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A rapid method for selection and cloning of stably transformed insect cell lines

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Abstract

Insect cell lines have been widely used for recombinant protein production due to their abilities in eukaryotic-type post-translationally modifications of proteins and simple culture process. Stably transformed insect cells can be engineered by delivery of the target gene to integrate randomly into the insect cell chromosome, they are then able to stably express this gene and continuously produce recombinant protein. To ensure maximum recombinant protein production, selection of the highest recombinant protein producing cells by cell cloning technique is recommended. A limiting dilution method is generally used for cell cloning, however it is inefficient and time consuming. A rapid cell cloning method for transformed cells selection was therefore developed. A bi-cistronic transfer vector was constructed for gene delivery and co-expression of fusion gene between the target gene, T2A peptides and red fluorescent protein (RFP) encoded genes in insect cells. T2A peptides induce ribosomal skipping during translation thus allowing separation of the target recombinant protein and RFP. Thus, the red florescent cell should be the recombinant protein producing cells and could be easily separated and selected. To validate this vector, an influenza virus neuraminidase gene (NA) was used as a target gene. After 24 hours post transfection, red fluorescent cells were readily observed and selected. Single target cell selection could be achieved within 7-10 days which was 2-3 times more rapid than the original cell cloning process. All cloned cells could stably express the NA gene for at least 22 passages.

Keywords: Cell cloning, Co-expression, N1 neuraminidase, Red Fluorescent Protein, Stably transformed insect cells, T2A peptides

1. Introduction

Insect cells are prominent and reliable cells for production of many important human recombinant proteins due to their abilities to perform eukaryotic-type post translation modifications and simple culture maintenance [1]. Insect cells can be genetically engineered to become a transformed cell line which is capable of stably expressing the target gene over many passages. By using an insect-specific promoter, a series of expression vectors for lepidopteran and dipteran cell lines has been developed and employed for high-level protein production [2]. Unlike a virus expression vector, proteolysis is limited in the plasmid transfected cells due to an absence of cell lysis by virus infection. Generation of stable transformed insect cell lines typically involves in plasmid transfection to deliver the target gene to integrate into the insect chromosome. This transfection usually results in a heterogenous polyclonal population that varies in the number of integration events and the site(s) of integration across cells. A cell pool could lead to reduced transgene expression over time, as the lower expressing clones take over the polyclonal cell pool. Generating a monoclonal cell line by the cell cloning method could result in cell populations that are more likely to retain stable transgene expression. Although single-cell cloning is an established method in mammalian cell lines [3] it is still challenging for insect cells. Cloning by limiting dilution method has been adopted for Sf9 insect cell lines from *Spodoptera frugiperda* [4] and *Drosophila* S2 cell lines [5,6]. Although it is a simple method without special requirements for equipment and reagent, there is no guarantee that the colonies arose from single cell. Re-cloning is usually advised to ensure that the cells originated from a

single cell. The obtained cloned cells are then further screened for the maximum producer of the target recombinant protein, using quantitative methods such as enzyme-linked immunosorbent assay (ELISA). All steps described are therefore laborious and time consuming.

To simplify the process for selection of the transformed cloned cell lines, a new method was developed for rapid cell cloning and selection. A bi-cistronic transfer vector was constructed by insertion of a self-cleaving T2A peptides and red fluorescent protein (RFP) coding sequences downstream from a multicloning sites of this plasmid [7,8]. The 2A peptides are usually 18-22 amino-acid (aa)-long viral oligopeptides with a highly conserved sequence GDVEXNPGP shared by different 2As at the C-terminus. These sequences are essential for the creation of steric hindrance and ribosome skipping that mediates cleavage of polypeptides during translation in eukaryotic cells. The ribosome skipping results in two "cleaved" proteins: the protein upstream of the 2A is attached to the complete 2A peptide except for the C-terminal proline, and the protein downstream of the 2A is attached to one proline at the N-terminus [9,10]. In this study, a T2A from *Thosea asigna* virus 2A which had been shown to have the highest cleavage efficiency close to 100% in mammalian cell and insect cell [11], was chosen for construction of a new bi-cistronic plasmid, namely pIZ-T2A-RFP. This plasmid aimed to constitutively express the gene of interest under the control of the *OpIE2* promoter derived from the baculovirus *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus (OpMNPV) [12]. An influenza H5N1 neuraminidase (NA) which exists as a tetramer of four identical monomers, each of approximately 433 amino acids, was used as a model for validation of this system. It is proposed that the pIZ-T2A-RFP could be used to co-express the NA gene, T2A peptides and RFP encoded genes in insect cells. Due to the ribosome skip mechanism caused by T2A peptides, recombinant NA protein and RFP will be independently translated in the same cell . Thus, transformed insect cells could be rapidly identified, selected and cloned by monitoring of RFP. This transfer vector also facilitates chromosome integration of the target gene, thus the NA gene expression should be stable and recombinant NA protein is expected to be continuously produced in many future generations. In addition, the recombinant NA protein is a naturally secreted protein. These cloned cell lines or stably transformed cell lines are therefore anticipated to have simple scale up and purification processes.

2. Materials and methods

2.1 Materials

pIZ/V5-His was purchased from Invitrogen, USA. NA gene (A/Thailand/1(KAN-1)/2004/H5N1) was kindly provided by Faculty of Medicine (Siriraj Hospital), Mahidol University, Thailand. T2A-*SacII*-Fwd and RFP-*Age*I-Rev primers for amplification of T2A-RFP and NA gene specific primers; NA-*Kpn*I-Fwd and NA-*EcoR*I2-Rev primers were purchased from 1st BASE Pte, Ltd, Singapore. Polyclonal antibody specific to the H5N1 Influenza A Virus NA was from Gene Tex., USA. *S. frugiperda* (Sf9) insect cell lines were from Invitrogen, USA. Zeocin™ antibiotic and CellFectin™II reagent were from Invitrogen, USA.

2.2 Construction of pIZ-NA-T2A-RFP co-expressing NA and T2A-RFP

T2A nucleotide sequences from *T. asigna* were fused to the RFP gene by Polymerase chain reaction (PCR) overlapping method using T2A-*Sac*II-Fwd (5'-CAA TCC GCG GGA GGG CAG AGG AAG-3') and RFP-*Age*I-Rev (5'-CCC ACC GGT TTA GGC GCC GG-3') primers. The PCR fragment was then ligated to pIZ/V5-His at the *Sac*II and *Age*I cloning site, and resulted in a pIZ-T2A-RFP. The NA gene was PCR amplified using NA-*Kpn*I-Fwd (5'-GGG GTA CCA AAA TGA ATC CAA ATA AG -3') and NA-*EcoR*I-Rev2 (GCG GAA TTC CTT GTC AAT GGT GAA-3') primers. The amplified NA gene fragment was ligated to the previously described pIZ-T2A-RFP. Ligate mixtures were transformed into competent *E. coli* DH5α cells by heat shock transformation. After overnight culture of transformed cells at 37ºC in Luria-Bertani (LB) agar plate containing 25 µg/mL Zeocin™ (Invitrogen, USA) as a selective marker. Nucleotide sequences of the insertion fragments in the new plasmid, pIZ-NA-T2A-RFP, were verified by DNA sequence analysis.

2.3 Transfection of recombinant pIZ-NA-T2A-RFP into Sf9 insect cells

The recombinant pIZ-NA-T2A-RFP was transfected into Sf9 insect cells by lipofection method. Sf9 insect cells at cell density of $1x10^6$ cells/mL were seeded into a 60 mm dish. Transfection mixtures containing 20 µL of CELLFECTIN II (Invitrogen, USA) and 10 µg of the pIZ -NA-T2A-RFP in 1 mL of insect cell Grace's culture medium were added onto the Sf9 cells and incubated for 4 hours at room temperature. The transfection medium was then replaced with 1 mL Grace's medium and further incubated at 27°C. Forty-eight hours post-transfection, the culture medium was replaced with a selective medium containing 350 µg/mL Zeocin™. The selective medium was replaced every 3 to 4 days.

2.4 Transfected and transformed Sf9 cells

2.4.1 NA and RFP gene expression analysis

Reverse transcription-PCR (RT-PCR) was performed to monitor co-expression of NA gene and RFP gene. Firstly, total RNA was extracted from pIZ-NA-T2A-RFP transfected Sf9 cells using Trizol reagent (Invitrogen, USA). Reverse transcription was performed for cDNA preparation according to the reverse transcriptase manufacturing instructions (Thermo Fisher, USA) and used as templates for PCR amplification of NA gene and RFP gene using their specific primers. NA gene specific primers were as described in 2.1 and RFP specific primers were RFP-*Age*I-Rev (5'-CCC ACC GGT TTA GGC GCC GG-3') and T2A-*Sac*II-Fwd (5'-CAA TCC GCG GGA GGG CAG AGG AAG-3'). RFP gene expression in transfected Sf9 cells could also be determined by observation of the cells under fluorescent inverted microscope (OLYMPUS DP74).

2.4.2 NA and RFP gene integration into Sf9 chromosome

The pIZ-NA-T2A-RFP integration into the Sf9 chromosome of transfected cells was confirmed by PCR analysis. Chromosomal DNA (cDNA) was extracted by Alkaline lysis method [13]. This DNA was used as a PCR template for detection of the NA gene and RFP gene, using specific primers to NA gene and RFP gene, respectively as described in 2.4.1.

2.4.3 Cloning of transformed cells using dilution method

Cell suspension at $1x10^4$ cells/mL in 10 mL Grace's medium were prepared and 100 µL/well were added into 24 wells of a 96-well plate. The remaining cell suspension were diluted at 1:1 with the same medium and seeded to the next 24 wells (100 µL/well) and dilution continued until a single cell/well was observed under microscope. Plates were incubated at 27^{o}C overnight. Single red fluorescent cells were marked and further incubated at 27°C until colonies were formed and individually expanded into larger culture volumes. These cloned cells were characterized for NA gene expression, genetic stability and recombinant NA protein production.

2.5 Recombinant NA Protein and RFP Detection

To obtain proteins produced by transfected Sf9 cells and secreted into the medium, a culture medium was harvested, overlayered onto a 40% sucrose cushion and centrifuged at 28,000 rpm for 2.5 h at 4^{o}C in a Beckman SW28 Swinging bucket and the pellet suspended in phosphate buffered saline (PBS). Protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) then transferred onto a nitrocellulose membrane. The membrane was blocked in 3% bovine serum albumin in PBS for 1 hour and incubated in anti-NA polyclonal antibody (1:500 dilution, Gene Tex., USA) at 4^{o}C overnight. After washing, the membrane was incubated with anti-rabbit IgG conjugated with horseradish peroxidase (1:5000 dilution, Thermo, USA) for 2 hours. The immunoblot was developed with 3,3′,5,5′-Tetramethylbenzidine (TMB) conjugate substrate kit (Bio-Rad, USA).

3. Results and discussions

3.1 Construction of pIZ-T2A-RFP

The T2A-RFP PCR product was amplified and ligated into the pIZ/V5-His at the *Sac*II/*Age*I cloning sites. The resulting plasmid, pIZ-T2A-RFP was a bi-cistronic vector for co-expression of target gene and RFP in Sf9 insect cells (Figure 1A). The pIZ-T2A-RFP provides multicloning sites for the insertion of the target gene in frame with T2A and RFP encoded nucleotide sequences. In insect cells, a transcript of target gene-T2A-RFP will be produced under the control of *OpIE2* promoter. T2A viral peptide sequence prevents the ribosome from covalently linking a new inserted amino acid due to inhibition of peptidyl transferase, and allows it to continue translation by restarting on Proline. This resulted in co-translational cleavage of the polyprotein. The apparent cleavage occurs between G and P (Figure 1C). In this study, the NA gene was inserted into *Kpn*I and *Eco*RI in frame with nucleotides coded for T2A and RFP of the pIZ-T2A-RFP. The recombinant plasmid, pIZ-NA-T2A-RFP was obtained and used for generating stably transformed Sf9 insect cells producing recombinant NA protein and RFP.

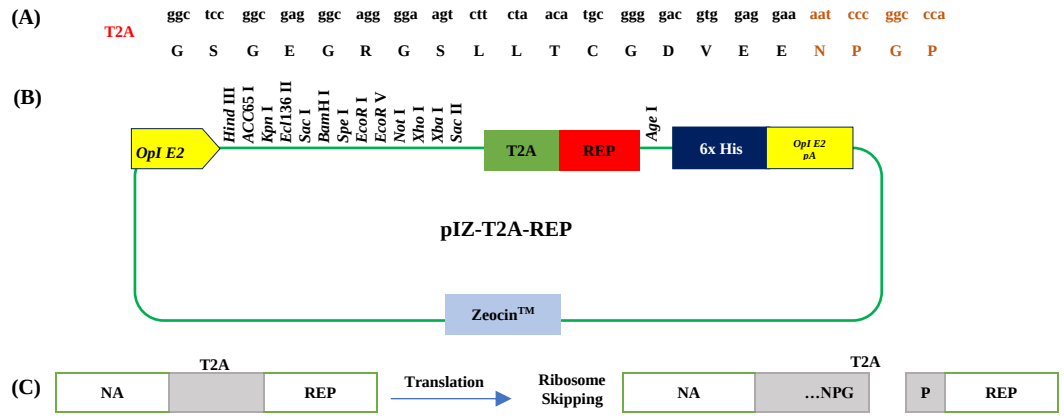

Figure 1 DNA and amino acid sequences of the T2A (A), a simplified map of the pIZ-T2A-RFP cloning vector showing T2A and multicloning sites (B), schematic representation of the mechanism of "self-cleaving" by ribosome skipping of T2A peptides (C).

3.2 Transient and transformed cells expressing NA and RFP

After an hour post-transfection with pIZ-NA-T2A-RFP, some of the red fluorescent Sf9 cells were readily observed under fluorescent microscope (Figure 2A) indicating successful transfection and expression of the RFP gene. Transfected cells were incubated for 24 hours then gene expression analysis by reverse transcription followed by PCR (RT-PCR) was performed to confirm that both genes were co-expressed. A PCR product was amplified from the reaction containing cDNAs template prepared from the pIZ-NA-T2A-RFP transfected Sf9 cells, its size corresponded to the NA-T2A-RFP transcript at approximately 2.1 kbp. In addition, NA specific primers and RFP specific primers detected NA gene and RFP gene in this cDNAs, respectively (Figure 2B). These results indicated that a single transcript product of the NA-T2A-RFP fusion genes was synthesized after the pIZ-NA-T2A-RFP transfection into insect cells. Thus, the pIZ-NA-T2A-RFP was an effective vector for driving transient expression of the inserted gene upon transfection. This vector could be useful for applications such as tagging of protein of interest for live cell imaging [14,15], creation of animal models of various hereditary diseases [16,17], etc. The NA-T2A-RFP fusion genes was also found to be integrated into insect chromosome since this part was detected from cDNA of transfected insect cells by PCR analysis (Figure 2C). Stable gene expression cell lines were therefore anticipated as they are incredibly useful to long-term genetic studies and for protein manufacturing in biotechnology and the pharma industry.

Mix population of transfected cells were next subjected to cell cloning to remove non-transfected cells and for selecting the stable clonal transformants that provide the highest protein production. RFP greatly facilitated the cloning as transformants were easily observed and separated from non-transfected cells. Figure 3 shows a clonal cell line originated from a single cell that grew and formed a red colony.

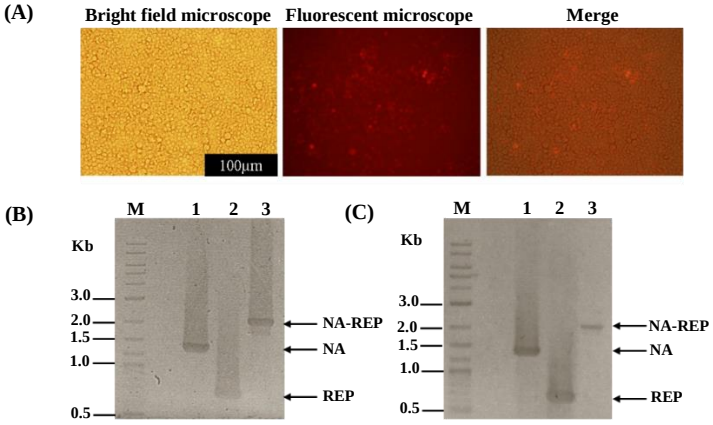

Figure 2 (A) RFP detection in the transfected cells by fluorescence inverted microscope, (B) NA gene and RFP gene expression analysis by RT-PCR using cDNAs template from pIZ-NA-T2A-RFP transfected Sf9 cells with different primers, (C) PCR analysis of cDNA obtained from pIZ-NA-T2A-RFP transfected Sf9 cells using different primers. Lane M: 2 log-DNA ladder, Lane 1: NA gene specific primers, lane 2: RFP gene specific primers, lane 3: forward primer: NA gene specific primer and reverse primer: RFP gene specific primer.

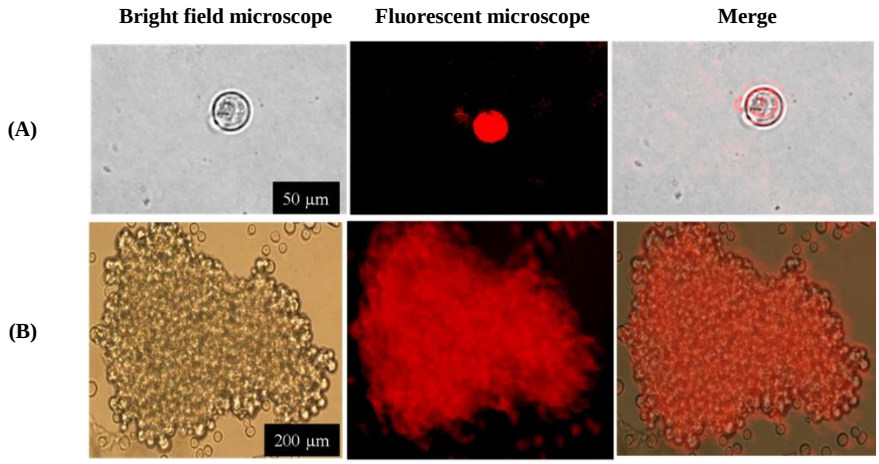

Figure 3 Cloned cells observed under fluorescence inverted microscope. A single cell separated from others by dilution method on day 1 (A), a colony was formed on day 7 (B).

3.4 Recombinant NA protein

Multimeric forms of recombinant NA protein were detected in the culture medium of transfected Sf9 cells by anti-NA antibody, as shown by Western blot analysis (Figure 4A). The molecular weight of monomer form was found at approximately 55 kDa while dimers, trimers and tetramers were at approximately 110 kDa, 165 kDa and 220 kDa, respectively. Another higher molecular weight than tetramers was also found in the most upper part of the blot. These are similar to native forms of influenza NA and corresponded to the recombinant NA expressed by stably transfected mammalian cell lines [18]. The transfected Sf9 cells produced recombinant NA protein in similar forms as mammalian cells. After cell cloning, a clonal cell line had been selected and cells were serially passaged for 22 passages. All cells produce RFP that can easily be observed under a fluorescent microscope throughout the sub-culturing process. In addition, the recombinant NA proteins secreted into medium from clonal cells were still detected by dot blot (Figure 4B). These corresponded to previous results showing that the NA gene and its fusion partners were found incorporated into the insect chromosome (Figure 2C).

NA-based influenza vaccine has been recently suggested to be included in the formular of both pandemic and seasonal vaccines since it has demonstrated independent antigenic drift. It is likely that NA-induced immunity will be an important piece of future influenza vaccine strategies [19]. Thus, a simple NA vaccine production process will certainly accommodate this new strategy. The stably transformed clonal cell lines developed in this study is a promising platform for NA vaccine production. The recombinant NA protein was continuously secreted into a medium which can be easily obtained and purified. Furthermore, these cells can be cultured as suspension cells which are convenient for large scale production.

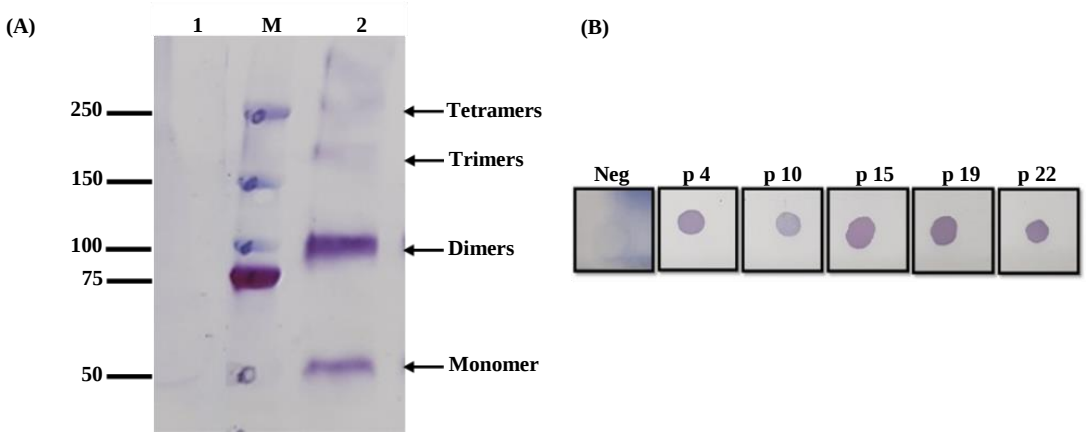

Figure 4 (A) Western Blot analysis for recombinant NA protein detection using anti-NA antibody in non-reducing condition. Lane M: protein marker, lane 1: negative control, supernatant from non-transfected insect cell culture medium, lane 2: pIZ-NA-T2A-RFP transfected Sf9 cells transfected cells culture medium, (B) Dot-Blot analysis using anti-NA antibody for detection of recombinant NA protein after serial passages.

3.5 A rapid method for selection and cloning of stably transformed insect cells lines

Cell cloning using limiting dilution method, which is laborious and time consuming, was improved in this study. A new bi-cistronic pIZ-NA-T2A-RFP was constructed and successfully used for selection of transformed Sf9 cells producing recombinant NA protein and RFP within 7-10 days by observing under fluorescent inverted microscope (Figure 5). RFP assists the selection of the transfected cells as quick as 24 hours post-transfection. The rest of the procedures including cell separation then move quickly and recombinant NA protein was detected from the first cell passage to the passage no. 22.

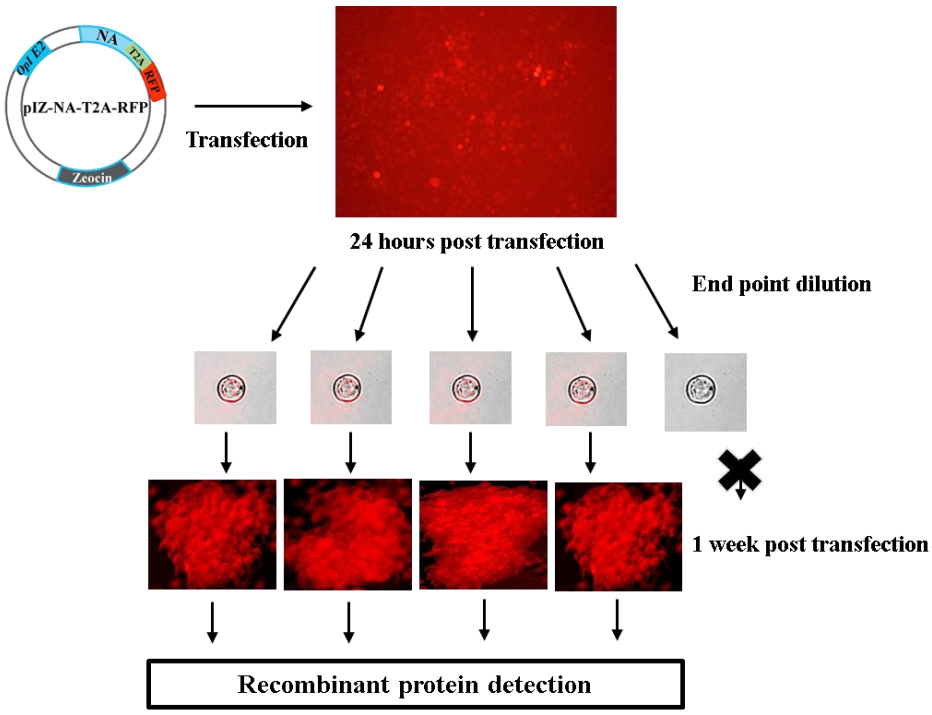

Figure 5 Schematic diagram showing process for screening and cloning of stably transformed insect cells using the bi-cistronic plasmid, pIZ-T2A-RFP.

4. Conclusions

A new bi-cistronic plasmid, pIZ-T2A-RFP was constructed for rapid identification of the transfected cells and facilitation of cell cloning. Results showed that insect cells transfected with this plasmid can be easily detected as RFP expressing cells under a fluorescence inverted microscope. In addition, cloning of target cells was made possible by selection of single red cells and homogeneity of cloned cells can also be monitored later after cell division. In this study, the transfected cells were transformed by plasmid integration into chromosome of insect cells. These stably transformed cells continuously produced recombinant NA protein up to 22 passages. Stably transformed cells can be selected and clonal cell lines producing recombinant NA protein within 7-10 days. Thus, this plasmid is an effective tool for rapid generation and cloning of a stably transformed insect cell line for production of the recombinant protein of choice.

5. Acknowledgement

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6. Conflict of interest

The authors declare that they have no conflict of interest.

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