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Research Article

Cloning of Anti-Human Epidermal Growth Factor Receptor 2 Nanobody Gene by pComb3xss Vector into ER2738 Bacterial System

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ABSTRACT
Antibody-mediated therapy for cancer treatment relies on the identification of selected molecular targets. This study is aimed to cloning monoclonal nanobodies into ER2738 (host bacterial cell), this antibody is derived from immunized camel with the purified recombinant HER2 protein. Following camel's immune response was measured by ELISA which showed the high titter (1/3200). Lymphocytes from the camel's blood were isolated, then total RNA was extracted, and first strand of cDNA was synthesized. The heavy chain antibody was amplified by nested PCR using (FPF-1 and FPRCH2) specific primers. The second PCR run was to amplify VHH gene (which is encoded for nanobodies) by using (VHH-F-PRIMER and MMA-VHH FOR 2) specific primers. Both of plasmid pComb3XSS and PCR production (V_{HH}) was digested using *sfil* enzyme (restriction enzyme) and both of them was ligated using T4DNA ligase enzyme and then transferred into *E.coli* ER2738 (host bacterial cell). The result of transformation efficiency showed (9×10^6 cfu/ml). The transformation was confirmed by PCR with specific primers.

Keywords: Nanobody, Cloning, pComb3XSS.

INTRODUCTION

Naturally produced immunoglobulin's (G, M, A, D, and E) are universal weapons against pathogenic threats. The predominant isotype in nature is IgG, a 150-kDa, IgG contributes up to 75% of all serums in Camelidae family [1], IgG is divided into subclasses are IgG_1, IgG_2, IgG_3. The immunoglobulin IgG_1 is a conventional antibody (two heavy chains and two light chains) with a molecular weight of ~150 kDa mammalian sera, in addition to conventional antibody IgG_1 is find in Camelidae family sera, the two other subtype immunoglobulin fractions are non-conventional antibodies are IgG_2 and IgG_3 (two heavy chains, and lack the light chains also lack the CH_1 domain); the absence of light chain and lacks of the CH_1 in heavy chain antibody are key characteristics of Camelid heavy-chain antibodies, "HCAb" and give small size ~90 kDa which are of the new discoveries in the recent time is called have heavy-chain antibodies, "HCAb" [2].

Nanobodies Structure

Nanobodies obtain from heavy chain only antibodies occurring naturally in Camelidae [3]. The heavy chain antibody has two antigen-binding sites (Fabs) which are called the variable domain of heavy-chain antibodies (V_{HH}), and referred to single domain antibody "sdAb" or "Nanobody a trading name of Ablynx" [4,5], this domain plays an important role for reaction with a different antigens. Cartilaginous fishes also have heavy-chain antibodies are called 'immunoglobulin variable new antigen receptor "IgVNAR fragments" [6] as showed in figure 1.

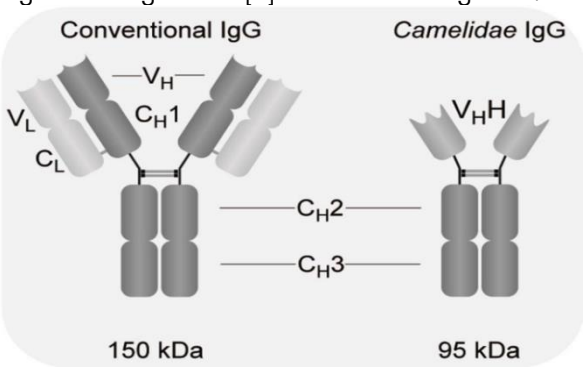


Fig.1: Schematic representation of antibodies in sera of Camelidae family.

Nanobodies Properties

Nanobodies have similar affinity and specificity of conventional antibodies [7] to different antigens, but it has unique features [8] are small proteins of 120 amino acids and, which is only tenth of a conventional antibody size that leads to more effective permeability to the tissues [7,9], nanbodies are easily eliminated renal system unlike whole antibodies [10,11,12], nanobodies can interact with a greater number and variety of epitopes than of conventional antibodies [13], they are easy on to be produce in yeast, mammalian cells, and bacteria, like *E. coli, Lactobacillus, P. pastoris, A. awamori, S. cerevisiae,* and tobacco plant with high yields [14], nanobodies are take into consideration non-immunogenic because of their high similarity with human V_H sequences; these rare properties triggered numerous applications in, diagnostics, therapy, and fundamental research [15,14], nanobodies can bind to their targets with a high affinity and can be output by simple methods with a lot of yields [16], nanobodies also demonstrate high fixity even at exposure to outside extreme conditions like very low/high pH and temperature [17], nanobodies are procured by cloning the V_{HH} gene and panning by phage display [18] by using M13KO7 helper phage, nanobodies are more heat-resistant survived at a temperature 90 ℃ for an hour without loss of their ability to bind antigens [19], nanobodies consist of 4 framework regions (FR_1, FR_2, FR_3, and, FR_4) and three complementarity-determining regions (CDRs), which are hyper variable loops responsible for antigen binding [20]. The antibodies are important tools can used to target a lot of antigens and good choice to cancer treatment with minimum toxicity.

MATERIALS AND METHODS

Camel Immunization

The camel was immunized with 10 ml subcutaneous injection of 100 μg of HER2 (Abcam), an equal volume of Freund's complete adjuvant (Santcruze). Booster injections for three times which were containing 100 μg [4] with amounting to volume of Freund's incomplete adjuvant (Santcruze) were given at intervals 21 days. Blood were collected 200 ml after the final injection after five days [21]. Blood samples were collected before each injection, and sera were used to evaluate the immune response by ELISA [22]. The ELISA was fulfilled: A total 20 wells of 96-well microplate were coated with 10 μg of HER2 in 100 μl of bicarbonate coating buffer (NaHCO3) (0.1 M Na2CO3, 0.1 M NaH-CO3, pH 9.5) and, incubation for 16 hours at 4 ℃. Next day, the antigen (HER2) was discarding and washed it three times by phosphate buffer (PB), then prepare blocking buffer (5% milk + PB) was added 250 μl and incubated it for one hour at 37 ℃. The wells were Wash three times with phosphate buffer (PB). The diluted serum (pre-immune and immune sera of Camel) 1/400, 1/800, 1/1,600 and 1/3,200 (added 100 μl for each well) were added. Two wells were without HER2 antigen and two wells were without antibody served as negative and incubated for 2 hours at 37 ℃. The wells were washed three times by Phosphate buffer. After that, anti-camel antibody (10μg/ml) were added 100 μl for each well and incubation for 1 hour at 37℃. Then anti-rabbit –HRP 100μl at a (1/1000 dilution) were added for each well incubation for 1 hour at 37 ℃. Substrate solution containing the chromogenic substrate 3, 3′, 5, 5′ Tetra-methyl benzidine (TMB), 100 μl were added incubated at 37 ℃ for 15-20 min. Finally the reaction stopped by adding 100μl /well of HCL and, the optical density was measured at 450 nm.

Camel Lymphocytes Isolation

A total of 200 ml of blood were collected from the cervical artery of the immunized camel, 5 days after the third injection. B- Lymphocyte cells were separated by Ficoll (Sigma-aldrich, Germany) according to Ivan *et al*; 2009. Interrupted gradient: 10 ml of blood were added with slowly into a tube which is containing 3 ml of Ficoll, and then the tube was centrifuged at 2000 g for 15 minutes. Then lymphocytes were washed with sterile phosphate buffer saline (PBS), then counted, and stored at -70 ℃.

RNA Extraction and cDNA Synthesis

The RNA extraction was performed by using high pure RNA isolation kit (Roche company kit, Germany), in accordance with the manufacturer's specifications, and assured by electrophoresis, and, RNA was then stored at -80 ℃. The first – strand cDNA was synthesized from a total RNA by using cDNA synthesis kit (Invitrogen, US), and the result of cDNA was stored at -20 ℃.

Nested PCR for Amplification V_{HH} gene

The V_{HH} was isolation from the variable domain of conventional antibodies (V_H) was carried out by using nested PCR on cDNA using the following primers: Forward primer: GAT GTG CAG CTG CAG GCG TCT GGR GGAGG. Reverse primer: CGC CAT CAA GGT ACC AGT TGA (South Korea, Pishgam)

First, the PCR reaction was performed with an initial denaturation step at 94℃ for 10 minutes followed by 30 cycles (at 94℃ for 30 seconds, at 66℃ for 35 seconds, and at 72 ℃ for 35 seconds), and a final extension at 72 ℃ for 10 minutes. Then, 600-bp and 700-bp bands (V_{HH}-hinge-C_{H2}) were purified by using the Gel Extraction kit (Roche, Germany) and used it as a

templates for the second PCR reaction by using specific primers for second PCR reaction are: Forward primer: CGT GGA GGCGGC CAT GGC CGA KGT SCA GCT. Reverse primer: ACT GGC CGG GGC CTG AGG AGA CGG TGA CCT G. (South Korea, Pishgam). The PCR product was analyzed by electrophoresis and purified with the gel extraction kit.

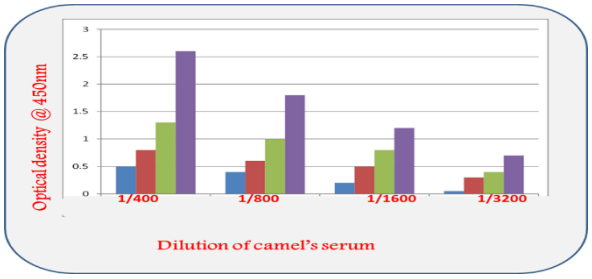
V_{HH} gene Cloning

After that purification pComb3xss vector (Addgene, US) from *E.coli* by using High pure plasmid Isolated kit (Roche, Germany) and digested with *sfil* restriction enzyme (Bio lab, England) and purified by the Gel Extraction Kit and then the digested *V_{HH}* genes was ligated with the plasmid Pcomb3x SS by T4 DNA ligase (Biolabs, England), and transferred into competent cells *E.coli* (ER2738) (NEB Company, Germany) used electroporation. The library size was evaluated by culturing 50 µl of the transforming on 100 µg/ml ampicillin LB agar plates. The colonies were counted to calculate the size of the library. V_{HH} cloning was confirmed by PCR on five randomly selected clones.

RESULT & DISCUSSION

Camel Immunization

The results of these experiments show strong reactivity of the post immunization (the antibody titer of immunization camel against HER2 reached a maximum 2.6 at a titter (1:400) dilution after the third injection, and that means the camel has good immune response for HER2 antigen figure 1, but for pre-immunization haven't as shown in figure 2.



	1/400	1/800	1/1600	1/3200
Normal serum	0.5	0.4	0.2	0.05
First injection	0.8	0.6	0.5	0.3
Second injection	1.3	1.0	0.8	0.4
Third injection	2.6	1.8	1.2	0.7

Fig.2: Evaluation of anti-HER2 antibodies in the camel's serum: A high signal was observed at 1:400 dilutions and gradually decreases with decreasing concentration of the antibody, and the camel have a good immune response for the HER2 antigen

PBMCs Isolation

To get peripheral blood mononuclear cell (PBMCs), the camel's blood was added to Ficoll density gradient media filled tubes and obtained many layers; one of them is called buffy coat. This layer contains peripheral blood mononuclear cell (PBMCs), and then PBMCs Count, by detecting the viable B lymphocytes or not viable, using 4% of trypan blue and the concentration B lymphocyte was calculated, and the viable cells/ml was 2×10^7.

RNA Extraction and cDNA synthesis of Camel's PBMCs

A Total RNA was purified from the prepared B lymphocyte by using a High Pure RNA Isolation Kit as shown in figure 2, and the RNA purified was used as a template for cDNA synthesis using oligo dT primers. A total RNA was used for cDNA synthesis by using cDNA synthesis kit and obtained a good band as illustrative up in figure 3, and it was separated on a 1 % agarose gel. The cDNA was used as a template for amplification genes which encoding to the variable domains (nanobodies) of the heavy chain antibodies (HCAb), and the yield of cDNA was stored at -20 ℃.

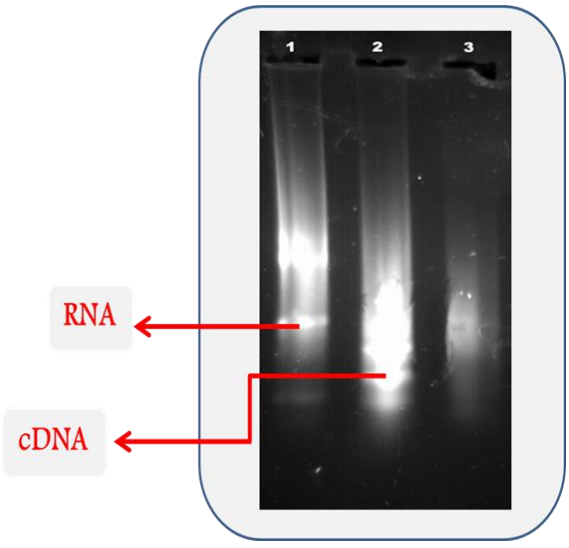


Fig.3: Lane 1: Analysis of the RNA extraction from camel's PBMCs. Lane 2: Analysis of cDNA from total RNA which used as a template for amplification genes encoding for the variable domains (nanobodies) for 20-30 min at 100 v.

The cDNA was used as template to amplify *V_{HH}* genes encoding nanobodies of the heavy chain antibodies (HCAb) by nested PCR reaction. The first PCR was done by using specific primers, and obtained three bands (figure 4), allowing for the discrimination between V_H and V_{HH} repertoire. This

PCR was for amplification V_H and V_{HH} fragments. The first band was a size of ~900 bp encoding the variable regions (V_H, C_{H1}, hinge and parts of the C_{H2} exons) of the heavy chain of conventional antibodies, the two bands (600 - 700 pb) were represented the variable regions (V_{HH}, hinge and part of the C_{H2} exons) of the heavy chain antibodies (HCAb) [22,23].

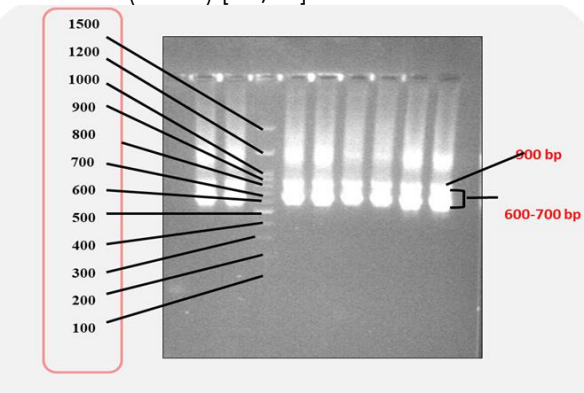


Fig.4: Analysis of the nested PCR product with agarose gel 1%: the first PCR for lane (1,2,3,4,5,6,7,8)with different sizes ~900 pb was a conventional antibody (V_H, C_{H1}, hinge region and C_{H2}), and lane (1,2,3,4,5,6,7,8) two bands were ~700 and 600bp (V_{HH}, hinge and C_{H2}). The whole PCR reaction sample was separated on a 1 % agarose gel for (20-30) min at 100 v.(gradient and optimal temp)

The bands were obtained as a heavy chain antibody (600 and 700 bp), purified from agarose gel, and used as templates for the second PCR with specific primers figure 5; these primers attach to framework 1 and framework 4 regions. V_{HH} fragment (Nanobodies band) was separation by used 1% (0.2 gr of agarose in 20 ml of TAE) agarose gel electrophoresis after staining with ethidium bromide.

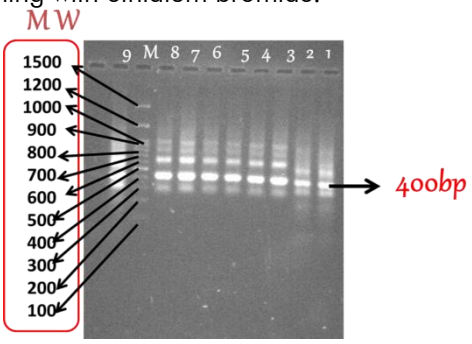


Fig.5: Analysis of the second PCR product with agarose gel (1%): In the second PCR, lane (1-9) appears band was 400pb (V_{HH} gene encoded for nanobodies). The whole PCR reaction sample was separated on a 1 % agarose gel for (20-30) min at 100 v.

Cloning of V_{HH} fragment (Nanobody) and Expression into pComb3X SS Vector.

At the beginning, the plasmid was purified from *E. coli* bacteria, and then the plasmid was digested by using restriction enzyme (*sfiI* enzyme) at the restriction site which presents in plasmid and band was obtained (3319 bp) as illustrative in figure 6, which appears linearized when separated on 1% agarose gel by using gel electrophoresis.

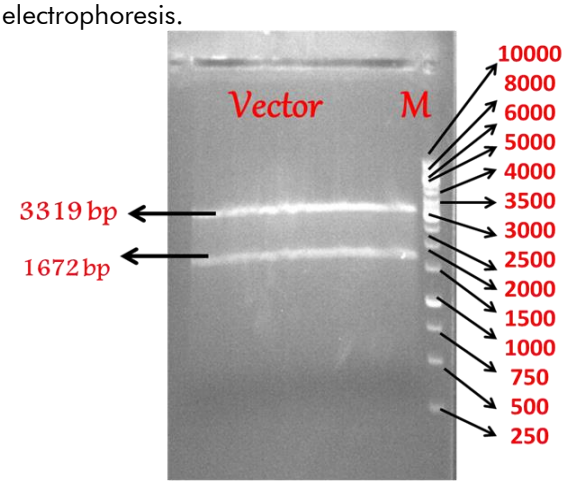


Fig.6: Gel electrophoresis of pComb3X SS plasmids digest: the plasmids were digested with *the sfiI* enzyme, and it was separated on a 1 % agarose gel for 20-30 min at 100 v.

The V_{HH} genes (Nanobodies) were successful cut with a restriction enzyme (*sfiI* enzyme) as show in figure 7 and get band was (400 bp), which appears linearized when separated by using gel electrophoresis 1% for 20-30 min at 100 V.

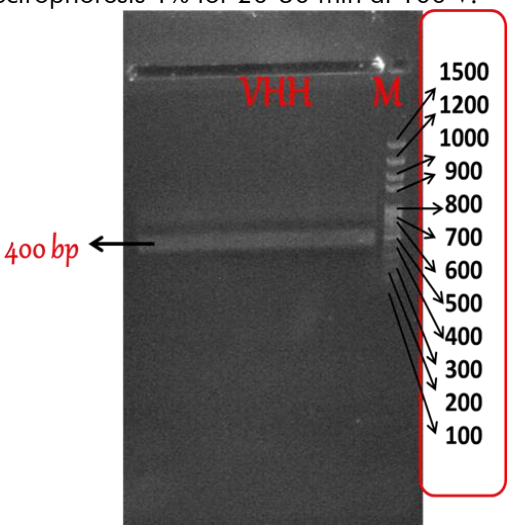


Fig.7: Gel electrophoresis of V_{HH} gene (nanobodies): the V_{HH} gene was digested with a *sfiI* enzyme; the V_{HH} gene was separated on a 1 % agarose gel for 20-30 min at 100 v. with used 100 bp ladder.

V_{HH} Gene (Nanobody) Cloning in pComb3X SS

The V_{HH} genes were ligated into the pcomb3X SS vector between sfil recognition sites which is catalysis in the formation of covalent phosphodiester bonds between the 3́ hydroxyl end and the 5́ phosphate end for transferred to ER2738 bacteria.

Electroporation

After ligation of V_{HH} gene with plasmid, both of them were transformed into suitable host cells (competent cell *E. coli* ER2738) is one of strain, then cultured on Luria-Bertani agar supplement media, and the bacterial cells were calculated and they gave a library size about (9×10^6 cfu/ml) as shown in figure 8.

Fig.8: Transformed cells which contain V_{HH} gene. ER2738 cultured on LB agar for 24 hr. at 37.

V_{HH} Gene Transform Confirmation

To verify whether the ligation was correctly done or not, a single colony of each constructed was selected randomly for V_{HH} gene detection by PCR, and a DNA fragment 580 bp was observed for the entire clone, as illustrative in figure 9.

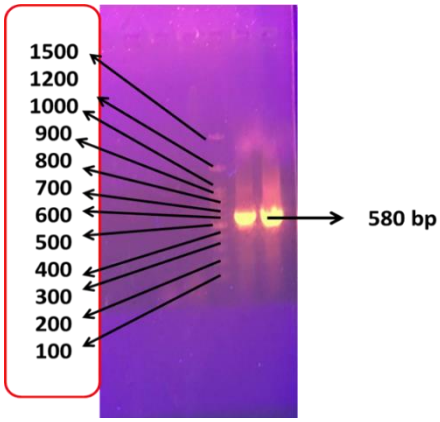


Fig.9: Gel electrophoresis to confirm colony PCR: Gel electrophoresis of colony PCR of constructs containing nanobodies (with ladder 100 bp), and a DNA fragment 580 bp was observed for the entire clone

DISCUSSION

Since the discovery of camelid's heavy chain antibodies about 20-25 years ago, naturally occurring heavy chain antibodies (hcAbs) derived from Camelidae family; antigen binding is mediated through a single domain, referred to as V_{HH} (variable domain of heavy chain antibodies) or single –domain antigen – binding fragment also called nanobodies. The main characteristics distinguishing VHH from other antibody fragments are a very small size (~ 15 kDa), extreme stability [24,25] and high solubility [26]. In addition, V_{HHs} have been shown to bind epitopes inaccessible for conventional antibodies [27,28]. Nanobodies can easily be generate in prokaryotes like *E. coli* [29,30]. Altogether, these features render V_{HHs} versatile tools for diverse applications in basic research, diagnostics and therapy, Nanobodies, because of their single domain nature, offer several advantages for biotechnological applications. Libraries of Nanobodies from immunized camels and llamas can be generated through a straightforward cloning procedure [25]. In a first step, the camel was a male (3 years) dromedary (*Camelus dromedarius*), and this age is optimal for immunization. For generation of anti-HER2 nanobodies, the camel was immunized with a HER2-Fc-recombinant protein is a weak immunogenic, this protein mixed with adjuvant is Freund´s adjuvant which contains heat-inactivated *Mycobacteria tuberculosis* in a mineral oil-surfactant mixture, this substance helped to stimulated the immune response and a raise both conventional antibodies (IgG_1 subclass) and heavy-chain-only antibodies (IgG_2 and IgG_3 subclasses) in response to the antigen [2]. A solid-phase ELISA on immobilized antigen using serum samples from day 0 (pre immunization) and post immunization [22] was used to monitor the humoral immune response of the camel, The camel's blood was added to Ficoll density gradient media filled tubes, and depended on Ficoll media, get many layers one of them is called buffy coat, this layer contain lymphocyte (PBMCs). A total RNA was extracted and used as template for cDNA synthesis. The cDNA was used as template to amplify genes encoding the variable domains (nanobodies) of the heavy chain antibodies (HCAb). In this study used nested PCR, to avoid the isolation of V_H genes. This approach can lead to mutagenesis and molecular diversity, which not only leads to library diversity but also prevents the contamination of the library with V_H genes [31], the second primers were designed in degenerated forms to allow the maximum isolation of V_{HH} genes, also the V_{HH} was distinguished from other antibody fragments are

its simple cloning procedure to obtain a single gene, and thus its easy selection from libraries [32,16].The main characteristics distinguishing V_{HH} from other antibody fragments are its simple cloning procedure to obtain a single gene, and thus its easy selection from libraries [32]. Many studies orientation to generation of monoclonal antibodies (mAbs) which is the one of the most advanced technologies and treated various diseases like autoimmune disease and infection disease and cancer [33]. Antibody fragments are now focus of most research since these small molecules have many advantages, and can be used for therapy purpose. There are a several monoclonal antibodies and single chain variable fragment (scFv) antibodies used against HER2 have been produced. To expression of nanobodies was carried out by using pComb3x SS. The pComb3X is the newest of the pComb3x vectors, and it's recommended for preparation of vector for library cloning. The expression of nanobodies was carried out by using pComb3X plasmid and under the control of lacZ promoter, and this plasmid vector was introduced and expressed in *E.coli* strain ER2738, (Figure 3.9), below show map of pComb3XSS plasmid The Pcomb3XSS vector has many features to be good vector for cloning and include increased stability and introduction of an asymmetric SfiI cassette for directional cloning of scFv, peptide, full Fab and other protein for phage display. 6xHis and HA tags permit for purification and detection. The double stuffer is rffer to "SS", a 1200 bp stuffer in the Fab light chain cloning region bounded by SacI and XbaI restriction sites and a 300bp stuffer in Fab heavy chain cloning region bound by XhoI and SpeI restriction sites. Also, the 1600 bp double stuffer (both stuffers plus the leader sequence between the Fab light chain and heavy chain cloning regions), can be eliminate by SfiI digest therefore; non-Fab genes of interest can be cloned [34] The purified plasmids and nanobodies were cut with sfiI enzyme to generate compatible sticky ends.

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