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Vishweshwar Kumar Ganji
FMD research lab, Indian Veterinary Research Institute, Bengaluru, Karnataka, India

Dechamma HJ
FMD research lab, Indian Veterinary Research Institute, Bengaluru, Karnataka, India

Correspondence
Vishweshwar Kumar Ganji
FMD research lab, Indian Veterinary Research Institute, Bengaluru, Karnataka, India

PCR amplification of cloned gene from recombinant baculovirus by direct PCR for sequencing

Vishweshwar Kumar Ganji and Dechamma HJ

Abstract
The present study describes a sophisticated technique to amplify gene fragment directly from the baculovirus without isolating genome. The study is carried out at FMDRL, IVRI, Bengaluru during January to March 2017. The study was performed with a recombinant baculovirus which was propagated in *Trichoplusia ni* (Tn5) insect cell lines. Here we PCR amplified the gene fragment directly from the recombinant baculovirus without the need for isolation of genomic DNA and sequenced it. Five out of five PCR fragments sequenced shows no changes in the nucleotide sequence which indicates that there was 100% efficiency of the direct PCR approach for amplifying gene fragment from recombinant baculovirus is high. The use of direct PCR approach for amplifying gene fragment from recombinant baculovirus helps to cut short the time and expenses for amplification of gene fragment from baculovirus for sequencing.

Keywords: Baculovirus, mutations, sequencing, PCR

1. Introduction
The combination of recombinant baculovirus and insect cell expression systems produced high levels of recombinant proteins that undergo eukaryotic type post-translational modifications such as glycosylation or phosphorylation [1]. The BEVS technology is developed by Summers, Smith and their colleagues at Texas A & M University [2]. In this, the foreign proteins are expressed under the control of polyhedrin or p [10] promoter from AcMNPV during the very late phase of baculovirus gene expression [3]. The most widely used lepidopteran cells for BEVS are Sf9, Sf21 and Tn5 cell lines. Baculovirus expression system has been successfully used to express VLPs from viruses such as poliovirus [4], rabbit hemorrhagic disease virus [5], severe acute respiratory syndrome (SARS) virus [6], enteroviruses [7], Norwalk like viruses [8], swine vesicular disease virus [9], FMDV serotype Asia 1 [10], O [11, 12] and A [13, 14] and bluetongue virus [15]. A report says they have found 60 high-frequency non-synonymous mutations under balancing selection distributed in all functional classes [16]. The mutation rate is mainly very high in the protein coding regions of baculovirus [17] and requires frequent screening for mutations. The present study has been taken up with an objective to design a rapid, easy and cost-effective way of amplifying gene fragment from the baculovirus genome for sequencing.

2. Materials and Methods
The present study was carried out at FMDRL, IVRI, Bengaluru during January to March 2017. The cell lines used were taken from FMDVP lab, IVRI, Bengaluru. All the chemicals used in the study are from Sigma Chemicals Co., St.Loius, USA, enzymes were procured either from New England Biolabs or MBI Fermantas. The HPLC grade primers obtained in lyophilized form from Bioserve, India were reconstituted as 1nM stocks in sterile TE buffer.

2.1. Cell Lines
Trichoplusia ni (Tn5) insect cell lines were maintained at 27ºC were used in the study. Tn5 cells maintained in SF-900 II SFM containing 1X antibiotic mix (100X antibiotic mix: - Penicillin: 300mg, Streptomycin: 500mg, Kanamycin: 250mg, Distilled water: to 50ml) were used for transfection, virus propagation.

2.2. Transfection of insect cells with recombinant bacmid DNA

In 24well culture plate $0.3x10^6$ cells/well Tn5 cells were seeded in antibiotic-free SF-900 II SFM (Invitrogen) media. The transfection mixture was prepared simultaneously when cells are allowed to adhere.

Mix A

SF-900 II SFM-50µl mixed with 3µl of Cellfectin-II reagent (Invitrogen) and incubated on ice for 5min.

Mix B

SF-900 II SFM-50µl mixed with 4µg of recombinant bacmid DNA.

Above A and B were mixed and incubated on ice for 15min and added with 400ul of SF-900 II SFM.

The cells were washed twice with SF-900 II SFM to remove dead cells. The transfection mix 500 µl was layered over the cells (care should be taken to avoid drying of cells). After the incubation at 27ºC for 5 hours, the transfection mixture was replaced with 750 µl of complete growth medium (SF-900 II SFM supplemented with antibiotic mix) and incubated at 27ºC for 72 hours, observed for the signs of viral infection. The virus supernatant was collected into sterile 1.5 ml Eppendorf tubes and centrifuged at 1000 rpm for 5 min to remove cell debris. This P0 viral stock was stored at 4ºC.

2.3. PCR amplification of gene insert from recombinant baculovirus for sequencing

The gene insert was amplified by PCR from recombinant baculovirus using a protocol described below

1. The harvested recombinant baculovirus culture was digested in single digestion buffer (KCl: 0.186g, Tris base: 0.079g, Gelatin: 5mg, Nonidet P-40: 225µl, Tween 20: 225µl, Distilled water: to 50ml). 20 µl of baculovirus was added to 80µl of digestion buffer and incubated at 56ºC for 1 hour and inactivated at 95ºC for 3 min. The digested mix was centrifuged at 12000 rpm for 2 min and the supernatant was used as a template DNA for PCR.
2. PCR was carried out using gene-specific VP4L (GGAGCCGGGCAATCCAG) and 3CR (TCTGCCGTCCAACATGATCT) primers in the reaction volume of 50 µl containing following reagents in 0.2 ml thin-walled PCR tubes

Reagents	Volume to be added
DNA template	15 µl
20 µM Forward primer	1 µl
20 µM Reverse primer	1 µl
dNTP	1 µl
Enzyme	0.25 µl (1U)
Buffer	5 µl
FQW	26.75 µl
Total reaction volume	50 µl

3. Amplification was carried out in following thermocycling condition

Step	Temperature	Time	No. of cycles
Initial denaturation	96ºC	3 min	1
Exponential amplification			
Denaturation	96ºC	20 sec	30
Annealing	55ºC	30 sec	
Extension	72ºC	2 min 30 sec	
Final extension	72ºC	10 min	1
Hold	4ºC	10 min	1

The PCR product was analyzed in 0.8% agarose gel alongside with a DNA molecular weight marker. The amplified PCR product was purified by GeneJET Gel extraction kit (Thermo Scientific).

2.4. Purification of PCR product by GeneJET Gel extraction purification kit (Thermo Scientific)

The PCR product was resolved in 0.8% agarose gel and exact size of the amplicon was sliced and purified using GeneJET Gel extraction kit (Thermo Scientific, catalog No. K0691) as per the manufacturer's protocol. An equal volume of binding buffer and the gel slice was heated at 65ºC for 10 min on the dry bath. The mix was transferred into the GeneJET spin column and kept for 1 min at RT. The spin column was Centrifuged at 12000 rpm for 1 min and the flow through was discarded. The column was added with 700 µl of wash buffer and centrifuged at 12000 rpm for 1 min. The flow-through was discarded and centrifuged at 12000 rpm for 1 min. Spin column was transferred onto a fresh 1.5 ml sterile Eppendorf tube and DNA was eluted in 20 µl of elution buffer by centrifuging at 12000 rpm for 2 min. DNA was quantified at λ_{260} using nanodrop. The sample was analyzed on 1% agarose gel alongside with a DNA ladder and sent for sequencing.

2.5. Sequencing and sequence analysis

The five PCR fragment amplified as mentioned above were sequenced using ABI 377 Perkin Elmer Automated DNA Sequencer at the sequencing facilities Eurofins, Bengaluru. The PCR fragment was sequenced from 5' end using a VP4L primer. The data obtained were analyzed using Bioedit and MEGA software. The data was aligned with the published sequence in NCBI (FMDV serotype O/IND/R2/75) and checked for the similarity in the nucleotide sequence.

2.6. Statistical analysis

The statistical analysis was done by checking the percentage of identity between the sequences after performing the BLAST at NCBI website. Fifteen passages of recombinant baculovirus were sequenced and observed for mutations in the gene inserted across the generations.

3. Results

As shown in the Fig. 1 are the Tn5 cells after the infection with wild-type baculovirus (A) and recombinant baculovirus (B-F). The images were taken under the inverted light microscope at 10x magnification 3 days post infection. The cells showing a characteristic cytopathic effect with rounding off, granulated appearance and detached from the surface of the flask. As shown in the Fig. 2 there was an amplification of 2.4 kb gene fragment from plasmid DNA control in lane 2, from five replicates of recombinant baculovirus in lane 3-7 and no PCR amplified fragments from wild-type Bacmid DNA control as evident from lane 8. As shown in Fig. 3 we could not see any change in the nucleotide sequence of the inserted gene, this was confirmed by cross-checking with chromatogram in Bioedit. Further, the sequence of the

inserted gene fragment is found to be intact with no mutations until 15 passages.

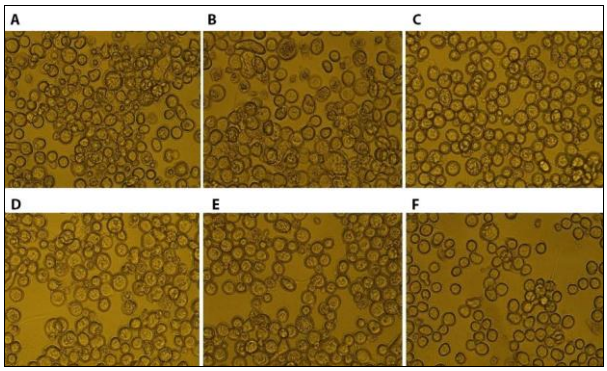

Fig 1: CPE of Tn5 cells infected with baculovirus (10x magnification). Fig. A shows the Tn5 cells infected with wild-type baculovirus Fig. (B-F) shows Tn5 cells infected with recombinant baculovirus (five replicates)

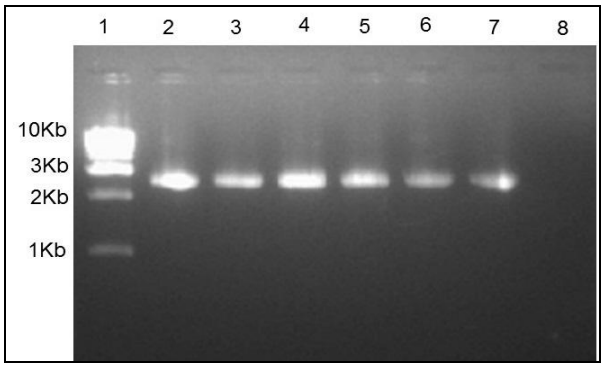

Fig 2: PCR amplification of a 2.4 Kb fragment from recombinant baculovirus using VP4L and 3CR primers (1% agarose gel) Lane 1: GeneRuler 1Kb DNA ladder (Fermantas) Lane 2: Plasmid DNA control showing 2.4 Kb amplified fragment Lanes 3-7: PCR amplified fragments from five replicates of recombinant baculovirus showing 2.4 Kb amplified fragment Lane 8: No PCR amplified fragments from wild-type Bacmid DNA control.

Fig 3: MEGA alignment of the sequence of PCR fragment against the published sequence

4. Discussion

The use of baculovirus expression systems was increasingly in demand because of the advantage of producing high levels of recombinant proteins that undergo eukaryotic type post-translational modifications such as glycosylation or phosphorylation [1]. In our study, the Tn5 insect cells infected with recombinant baculovirus shown a similar pattern of CPE as reported by others [2, 18]. However, the major problem associated with BEVS is that the rate of mutation accumulation was very high in the protein coding regions of baculovirus [17]. It requires screening the gene insert of recombinant baculovirus after each subsequent passaging to have a check on mutations. But the process involves a time consuming, cumbersome genome extraction [19] followed by sequencing the genome [20]. So, we have used a direct PCR approach by digesting the capsid of baculovirus in the buffer and PCR amplified the gene fragment. The quality of PCR fragment is as similar to the gene fragment amplified from the bacmid DNA [1]. The sequence quality of the PCR fragment is good and was evident from chromatogram [21]. In contrary to what others reported we have found that the sequence data of PCR fragment from baculovirus is even intact with no mutation even after 15 passages [17, 22]. But we cannot say that the statement of mutations in the baculovirus is wrong as we have sequenced only the gene insert region.

5. Conclusion

In conclusion, we have come up with a more sophisticated technique for screening recombinant bacmids using a direct PCR approach. This method helps to save time, more efficient and cost-effective. Further, the steps in isolation of bacmid DNA and use of genome extraction kits can be reduced.

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