA generated using a primer on the CH2 region of the immunoglobulin heavy chain, which is common to VHH antibodies and antibodies of traditional structure. Sequencing of individual clones showed that each of the primer variants specifically amplifies the sequence variants belonging to the corresponding family.

Each additional stage of amplification of the library of VHH fragments leads to a change in the representation of the variants and the leaching of rare sequences from it. To avoid reducing the quality of libraries when combining ribosome and phage displays, we modified the pHEN2 phagemid and introduced restriction sites into primers to create ribosome display constructs, which allowed us to directly clone the VHH fragments into the phagemid library (Fig. 1).

In order to check whether the combination of two selection methods can lead to an increase in its efficiency, two libraries of VHH fragments were constructed from a common initial alpaca cDNA: library 1 consisted solely of sequences belonging to the VHH3 family, library 2 consisted of sequences included in the VH3, VHH3 and VH4 families. Each library was cloned into a phagemid, and also used to generate a linear construct for selection in a cell-free system. Phage libraries were subjected to three rounds of selection, and libraries of linear constructs after the first round of selection by ribosome display were

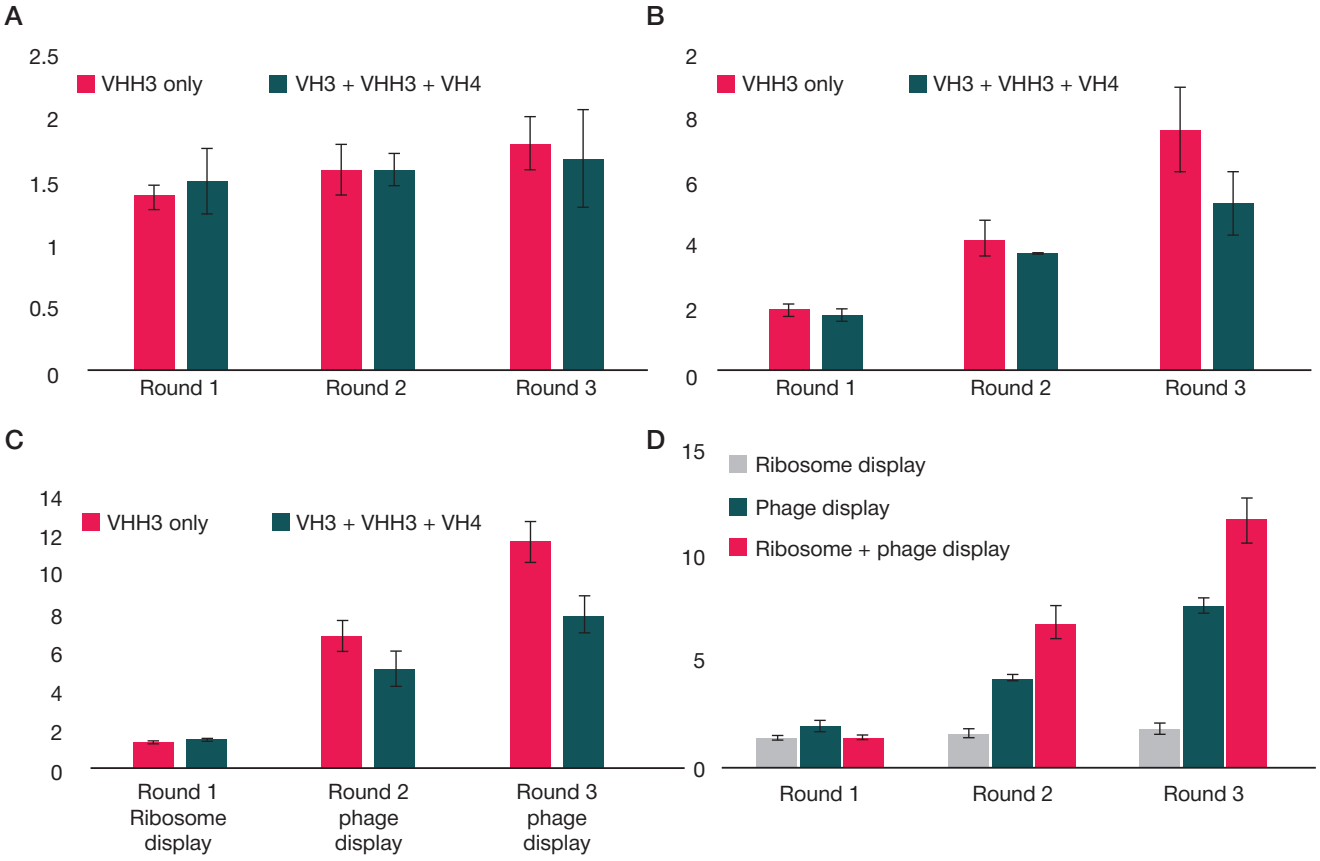

Fig. 2. Enrichment of libraries of VHH fragments during selection: with ribosome display (**A**); with phage display (**B**); with combination of ribosome and phage display (**C**). Comparison of enrichment by all methods for VHH3 library (**D**)

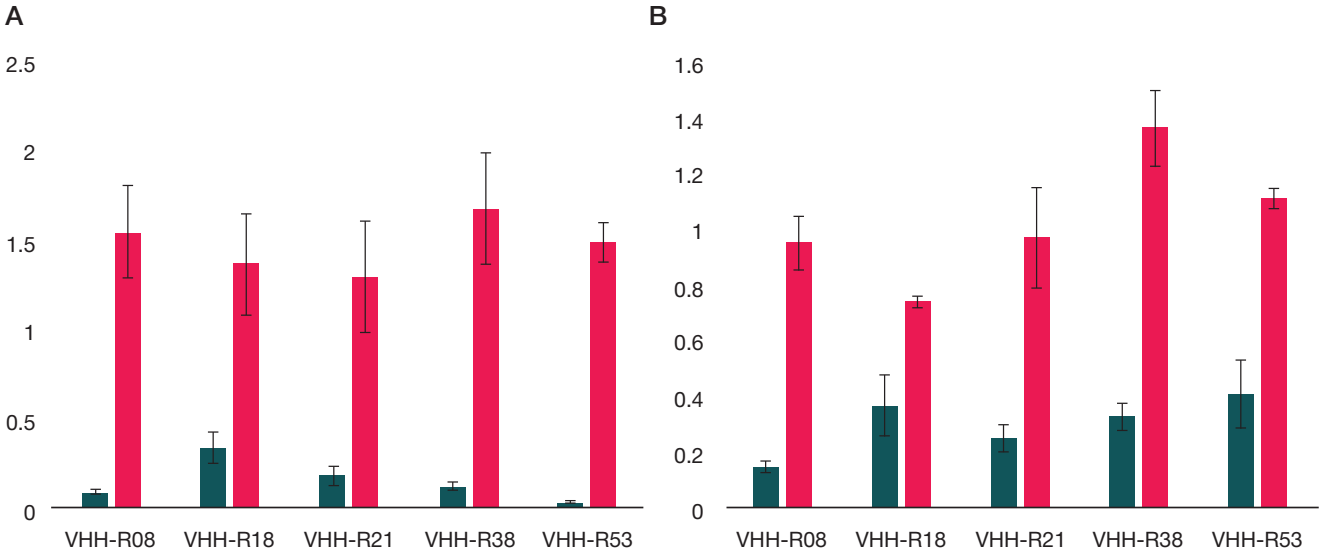

Fig. 3. Comparison of binding properties of five selected VHH fragments in ELISA

divided into two parts: one was subjected to two more rounds of selection, and the second was cloned into phagemid and selected by phage display. After each round, the level of enrichment of affinity fragments was estimated for each library using direct ELISA against negative control (BSA) and the target (PDLIM4 protein linker region). The ratios of signals obtained in the reactions vs control were used to assess the presence of desired VHH sequences in the library (Fig. 2).

The results showed that the library, consisting solely of sequences of the VHH3 family, demonstrated high rates of enrichment with affine variants in all three selection modes. Based on this, we can conclude that the VH3 and VH4 families do not make a significant contribution to the formation of a variety of single-chain VHH antibodies, and their inclusion in the resulting library is impractical. If we compare the efficiency of selection with different methods from the library of sequences of the VHH3 family, the selection in the ribosomal display was much less effective than in the phage display. On average, enrichment from round to round was 26.45% for ribosomal and 220.4% for phage displays. At the same time, the use of a combination of selection methods made it possible to increase the average enrichment to 355%.

After three rounds of selection using the phage display and the combined method, 35 individual clones were isolated and analyzed from the final enriched libraries. Contrary to expectations, the variety of sequences in the final library after combined selection was higher than after selection using the phage display method — 18 different types of sequences were found, while in phage selection there were only 11.

To assess specificity and affinity of interactions between selected VHH fragments and the target, five most represented variants obtained after the combined selection were expressed as monomers in strain HB-2151; purified using affinity chromatography and tested by ELISA. The target was either the PDLIM4 antigen preparation (the central part of the protein, unique to this member of the PDLIM family), or the total protein extracts of the MDA-MB-231 and T47D cell lines characterized by the normal and knockout status of the PDLIM4 gene [12], respectively (Fig. 3).

The experimental results showed that all five variants of VHH fragments are capable to specifically bind the PDLIM4 protein. Variant VHH-R53 possess the maximum specificity in the experiment with purified PDLIM4 protein, but at the same time, it intensely stained a PDLIM4-negative sample of the protein extract from T47D cells. VHH-R08 had a slightly worse specificity when tested on the purified protein preparation, but showed the greatest difference in signals in experiments with total protein extracts.

DISCUSSION

Regardless of the method of selection used, we observed differences in the dynamics of the enrichment of functionally active VHH fragments. It can be assumed that the proportion of VHH antibodies for the VH3 and VH4 gene families is insignificant, and that the combined library may contain a large number of defective variants of VH fragments that could specifically bind the antigen only in conjunction with the VL fragment and make selection difficult. Perhaps some tasks may require the use of such a combination of VHH and VH fragments. For example, since the framework structure of antibodies of the VH4 family of alpaca is very similar in structure to human antibodies, such antibodies are well suited for further humanization [13]. However, for routine selections, it is preferable to use a library containing only members of the VHH3 family.

The relatively low selection efficiency when using a ribosome display can be attributed to two factors: the use of too mild washing conditions that prevent the removal of low-affinity sequences, or the systematic cross-contamination of libraries with DNA fragments that are not completely removed at the stage of preparation of functional units of the library. At the same time, it should be noted that the use of more stringent conditions for the selection and DNA removal can lead to the destruction of the target complexes of mRNA–ribosome–protein. It seems that further optimization of the conditions of this selection method can increase the efficiency of selection, but it is unlikely to improve the result qualitatively. Interestingly, although in the combined selection method, the efficiency of the first round, which used the ribosome display, was relatively low (37% enrichment versus 97% in the first round of phage display), the final efficiency of the combined selection was improved dramatically. Probably, this combination made it possible to increase the representation of most capable variants of VHH fragments at the library cloning stage into a phagemid, while the “parasitic” sequences that persisted from round to round when selected in the ribosome display were removed during the subsequent round of phage display. As a result, the combined selection method led to the elimination of the

bottleneck which is characteristic of the phage display method. This is also supported by the greater variety of different VHH fragments found as a result of combined selection.

The selected variants of VHH fragments demonstrated high specificity for the PDLIM4 protein in *in vitro* experiments. However, in experiments with total protein extracts of cells, some variants, apparently, were able to bind to other proteins of the PDLIM family. For purpose of detecton of the status of intracellular PDLIM4, the most promising is the selected variant VHH-R08, for which non-specific binding was minimal.

It can be concluded that the developed for combined selection method makes it possible to achieve better results, without increasing labor costs and selection time, whithout the requirement to introduce additional amplification stages when constructing phagemid libraries.

CONCLUSIONS

A new primer system has been successfully tested for amplifying all members of the VHH3 family and creating libraries of VHH antibody fragments for combined selection using the ribosome and phage display methods. Comparison of the efficiency of selection showed that the combined method allows to achieve greater enrichment of the library with high-affinity fragments, and to reduce the loss of the initial diversity of the repertoire during cloning into a phagemid vector. By selecting using the combined method, it was possible to obtain a greater number of variants of high-affinity sequences, and all the VHH fragments subjected to individual testing were able to specifically recognize the target (PDLIM4 protein fragment) both in reactions with a purified protein preparation and in reactions with total protein extracts of cells.

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