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New Method for Rapid Screening of VHH clones Selected by Ribosome Display

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Abstract

Ribosome display is an advanced method for cell-free in vitro selection and directed evolution of antigen-binding molecules from large DNA libraries of VHVL and VHH antibody fragments. Being the most efficient and high-throughput method for selection of high affinity binders, ribosome display has some drawbacks in its practical use. Selection and development of affinity ligands using ribosome display typically include cloning of a pool of selected sequences in the expression vector and subsequent evaluation of their individual properties, which is sometimes very inefficient. In this work, we developed a protocol that allows to select and screen for highly specific clones much faster, before cloning them to expression vectors and setting up bacterial expression. For this purpose, we used immunoassay on individual in vitro generated affinity fragments which are evaluated for interaction potential with the immobilized antigen. Our method was tested using individual VHH fragments from ribosome display library which was obtained after six round of panning on CD44 protein.

Keywords: ribosome display, VHH antibody fragments, DNA libraries, antibody selection

INTRODUCTION

Ribosome display is an advanced method for cell-free in vitro selection and directed evolution of antigen-binding molecules from large DNA libraries of VHVL and VHH antibody fragments [1, 2]. This method is based on the principle of coupling individual nascent proteins to their corresponding mRNA, through formation of stable protein–ribosome–mRNA (PRM) complexes. This permits for simultaneous isolation through affinity to ligand of functional proteins together with their encoding mRNAs. Unlike phage display, where selection is limited by efficiencies of bacterial cell transformation and phage infection process, ribosome display is devoid of these restrictions. This advantage allows to construct and use for selection RNA libraries with complexity of up to 10^{13} individual variants [3]. Selection by ribosome display is much faster than phage display due to the same principle being used both for selection and expression of selected protein molecule. A cycle of the eukaryotic ribosome display includes the following steps: formation of protein-ribosome-mRNA complexes based on DNA libraries in cell-free transcription-translation system, degradation of the original DNA, selection of complexes by binding them to ligands, *in situ* cDNA generation by reverse transcription, and full length DNA fragment regeneration by subsequent PCR [4,5,6].

Being the most efficient and high-throughput method for selection of high affinity binders, ribosome display has some drawbacks in its practical use. In spite of selection with ribosome display being potentially faster compared to phage display or cell display, the final stage of selection comprises rather laborious search for individual high affinity clones. Published studies describing selection and development of affinity ligands using ribosome display typically include cloning of a pool of selected sequences in the expression vector and subsequent evaluation of their individual properties, which is sometimes very inefficient [7]. The aim of this work was to develop a protocol that allows to select and screen for highly specific clones more rapidly, before cloning them to expression vectors and setting up bacterial expression. For this purpose, we used immunoassay on individual in vitro generated affinity fragments which are evaluated for interaction potential with the immobilized antigen [8,9].

MATERIALS AND METHODS

Primer design

To assemble VHH libraries for subsequent rounds of selection and evolution of VHH fragments on the ribosome display, it was necessary to synthesize the optimal set of primers according to developed scheme (Fig.1).

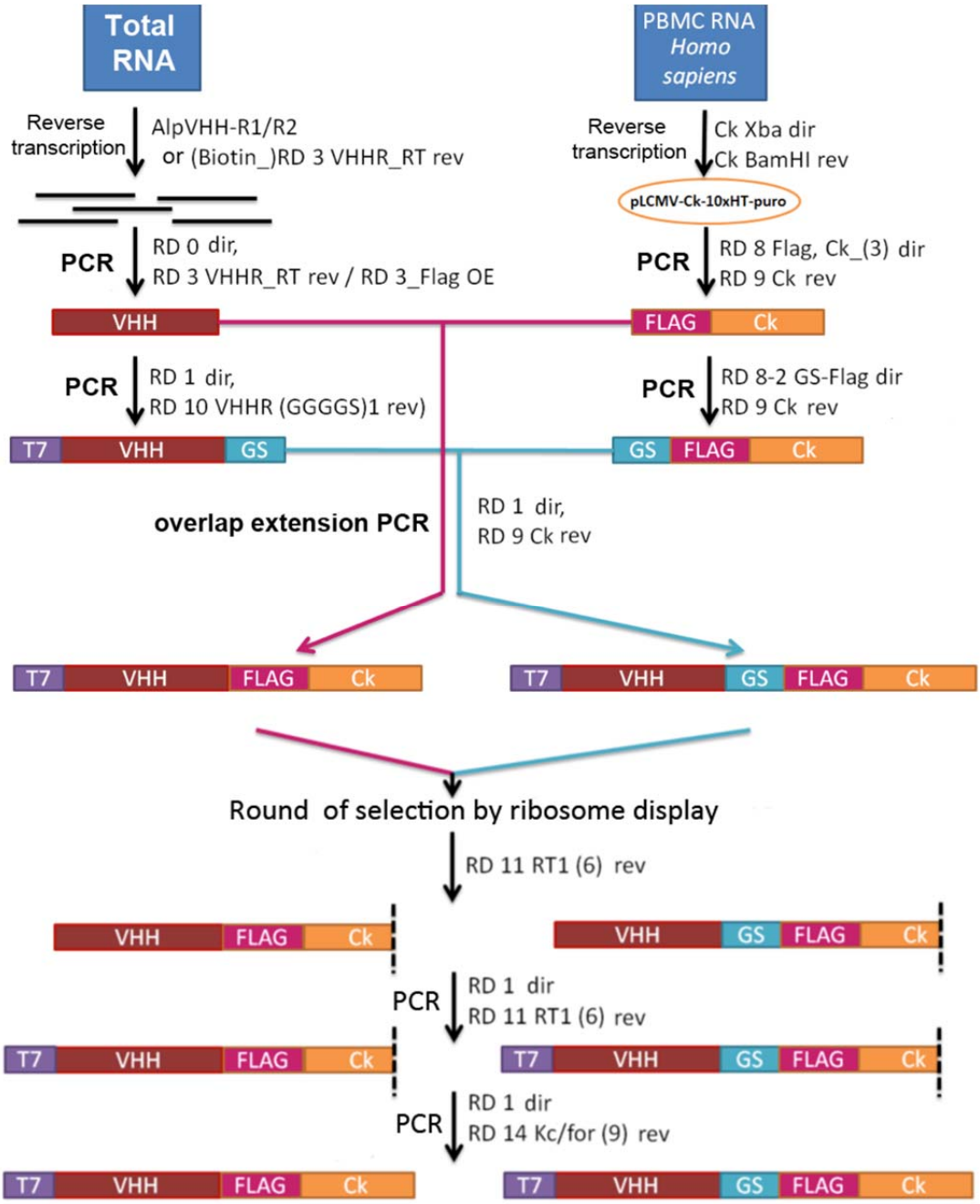

Figure 1 – Schematic representation for construction of VHH fragments library for ribosome display

To amplify and insert VHH fragments into expression cassette we used primers specific to VHH fragments of Camelidae [4]. These primers are efficient for creation of high diversity DNA library; being non-species-specific, they also efficiently amplify non-standard VHH fragments from alpaca (*Vicugna pacos*) genetic material.

For reverse transcription we used a mix of two reverse primers AlpVHH-R1, AlpVHH-R2. For further amplification we added direct primer AlpVh-F1 to the reaction mixture which is annealed at the 5'-terminal end of VHH [10]:

AlpVh-F1:
5’-GATCGCCGGCCAGKTGCAGCTCGTGGAGTCNGGNGG-3’

AlpVHH-R1:
5’-GATCACTAGTGGGGTCTTCGCTGTGGTGCG-3’;

AlpVHH-R2:
5’-GATCACTAGTTTGTGGTTTTGGTGTCTTGGG-3’.

We also used an alternative set of primers for reverse transcription [6]. Degenerate primers sequences allow to amplify VHH from genetic material of different species like *Vicugna pacos/Lama pacos*, *Lama glama*, *Camelus dromedarius* and *Camelus bactrianus*. These primers allow to produce a library which captures wider VHH antibody diversity and increases probability of detection highly specific VHH molecule.

RD 3 VHHR_RT rev:
5'-TSWGGAGACRGTGACCWGGGTCCC-3'

RD 3_Flag OE:
5'-CTTATCGTCGTCGTCCTTGTAATCTSWGGAGACRGTGACCWGGGTCCC-3'

RD 10 VHHR (GGGGS) 1 rev:
5'-GCTTCCACCGCCTCCTSWGGAGACRGTGACCWGGG -3'

RD 11 RT1 (6) rev:
5'- ACTTCGCAGGCGTAGAC -3'

Complete expression constructs for ribosome display selection of VHH fragments were obtained by using following primers:

RD 0 dir:
5'-GAACAGACCACCATGGCCSAGGTGSAGSTSSWGSMGTC-3' – annealed at the 5'-terminal end of VHH

RD 1 dir:
5'-GGATCCTAATACGACTCACTATAGGGAACAGACCACCATG-3'

RD 1 long sup:
5'-GCAGCTAATACGACTCACTATAGGAACAGACCACCATGGCCSAGGTGS-3'

RD 8 Flag, Ck_(3) dir:
5'-GATTACAAGGACGACGACGATAAGACTGTGGCTGCACCATCTGTCT-3'

RD 9 Ck/for (4) Ck rev:
5'-AACACTCTCCCCTGTTGAAGCT-3'

RD 14 Kc/for (9) rev:
5'-AACACTCTCCCCTGTTGAAGCTCTTTGTGACGGGCGAGCTCAGGCCCTGATGGGTGACTTCGCAGGCGTAGACTTTG-3'.

Additionally, to isolate VHH coding mRNA from total RNA pool we used biotinylated reverse primers on 3'-terminal region of the VHH genes, Biotin_RD 3_Flag OE and Biotin_RD 10 VHHR (GGGGS).

Library construction

Naïve library was constructed from total RNA derived from alpaca lymph nodes. Two peripheral lymph nodes from non-immunized adult alpaca were homogenized and RNA was isolated with Trizol reagent.

To enrich the obtained sample with VHH-coding mRNA sequences, total RNA was preincubated at 65C with Biotin_RD 3_Flag OE and Biotin_RD 10 VHHR (GGGGS) 1 reverse primers; hybridized mRNA was immobilized by addition of hydrophilic streptavidin superparamagnetic particles (NEB, USA) and unbound mRNA was removed during washing. VHH-enriched samples were then reverse transcribed and amplified using OneTaq One-Step RT-PCR Kit (NEB, USA). For reverse transcription, an alternative set of primers AlpVHH-R1 and AlpVHH-R2 also was used. Further amplification was performed using a mixture of these reverse primers and the forward primer at the 5'-terminal region (AlpVh-F1).

For selection with ribosome display, a crucial step is protein-ribosome-mRNA (PRM) complexes formation, to link phenotypic features - affinity VHH fragments and corresponding genotype (mRNA). To avoid dissociation of ribosome and to extend newly synthesized protein chain further away to ensure proper folding, mRNA sequence encoding VHH should be followed by linker without stop codon. As a result, VHH fragments and corresponding genetic material remain bound together, which results in selective amplification of sequences linked to most favorable phenotype. We used constant fragment of the light kappa chain of human immunoglobulin (Ck) as a linker. Ck fragment was amplified by PCR from pLCMV-Ck-10xHT-puro plasmid which was constructed previously, with a FLAG tag fused to 5'-end.

Next steps of library construction include several sequential PCR reactions. First VHH fragments were coupled with T7-RNA polymerase promoter using RD1 dir and RD 10 VHHR (GGGGS) 1 rev primers. Then overlap extension PCR was performed with a mixture of RD 1 dir and RD 9 Ck/for (4) Ck rev oligonucleotides. Resulting library consisted of constructs that included the following key elements: VHH- fragments, T7 RNA polymerase promoter, GS linker and Ck fragment, fused with FLAG tag (Fig.1)

Ribosome display selection

Each round of selection by ribosome display includes two consecutive PCR reactions resulting in full-length constructs regeneration.

First, we performed amplification using a mixture of

RD1 dir and RD 11 RT1 (6) rev primers at the 5'- terminal region to add T7 promoter, that was lost during previous step of selection. Second round of PCR introduced region of Ck at the 3'-terminal region using combination of oligonucleotides RD1 dir and RD14 Kc/for (9). Further, PCR products were directly used for either in vitro transcription or coupled transcription/translation (Fig.1).

pAL-T clone selection

For cloning individual VHH fragments after selection, we used pre-cut pAL-T vector (Evrogen) [11]. This vector is well suited for cloning of DNA libraries, as does not require digestion with restriction endonucleases. Also, a blue and white selection of clones with inserts is possible. Transformed clones were spreaded on 25 cm Xgal/IPTG LB agar plate and incubated overnight at 37C. Next day, white colonies were screened by PCR for VHH inserts, insert-containing samples with then used for coupled transcription-translation for individual screening of VHH clones affinity.

Immunoassay

Immunoassay testing was performed on 96 well plate. 46 individual PCR fragments were randomly chosen and tested against CD44-coated wells with BSA-coated wells used as a control. 96-well plate (Maxisorp, Nunc) was precoated overnight with CD44 at 250 ng per well. For coupled transcription/translation reactions we used TnT T7 Quick for PCR DNA System (Promega). A 20 ul reaction mixture, contained 10 ul master mix, 1 mM methionine and 100 ng of PCR product incubated at 30C for 60. After blocking, wells were coated with ½ of TnT reaction mix and incubated for 1.5 hours at room temperature. VHH binding was detected by primary anti–Flag antibody (Sigma) and secondary anti-rabbit HRP antibody (Santa Cruz Biotechnology).

RESULTS AND DISCUSSION

Pools of total RNA were isolated from the lymph nodes of alpaca (Fig.2).

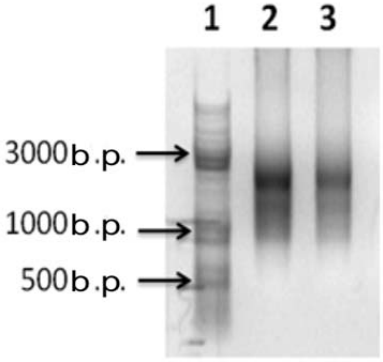

Figure 2 – Total RNA isolated from lymph nodes of alpaca. 5 µl of material was applied to each well. From left to right: 1 - GeneRuler 1 kb DNA Ladder mix 100 to 10,000 bp (250 ng); 2 - Fraction of total RNA isolated from the peripharyngeal lymph node; 3 - Fraction of total RNA isolated from the lymph node of the base of the neck.

Fractions of mRNA molecules encoding VHH were obtained from total RNA pool using the biotin-streptavidin system. Enriched fraction of VHH-coding mRNA was used in one step reverse transcription – polymerase chain reaction with primers RD 0 dir and RD 3_Flag OE (Fig.3). mRNA isolation from pools of total RNA using biotinylated primers, reverse transcription and amplification were carried out twice. Linear expression constructs were generated, comprising of VHH fragments with T7-RNA fused with polymerase promoter and Kozak consensus sequence fused at 5'-terminus and 3'-terminal region consisting of a GS-linker, FLAG tag and Ck fragment without stop codon. Resulting library was subject to 6 rounds of selection against CD44-coated magnetic microbeads.

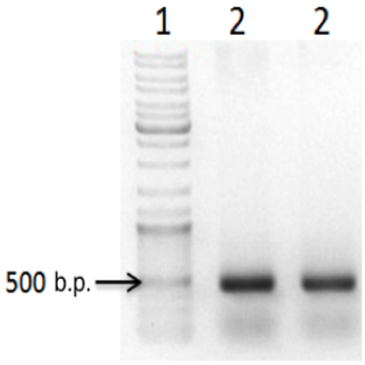

Figure 3 – PCR fragments amplified from cDNA of VHH genes. All fragments are ~500 bp length.

To estimate diversity of VHH library after 6 rounds of selection, we cloned a portion of selected library into pAL-T vector and randomly chose individual colonies. Clones were tested by PCR and fingerprinted by HaeIII restriction as it shown on the Fig.4.

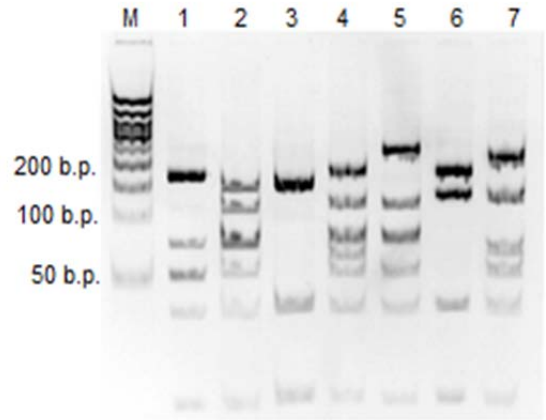

Figure 4 – Fingerprint of seven variants of PCR fragments after HaeIII restriction on 12% PAAG gel. M- 50 bp DNA ladder (Evrogen), 1-7 - PCR fragments.

After initial testing for VHH insertion and fingerprinting, unique VHH-bearing clones were used to generate truncated expression constructs devoid of Ck and containing stop-codon after Flag tag. These constructs were then subjected coupled transcription-translation reaction, and generated individual Flag-tagged VHH variants were assessed for their binding properties (Fig.5).

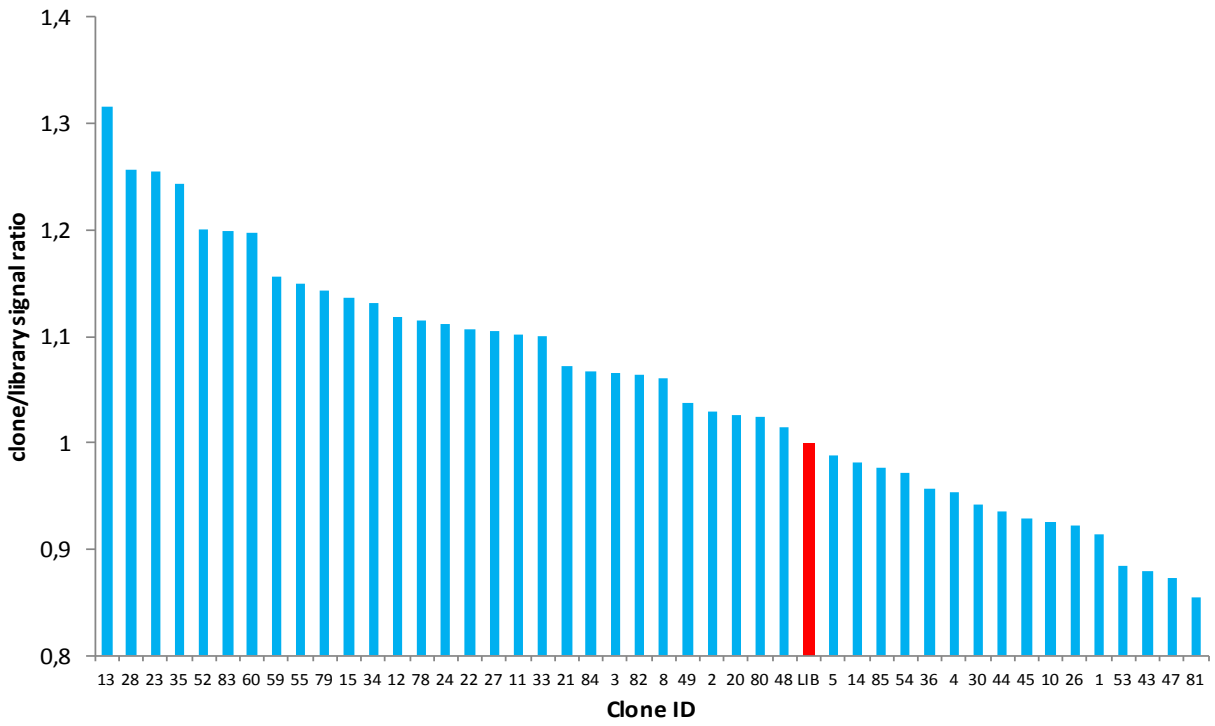

Figure 5 – Affinity assessment of VHH clones. X axis – VHH clone ID, Y axis - signal ratio between clone's and reference control (CD44/BSA).

For reference control, we used total VHH library generated after 6-th round of selection. HRP activity was measured for each clone and total library in BSA-coated and target protein (CD44) coated wells. We calculated target-to-control values (signal with CD44 / signal with BSA), and then compared each clone to reference control (total VHH library). Resulting ratios are presented in figure 5.

Analysis of 46 individual clones revealed substantial variations in their binding affinity. Around 40% performed worse than total VHH library, among others, 7 had over 20% higher binding properties than reference, and only 1 had 30% higher performance.

The developed screening method was not previously described, aim of the study was to search for individual affinity variants of VHH antibodies. Previously, when working with the phage library of VHH antibodies, screening was carried out either on monoclonal phages presenting antibodies on their surface or soluble antibody fragments expressed in bacteria. The latter is especially difficult in the case of screening large amounts of clones. Screening performed on soluble VHH fragments generated in vitro allows it to be carried out immediately after selection and is compatible with libraries selected by both phage and ribosome display.

CONCLUSION

In this work, we developed and tested rapid method of affinity testing of individual VHH fragments from ribosome display library which was obtained after six round of panning on CD44 protein.

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REFERENCES:

[1] Hanes, J., Jermutus, L., Weber-Bornhauser, S., Bosshard, H.R., Plückthun, A. Ribosome display efficiently selects and evolves high-affinity antibodies in vitro from immune libraries. Proc. Natl. Acad. Sci. U. S. A. 1998; 95: 14130-14135.

[2] Hamers-Casterman, C., Atarhouch, T., Muyldermans, S., Robinson, G., Hamers, C., Songa, E.B., Bendahman, N., Hamers, R. Naturally occurring antibodies devoid of light chains. Nature. 1993; 363: 446-448

[3] Dufner, P., Jermutus, L., Minter, R.R. Harnessing phage and ribosome display for antibody optimization. Trends Biotechnol. 2006; 24: 523-529.

[4] He, M., Liu, H., Turner, M., Taussig, M.J. Detection of protein-protein interactions by ribosome display and protein in situ immobilisation. N Biotechnol. 2009; 26: 277-281.

[5] He, M., Taussig, M.J. Ribosome display: Cell-free protein display technology. Technique review. 2002; 1: 204-212.

[6] He, M., Taussig, M.J. Eukaryotic ribosome display with in situ DNA recovery. Nat Methods. 2007; 4: 281-288.

[7] Li, T., Vandesquille, M., Bay, S., Dhenain, M., Delatour, B., Lafaye, P. Selection of similar single domain antibodies from two immune VHH libraries obtained from two alpacas by using different selection methods. Immunology Letters. 2017; 18: 89-95.

[8] Pelham, H.R.B. and Jackson, R.J. An Efficient mRNA-Dependent Translation System from Reticulocyte Lysates. Eur. J. Biochem. 1976; 67: 247–56.

[9] Krieg, P.A. and Melton, D.A. Functional messenger RNAs are produced by SP6 in vitro transcription of cloned cDNAs. Nucl. Acids Res. 1984; 12: 7057–70.

[10] Maass, D.R., Sepulveda, J., Pernthaner, A., Shoemaker, C.B. Alpaca (Lama pacos) as a convenient source of recombinant camelid heavy chain antibodies (VHHs). J Immunol Methods. 2007; 324: 13-25.

[11] Holton, T.A.; Graham, M.W. A simple and efficient method for direct cloning of PCR products using ddT-tailed vectors. Nucleic Acids Research. 1991; 19 (5): 1156.