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Research Article

# Expressing of Recombinant VEGFR2-specific Nanobody in Baculovirus Expression System

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**Background:** Baculovirus expression system, introduced more than 20 years ago, is considered as a useful tool for large and complex eukaryotic recombinant protein production. A baculovirus expression vector is a recombinant virus which desired foreign protein coding sequences is under control of the virus gene promoter. Baculovirus only infects insect cells and do not normally infect vertebrates therefore, they possess no risk of biological risks for human.
**Objectives:** The aim of this study was to recombinant expression of vascular endothelial growth factor (VEGF) reseptor-2 specific Nanobody in the baculovirus expression system.
**Materials and Methods:** Gene of specific Nanobody against the VEGF reseptor-2 that called 3VGR19 was cloned and expressed in baculovirus system.
**Results:** 3VGR19 Nanobody gene was amplified by Polymerase Chain Reaction (PCR) using the specific primers, and was cloned in pFastBac HTA plasmid. *DH10Bac* bacteria was transformed with resulted donor plasmid. The cultured Sf9 insect cell line was transfected with recombinant bacmid, and finally, the expression and purification of 3VGR19 was confirmed in insect cells.
**Conclusions:** In conclusion, Transient infection of insect cells with baculovirus can be a promising technology for expression of antibody fragments.

*Key words*: Angiogenesis, Baculovirus expression system, Nanobody, Sf9 insect cell.

## 1. Backgroud

Recombinant Protein production is the basic foundation for drug production in Pharmaceutical industries (1). Among eukaryotic expression systems, Baculovirus has been a great platform for recombinant protein production in research laboratories or company (1). This system rely on high-level heterologous protein production in insect cells cultures (2). A baculovirus expression vector is a recombinant virus which desired foreign protein coding sequences is under control of the virus gene promoter (3). Common baculovirus expression vectors are based on the use of P10 and polyhedron promoters [3]. Since this system use eukaryotic cells, proper protein folding, oligomerization and post-translational modifications is expected, therefore, the expressed proteins are almost biologically active (4).

The formation of blood vessels is implemented by two mechanisms, vasculogenesis and angiogenesis(5). Angiogenesis is responsible for remodeling and developing these blood vessels network (5). It has been shown that angiogenesis is essential for tumor, growth and metastasis, therefore, by inhibition of angiogenesis progression of cancer can be prevented(6). Formation of new blood vessels are stimulated by angiogenic factors which attached to their receptors on endothelial cells (7). Among angiogenic factors, vascular endothelial growth factor (VEGF) (8, 9), and its receptors(VEGFR1, VEGFR2, VEGFR3) (10), has been shown to be the most important player in angiogenesis. As the reaction of VEGF receptors is necessary for angiogenesis, the inhibition of VEGF receptor signaling may inhibit new angiogenesis and tumor metastasis (11-13).

Antibodies consist of two heavy chains and two light chains. Heavy chain has one variable domain and 3-4

constant domains, while a sub-class of IgGs in camelid has shown to lack light chains and named heavy chain antibodies (HcAbs) (14, 15). In HcAbs the CH1 domain is also missing. Antigen-binding site of HcAbs is composed of only one variable domain referred to as VHH or Nanobody (Nb) (14).

## 2. Objective

In our previous study VEGFR2 specific Nanobody (3VGR19) was developed and was shown to have affinity for VEGFR2 on the cell surface and prevents the formation of capillary-like structures in endothelial cells (16). In present paper, baculovirus expression system was used for recombinant expression of 3VGR19. We showed this expression system can be used for recombinant Nanobody production.

## 3. Materials and Methods

### *3.1. Cell Culture*

*Spodoptera frugiperda* (Sf9) was cells purchased from National Cell bank of Iran (Pasteur Institute of Iran) and used for virus transfection. Sf9 cells adapted to grow in Grace's insect cell medium (Invitrogen, USA) containing 10% fetal bovine serum (FBS) (Gibco, USA) and 1% penicillin-streptomycin (Gibco, USA). Cells were maintained at 27 °C in a non-humidified incubator. Sf9 cells cultured in T-25 flask and after 3-4 days reached 90-100% confluency. Cells were washed with PBS and detached with a scraper before transfection.

### *3.2. Gene Construction*

3VGR19 gene was amplified by PCR with specific primers containing *BamH*I and *Xho*I restriction sites (**Table 1**), using *Pfu* DNA polymerase. PHEN6C-3VGR19-Dia plasmid was used as PCR template . PCR condition was as follow: initial denaturation cycle at 95 °C for 2 min; 30 cycles at 95 °C for 30s, 60 °C for 30s and 72 °C for 60s, the final elongation step at 72 °C for 10 min. PCR products was gel extracted and digested with *BamH*I and *Xho*I restriction enzymes. Digested PCR product was cloned into pFastBacHTA donor vector, which was digested with same restriction enzymes. The resulted construct entitled pFast-VGRNb was transformed into *E.coli* TOP10F′. The grown colonies were then verified by colony-PCR with polh-Forward and PFSBC- Reverse primers (**Table 1**) and DNA sequencing.

### *3.3. Recombinant Baculovirus Generation*

Purified pFast-VGRNb was transformed into *E.coli* DH10Bac by calcium chloride method and plated onto LB agar containing kanamycin (50 $\mu g.mL^{-1}$), gentamicin (7 $\mu g.mL^{-1}$), tetracycline (10 $\mu g.mL^{-1}$), IPTG (40 $\mu g.mL^{-1}$), X-gal (100 $\mu g.mL^{-1}$). Identification of the recombinant colonies was performed with blue-white colony selection. As a result of this transposition, the desired sequence was transposed into the bacmid. For confirmation of transposition, colony-PCR was performed with M13 universal primers (**Table 1**). The recombinant bacmid was purified by Carmo's method (17).

### *3.4. Sf9 Cells Transfection and Recombinant Virus Harvesting*

The recombinant bacmid (Bac-VGRNb) was transfected according to Bac-to-Bac expression system manual into insect cells (Invitrogen, USA). Briefly, $8\times10^5$ Sf9 cells were seeded in each well of a 6-well plate and incubated at 27°C for an overnight. Transfection buffer A and B was prepared (buffer A: 1 microgram of pFast-VGRNb in 100 µL of Grace's insect cell culture, buffer B: 6 µL of Cellfectin (Invitrogen, USA) in 100 µL of Grace's insect cell culture) and mixed with each other. After 20 min of incubation, 800 µL of Grace's insect cell culture was added to the mixture. While cells are attaching to the plate, transfection mixture was added to cells. After 3 days of incubation the signs of infection were investigated under the light and fluorescent microscope. The Bac-copGFP bacmid was used for transfection process control as described by Shokrollahi et. al (18). The recombinant baculovirus begins accumulating in the medium 8 to 10 hours' post-infection. The supernatant containing virus was collected from each well and stored at 4°C or -80 °C (for long-term) and protected from light, Sf9 cells harvested and the cell

**Table 1.** The specific and universal primers sequence. The underlines indicated restriction enzyme sites.

| Name | Sequence |
|---|---|
| Nb-BacF (*BamH*I) | 5'-ACGGGATCCACAGGTGCAGCTGCAGGAGTCTGG-3' |
| Nb-BacR (Stop-*Xho*I) | 5'-ACGCTCGAGTTATGAGGAGACGGTGACCTGG-3' |
| polh-Forward | 5'-AAATGATAACCATCTCGC-3' |
| PFSBC-Reverse | 5'-CCTCTACAAATGTGGTATGGC-3' |
| pUC/M13-Forward | 5'-CCCAGTCACGACGTTGTAAAACG-3' |
| pUC/M13-Reverse | 5'-AGCGGATAACAATTTCACAGG-3' |

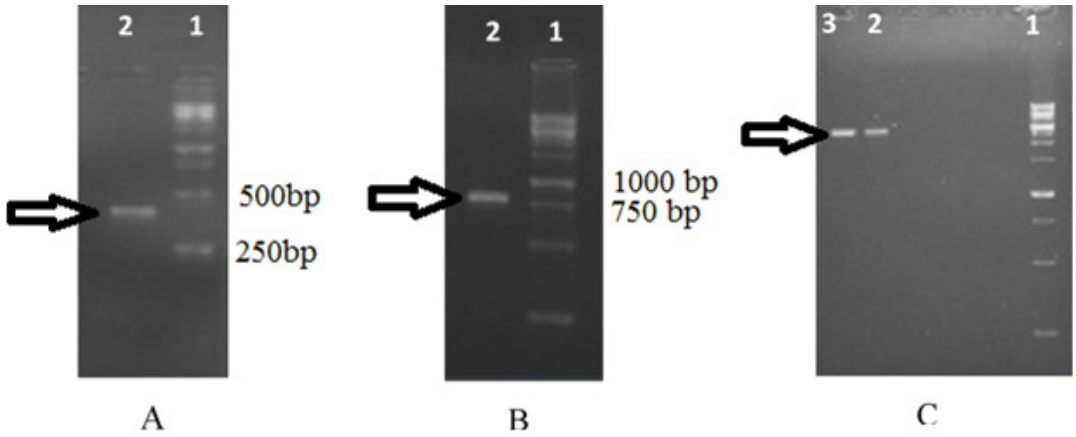


**Figure 1.** Generation of baculovirus vectors expressing 3VGR19 Nanobody. **A)** 3VGR19 amplification PCR product. Lane1: 1Kb ladder, lane 2: 3VGR19 genes (400bp). **B) S**ub-cloning confirmation. Colony-PCR with universal primers was done. Lane1: 1 Kb DNA ladder, lane2: 3VGR19 PCR product (750bp). **C)** Transposition confirmation in DH10Bac. Presence of 3VGR19 gene within bacmids was confirmed by colony-PCR with M13 universal primers (2740bp band). Lane1: 1Kb DNA ladder, lane 2,3: confirmed colonies.

plate stored at -20°C for recombinant protein expression assessment.

*3.5. SDS-PAGE and Western Blot for Expression Analysis*

Sf9 cells were used as baculovirus sensitive cells with transient transfection of recombinant bacmid. Virus pellet was obtained by centrifuging at 3000 rpm for 3 min. Cell pellets were lysed by freeze-thaw with liquid Nitrogen and boiling for 5 minutes. Cell lysate was subjected to 12% SDS-PAGE and transferred to nitrocellulose membrane. After blocking with 4% skim milk/PBST (PBS with 0.05% Tween 20) for 12-16 h, Immunolabeling was done with 1:2000 mouse anti-His (Roche, Switzerland), followed by 1:3000 anti-mouse HRP conjugated (Sigma, USA). The bands were appeared after using the developer (4-chloro-1-naphtol (Sigma, USA)).

*3.6. Purification of Recombinant Nanobody*

Affinity chromatography by nickel-nitrilotriacetic acid (Ni-NTA) was used for Nanobody purification. The infected Sf9 cells were harvested from a 250 mL culture flask by centrifugation (1200 rpm for 10 minutes). Binding buffer contains 300 mM NaCl, 50 mM NaH2PO4, 10 mM imidazole, pH 8. Washing and elution buffer were the same as binding buffer with the exception that these contained 20 and 250 mM imidazole, respectively. Cells were resuspended in 10 mL of binding buffer and cells were lysed with sonication. The lysate was centrifuged at 12000 rpm for 10 min to pellet the cellular debris. The supernatant was loaded on Ni-NTA affinity column according to the instructions supplied by the manufacturer (QIAGEN, Germany). After washing, the recombinant Nanobody was eluted with elution buffer. The result was analyzed with SDS–PAGE.

## 4. Results

*4.1. Mediator Plasmid Construction*

3VGR19 gene was amplified by PCR with specific primers. A 400 bp bond was observed in 1.5% agarose gel (**Fig. 1A**). Consequently, PCR product and pFastBacHTA plasmid were digested, ligated and transformed in *E.coli* TOP10F′. The Final construct (pFast-VGRNb) was confirmed with colony-PCR using universal primers (**Fig. 1B**) and sequencing.

*4.2. Generation of Recombinant Bacmid*

PFast-VGRNb donor plasmid was transformed into DH10Bac *E.coli*, so desirable gene transposed into the bacmid and disrupted of lacZ gene. 3VGR19 gene was transferred into a mini-attTn7 target site of the bacmid by gene transposition. After 48 hours, the white colonies were confirmed by colony-PCR using M13 universal. As shown in **Figure 1C**, a 2830 bp band (Bac-VGRNb) was observed.

*4.3. Insect Cells Transfection*

In order to produce recombinant baclovirus, Sf9 cells were transfected with Bac-VGRNb using liposome mediated method. Bac-copGFP was used as positive transfection control. Detection of cells expressing of

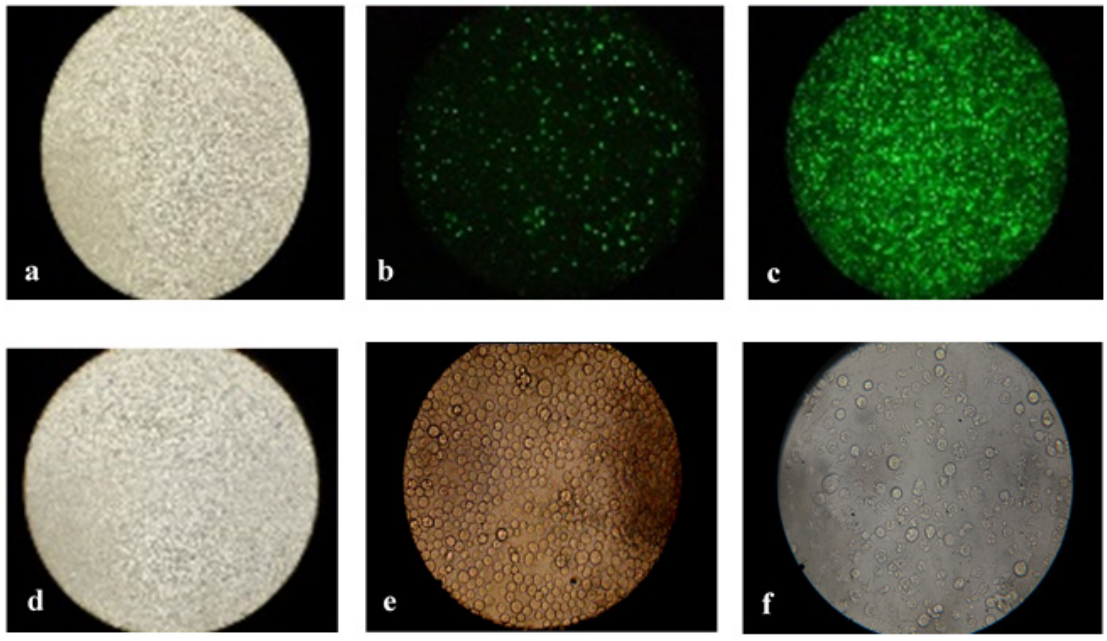


**Figure 2.** Sf9 cell transfected with Bac-copGFP, (a) before transfection (×10), (b) after 48 h (×10), (c) after 72 h (×10). Sf9 cell transfected with Bac-VGRNb, (d) before transfection (×40), (e) after 48 h (×40), (f) after 72 h (×40).

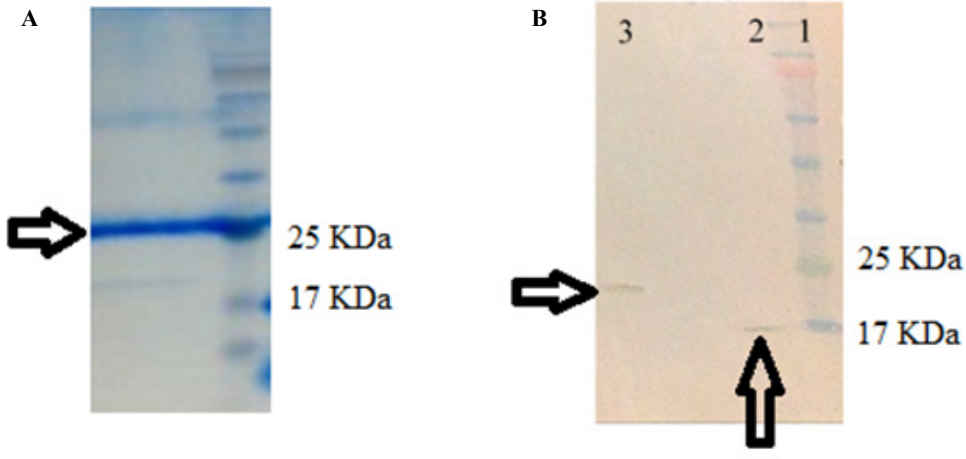


**Figure 3.** Purification of 3VGR19 Nanobody by Ni-NTA affinity chromatography and its confirmation by SDS-PAGE and western blot analysis. A) The SDS–PAGE gel was coomasie blue-stained. A band with molecular mass of about 20 kDa was observed**.** B) western blot analysis was performed with anti-his taq antibody. Lane 1: protein marker, lane 2: contol protein for western blot, lane 3: 3VGR19 Nanobody.

recombinant protein was performed with fluorescent microscope following 3 days of transfection. At 48 h post inoculation, cells were analyzed visible hallmarks of AcMNPV infection which include: inhibition of cell growth, enlargement in cells' diameter and detachment of cells from the flask (**Fig. 2**). About 70% of the Bac-copGFP transfected cells were observed a bright green color (**Fig. 2**).

*4.4. Purification of 3VGR19*

3VGR19 was purified by Ni-NTA affinity chromatography, and Subsequently, purified fractions were analyzed by 15% SDS–PAGE (**Fig. 3A**), and western blotting (**Fig. 3B**).

## 5. Discussion

Over the last decade, the use of monoclonal antibodies

(mAbs) has extensively grown in the fields of research, diagnostics and cancer therapy but these antibodies have problems such as mAb dimensions (for IgG; ~ 150 kDa, 10–15 nm long and 7–9 nm wide) and their long half-life (ranging from days to up to 4 weeks) during molecular imaging. While the Nanobody have advantages such small size (~15 kDa, 4 nm long and 2.5 nm wide), high solubility, stability, specificity and affinity, ease of cloning as well as thermal and chemical resistance (19). On the other hand, Nanobodies because of their high similarity with human VH sequence may not cause immune response (20). In this regard, it should be mentioned they are stable in the reducing cytoplasmic environment (20). According to the insight into the current status, ongoing developments and future challenges towards successful implementation of Nanobodies in the diagnosis and treatment of different disease can be hoped (20-22).

Several studies based on Nanobodies indicate their effects on delay of tumor growth or inhibition of angiogenesis (20, 23-26). 3VGR19 Nanobody inhibits angiogenesis via suppression of VEGF/VEGFR2 signaling and because the presence of this Nanobody hampered angiogenesis and metastasis (16). Our team was developed a VEGFR2-specific Nanobody from a hyper immune camel library which can inhibit formation of capillary-like structures *in vitro* (16).

Several recombinant expression systems can be used for Nanobody expression (27-31). The yields of recombinant proteins obtained in baculovirus expression vector system and bacterial systems is almost equal but due to BEVS provides a proper folding and post-translational processing of eukaryotic proteins has been widely used by many laboratories.

To the best of our knowledge, no *sf9* cell was used for Nb production, one study, however, used insect larva as a living biofactory for Nb production(32). But several studies have used insect cells as a host for antibodies and its derivatives production such as IgG(33), IgM(34), IgE(35, 36) and Fab fragments(37-39). In a study by Furuta *et al*.(37) Fab fragment of 6D9 antibody was cloned into baculovirus and this recombinant virus was used to infect Trichoplusia ni BTI-TN-5B1-4 (High Five) cells. In our study, transfection of the *sf9* cell was high (**Fig. 2**). These results show that the baculovirus system can be used to successfully infect insect cells with high efficiency. Sf9 cells are capable of producing full length, functional antibodies. Lee *et al*.(40) showed that anti-breast cancer (BR55) antibody can be purified from *sf9* cells and is functional at binding to the MCF-7 cell line. In our study, the anti-VEGFR2 specific Nb was cloned into Bac-VGRNb bacmid. Sf9 cell was infected with this plasmid and expression of 3VGR19 was confirmed with western blot analysis. Subsequently, purification of Nb from the lysates of infected cells was performed with Ni-NTA chromatography. In conclusion, Transient infection of insect cells with baculovirus can be a promising technology for high-yield expression of antibody fragments. Using insect cells as a host for protein production platform has also the advantages like proper folding, higher yield and proper post-translational modification pattern (41).

## Conflicts of interest

The authors declare to have no conflicts of interest.

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