Video Article

Transient Expression of Foreign Genes in Insect Cells (sf9) for Protein Functional Assay

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Abstract

The transient gene expression system is one of the most important technologies for performing protein functional analysis in the baculovirus *in vitro* cell culture system. This system was developed to express foreign genes under the control of the baculoviral promoter in transient expression plasmids. Furthermore, this system can be applied to a functional assay of either the baculovirus itself or foreign proteins. The most widely and commercially available transient gene expression system is developed based on the immediate-early gene (IE) promoter of *Orgyia pseudotsugata* multicapsid nucleopolyhedrovirus (OpMNPV). However, a low expression level of foreign genes in insect cells was observed. Therefore, a transient gene expression system was constructed for improving protein expression. In this system, recombinant plasmids were constructed to contain the target sequence under the control of the *Drosophila* heat shock 70 (Dhsp70) promoter. This protocol presents the application of this heat shock-based pDHsp/V5-His (V5 epitope with 6 histidine)/*Spodoptera frugiperda* cell (sf9 cell) system; this system is available not only for gene expression but also for evaluating the anti-apoptotic activity of candidate proteins in insect cells. Furthermore, this system can be either transfected with one recombinant plasmid or co-transfected two potentially functionally antagonistic recombinant plasmids in insect cells. The protocol demonstrates the efficiency of this system and provides a practical case of this technique.

Video Link

The video component of this article can be found at https://www.jove.com/video/56693/

Introduction

Two protein expression systems have been commonly used for producing proteins: prokaryote protein expression systems (*Escherichia coli* gene expression system) and eukaryote protein expression systems. One popular eukaryote protein expression system is the baculovirus expression vector system (BEVS)[1]. Baculoviruses were first used as worldwide biological control agents of agricultural and forest pests. In the last few decades, baculoviruses were developed as biotechnological tools for protein expression vectors as well. The genomes of baculoviruses consist of double-stranded circular DNA and enveloped nucleocapsids[2]. To date, more than seventy-eight baculovirus isolates have been sequenced[3]. Based on the temporal cascade of baculoviral gene expressions in host insect cells, the gene transcription could be classified into four temporal cascades, including immediate-early, delayed-early, late, and very late genes[4].

BEVSs were designated so that the very late gene promoters (*i.e.*, polyhedron or p10 promoter) were used to drive the target genes, while the recombinant baculovirus was generated by homologous recombination. Expression of foreign proteins in insect cells by recombinant baculovirus is similar to that of mammalian proteins in post-translational modifications (suited for glycoprotein production). Thus, the baculovirus has been widely used[5,6,7]. However, one limitation is the presence of different N-glycosylation pathways in insect cells[7].

Therefore, a new baculovirus expression system, the transient gene expression system, was developed. This system expresses foreign genes under the drive of baculoviral immediate-early promoters (*ie-1* promoter) in insect cells. By using this system, the target protein can be immediately expressed under the control of *ie-1* promoter while modifying the N-glycosylation pathway in insect cells, resulting in better N-linked oligosaccharides[7]. Moreover, baculoviral immediate-early genes are transcribed by the host cell RNA polymerase II and do not require any viral factor for activation[4]. Therefore, foreign proteins can be expressed in insect cells within a short time. To date, the transient gene expression system is one of the most important technologies for performing protein functional assays in the baculovirus *in vitro* cell culture system. The system can be applied to analyze the function of either baculovirus or foreign proteins. One of the commercially available transient gene expression systems is based on the immediate-early gene (IE) promoters of *Orgyia pseudotsugata* multicapsid nucleopolyhedrovirus (OpMNPV) (OpIE2 and OpIE1 promoters).

However, the lower expression level of foreign genes in insect cells was still a problem when the OpIE promoter-based transient gene expression system was used[8,9,10]. Thus, another transient gene expression system was constructed based on the promoter of the *Drosophila* heat shock

protein 70 (hsp70) gene[8,9]. The promoter of hsp70 works more efficiently than baculoviral IE promoter when induced by heat shock in insect cells[10]. In this system, the target genes were expressed under the drive of *Drosophila* heat shock 70 (Dhsp70) promoter. Foreign genes can be easily cloned into the transient gene expression plasmid by PCR-based cloning methods. Furthermore, the control of timing for gene expression can be performed by heat shock induction.

In this report, we follow the approach and express three different truncations of the baculoviral gene (*inhibitor of apoptosis 3*, *iap3* from *Lymantria xylina* MNPV) by using the heat shock-based transient protein expression system and further apply these expressed proteins on anti-apoptotic activity analysis. This system can either express foreign proteins quickly or be further applied to the evaluation of protein anti-apoptotic activity in sf9 cells, while also having the potential to be applied to other protein activity assays.

Protocol

1. Preparations

1. Insect cell culture
    1. Prepare 50 mL of cell culture medium. To do so, add 500 µL of antibiotics (Amphotericin B = 0.25 µg/mL, Penicillin = 100 unit/mL, Streptomycin = 100 µg/mL) and 5 mL of heat-inactivated fetal bovine serum in serum-free cell culture medium (without FBS or antibiotics).
    NOTE: Heat the fetal bovine serum at 65 °C for 30 min in a water bath before use.
    2. Maintain *Spodoptera frugiperda* (Lepidoptera: Noctuidae), sf9 insect cells. Detach ca. 80% of the cells from 25 cm^2 cell culture flask by shaking the flask and check under light microscopy. Then, transfer 50% of the cell suspension to a new 25 cm^2 cell culture flask and allow the cells to attach for 15 min at room temperature. Replace the medium with 5 mL fresh cell culture medium and grow in an incubator at 28 °C. Passage cells every 2 to 3 days depending on the cell growth.

2. Preparation of plasmids for cell transfection
    1. Insert the target DNA fragments (*e.g., Lymantria xylina* MNPV (LyxyMNPV) *iap3* gene and its deletion constructs) into pDHsp/V5-His by PCR-based cloning method[11]. Check the growth of transformed colonies by colony PCR using PCR Master Mix (2X) and the pDhsp-F2/Op-IE2R primer set. Confirm the plasmid sequences by commercial sequencing service.
    NOTE: Table 1 lists the primers used for PCR and the corresponding constructs.
    2. Culture single sequenced bacterial colonies, which contain the aforementioned plasmid constructions (step 1.2) in 200 mL LB medium containing selected antibiotics (50 µg/mL), respectively.
    3. Extract the plasmids from the cultured *E. coli* using Midi Plasmid Kit according to manufacturer's instructions[12].

3. Prepare plating medium by mixing 1.5 mL of cell culture medium and 8.5 mL of serum-free cell culture medium.
4. Prepare Actinomycin D (ActD) cell culture medium by adding 1.5 µL of ActD stock (1 mg/mL) into 10 mL of cell culture medium (final concentration = 150 ng/mL). Store at 4 °C.

2. Protein transient expression

1. Cell seeding
    1. Harvest the sf9 cells by shaking culture flask, topple and fall the cell suspension to 50 mL tube, and transfer 10 µL to hemocytometer by a P10 pipette. Count the cell number under a light microscope.
    2. Plate 3 × 10^5 sf9 cells into each well in a 24-well plate for 15 min at room temperature. Replace the medium with 0.5 mL of plating medium.

2. Transfection of plasmids
    1. Dilute cell transfection reagent: Dilute 8 µL of cell transfection reagent in 100 µL serum-free cell culture medium and mix by vortexing for 1 s.
    2. Add 2 µg of plasmid DNA (pDHsp70-Ac-P35/V5-His or pDHsp70-Ly-IAP3/V5-His or pDHsp70-Ly-IAP3-BIR/V5-His or pDHsp70-Ly-IAP3-RING/V5-His) into 100 µL of serum-free cell culture medium and mix by vortexing for 1 s (**Figure 2**).
    3. Combine the diluted plasmid DNA and diluted cell transfection reagent (210 µL), and mix by vortexing for 1 s. Incubate for 30 min at room temperature.
    4. Add 210 µL of DNA transfection reagent mixture dropwise onto the cells by a P1000 pipette. Incubate at 28 °C for 5 h.
    5. Replace the plating medium with 0.5 mL of cell culture medium using a P1000 pipette. Seal the 24-well plate with tape and incubate the cells at 28 °C for 16 h.

3. Heat shock the transfected cells: Put the plate in a 42 °C water bath (floating on the water surface). Heat for 30 min and return the 24-well plate to the 28 °C incubator.
4. Detection of protein expression
    1. After 1 h or 5 h heat shock,wash the cells with 0.5 mL of 1x PBS buffer briefly three times.
    NOTE: Dilute 10x PBS buffer to 1x PBS buffer by adding 1 mL of 10x PBS buffer to 9 mL sterilized ddH_2O
    2. Lyse the cells with 40 µL of 1x SDS Loading Dye by pipetting up and down.
    NOTE: Dilute 4x SDS Loading Dye by mixing 30 µL of 1x PBS buffer and 10 µL of 4x SDS Sample Buffer.
    3. Heat the protein samples at 98 °C for 10 min in a heat block, spin down for 1 min, and put on ice for Western blot assay.
    4. Western blot assay
    Follow the procedure of Western blot assay from Eslami and Lujan[13]. Run SDS-PAGE gels[14]: one gel subjected to Coomassie blue staining (loading control for checking that the quantity of protein samples loaded in each well is equal in amount) and the other subjected to Western blot assay, according to Eslami and Lujan[13].

5. Detect V5-tagged fusion proteins with rabbit anti-V5 antibody (5 mg/mL) (1:5000 dilution in TBST buffer to working concentration 1 µg/mL) and goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugate (0.8 mg/mL) (1:10000 dilution in TBST buffer to working concentration 0.08 µg/mL).
   NOTE: Adjust the percentage of polyacrylamide to 17.5% when the protein molecular weight to be <17 kDa.

3. Anti-apoptotic activity assay

1. Gene-induced cell apoptosis: Repeat the aforementioned procedure from 2.1 to 2.3. Co-transfect 1 µg of pDHsp/D-rpr/FLAG-His plasmid DNA (containing apoptosis inducer gene) with 1 µg of plasmid DNA [pDHsp70/V5-His vector (negative control), pDHsp70-Ac-P35/V5-His (positive control), pDHsp70-Ly-IAP3/V5-His, pDHsp70-Ly-IAP3-BIR/V5-His or pDHsp70-Ly-IAP3-RING/V5-His, respectively.]. At 5 h post-heat shock treatment, conduct the cell viability assay (**Figure 2**).
2. Chemical-induced cell apoptosis: Repeat the aforementioned procedure from 2.1 to 2.3. In step 2.1.2, plate 1 x 10^6 sf9 cells into each well in a 6-well plate. Transfect 4 µg of plasmid DNA [pDHsp70/V5-His vector (negative control), pDHsp70-Ac-P35/V5-His (positive control), pDHsp70-Ly-IAP3/V5-His, pDHsp70-Ly-IAP3-BIR/V5-His or pDHsp70-Ly-IAP3-RING/V5-His, respectively.]. At 5 h post-heat shock, treat sf9 cells with 2mL of ActD cell culture medium for 16 h and conduct the cell viability assay (**Figure 2**).
   NOTE: Minimum volume to cover one well of 6-well plate is 1 mL.
3. Perform the above anti-apoptotic activity assay experiments, including 3.1 and 3.2 in triplicates.

4. Cell viability assay

1. Wash the treated cells by adding 1 mL of 1x PBS buffer for 1 min 3 times. Resuspend the cells by pipetting 1 mL 1x PBS buffer containing 0.04% trypan blue and stain for 3 min at room temperature. Transfer the cell suspension into a 1.5 mL microtube.
   NOTE: Dilute 0.4% trypan blue solution by mixing 9 mL of 1x PBS buffer and 1 mL 0.4% trypan blue solution.
2. Transfer 10 µL trypan blue-stained cell suspension to the hemocytometer with a P10 pipette and count the viable intact cells under a light microscope.
3. Statistical analysis
   1. Calculate the recorded data and present as the means ± S.D. for all counts.
   2. Analyze the data using the Student's two-tailed t-test by with Microsoft Excel. Define statistically significant data as *P*-value <0.05.

Representative Results

The full length and other two truncations (BIR and RING domains) of Ly-IAP3 from LyxyMNPV were overexpressed in sf9 cells, based on the heat shock-based pDHsp/V5-His/*Spodoptera frugiperda* cell (sf9 cell) system. The pDHsp/V5-His contained a *Drosophila* heat shock protein promoter, which drives downstream gene expression at a temperature of 42 °C condition by using cellular transcriptional factors and the translation system (**Figure 1**)[8,9,11]. The whole technological flowchart is shown in **Figure 2**. After 1 h or 5 h heat shock, the cells were lysed with protein sample buffer and subjected to Western blot assay to confirm the protein expression. The results indicated that the full-length proteins, AC-P35, Ly-IAP3, Ly-IAP3-BIR, and Ly-IAP3-RING, could be detected in transfected cells at either 1 h or 5 h post-heat shock. Moreover, the protein accumulations were found at 5 h post-heat shock (**Figure 3**). Thus, according to the data, the protein functional assay was performed at the maximum protein expression time point (5 h post-heat shock).

Both gene-induced cell apoptosis and chemical-induced cell apoptosis could be adapted to this system for evaluating anti-apoptotic activity (**Figure 2**). For gene-induced cell apoptosis, two constructs (apoptosis inducer and target gene plasmid constructs) were co-transfected into sf9 cells and then heat shocked to activite gene expression. Moreover, the anti-apoptotic protein Ac-P35 (positive control) and an apoptosis-inducer protein (D-RPR) were also expressed using this heat shock transient expression system.

After heat shock, both genes began to be expressed and translated into proteins. Compared to the vector/D-RPR, the positive control (Ac-P35/D-RPR) showed high anti-apoptotic activity, which reached up to 80% of the viability rate. From the cell viability results of the other constructs, the researchers could compare the anti-apoptotic activity to each other or the positive control (**Figure 4A**). For chemical-induced cell apoptosis, only one construct was transfected into sf9 cells. After 5 h post-heat shock, the chemicals (ActD) were added, and cell viability was measured after 16 h (**Figure 2**). Compared with those of the positive control (AC-P35) or the negative control (vector), each construct showed the various effects on anti-apoptotic activity (**Figure 4B**).

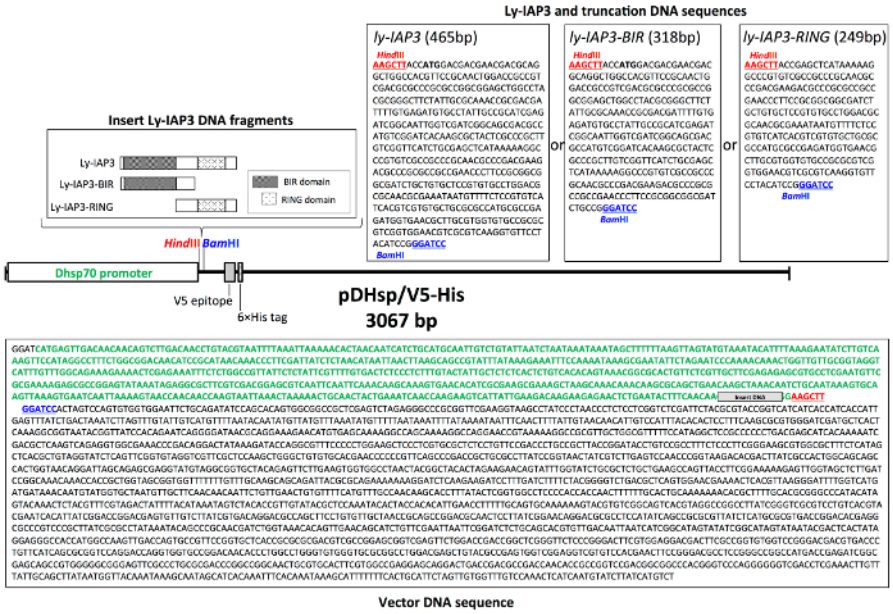

Figure 1: Diagram and nucleotide sequences of *ly-iap3* relative plasmid constructs based on pDHsp/V5-His vector. The start codon (ATG) is shown in bold. Underlining indicates the restriction enzyme cutting sites (*Hind*III in red color; *BamHI* in blue color); the sequence in green color indicates the Dhsp70 promoter region. Please click here to view a larger version of this figure.

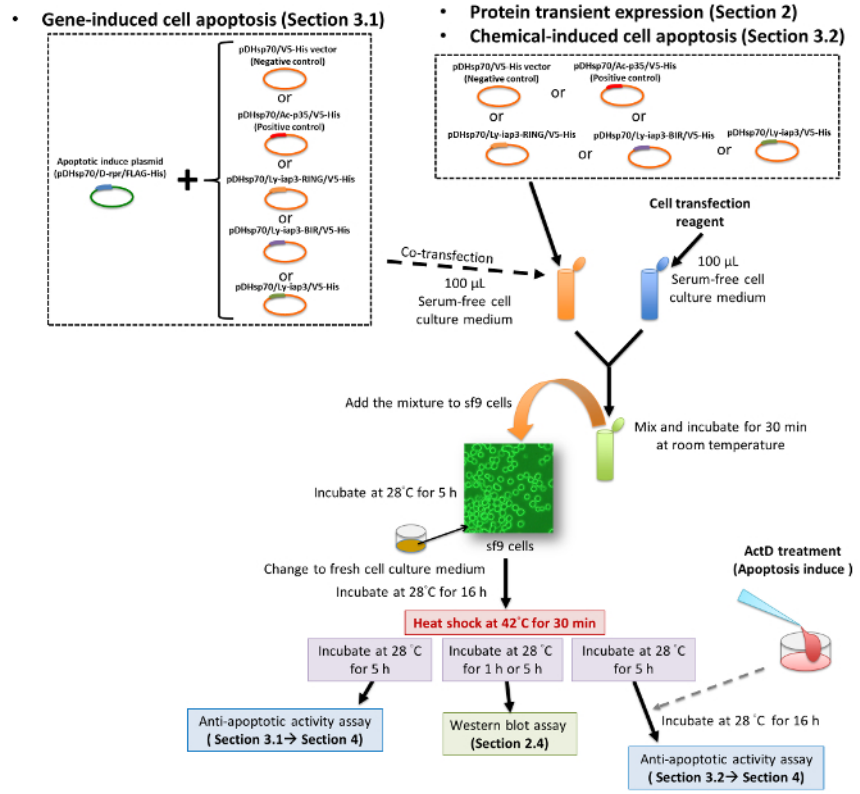

Figure 2: The flow chart of heat shock induced protein expression system (pDHsp/V5-His/sf9 cell system). Gene-induced cell apoptosis and chemical-induced cell apoptosis treatments were indicated to the initial step (co-transfection with apoptosis inducer gene plasmid) or to the later step after heat shock (Chemical-induced apoptosis), respectively, for anti-apoptosis assay. Modified figure and legend reproduced with permission of Springer[11]. Please click here to view a larger version of this figure.

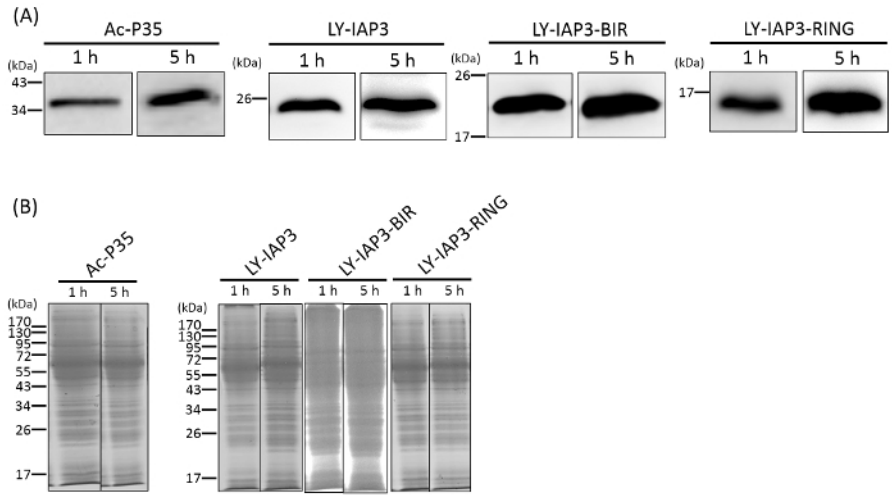

Figure 3: Overexpression of AC-P35, Ly-IAP3, Ly-IAP3-BIR and Ly-IAP3-RING using heat shock induced protein expression system (pDHsp/V5-His/sf9 cell system). At 1 h or 5 h post heat shock, the cell lysates were harvested and subjected to Western blot assay and SDS/PAGE. (**A**) Western blot assay with α-V5 antibody and (**B**) SDS/PAGE stained with Coomassie blue as the loading control. Please click here to view a larger version of this figure.

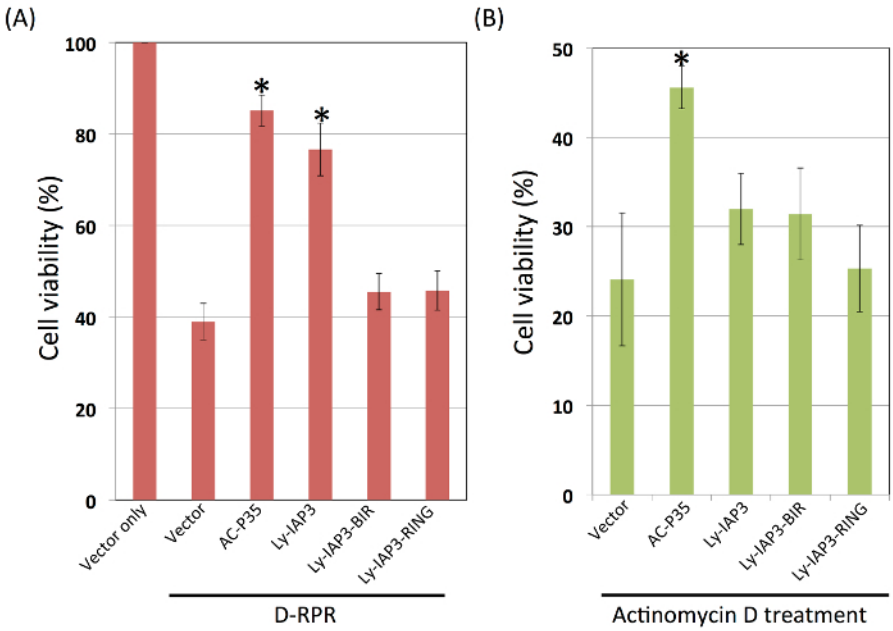

Figure 4: Viability assays for *D-rpr* or Actinomycin D-induced apoptosis. (**A**) sf9 cells were transfected with pDHsp70/V5-His vector (Vector only) and co-transfected pDHsp70/drpr/FLAG-His with pDHsp70/V5-His vector, pDHsp70/Ac-P35/V5-His, pDHsp70/Ly-IAP3/V5-His, pDHsp70/Ly-IAP3-BIR/V5-His, pDHsp70/Ly-IAP3-RING/V5-His, respectively. The differences between vector and P35 and between vector and Ly-IAP3 are statistically significant (*t*-test; $P < 0.05$). (**B**) sf9 cells were transfected with either pDHsp70/V5-His vector or pDHsp70/Ac-P35/V5-His, pDHsp70/Ly-IAP3/V5-His, pDHsp70/Ly-IAP3-BIR/V5-His, pDHsp70/Ly-IAP3-RING/V5-His, respectively, at 5 h after heat shock, ActD was added and 16 h later, the cell viability was measured. The difference between vector and P35 is statistically significant (*t*-test; $P < 0.05$). Modified figure and legend reproduced with permission of Springer[11]. Please click here to view a larger version of this figure.

Name	Sequences
pDHsp-IAP3-HindIII-F	5´- GGAAGCTTACCATGGACGACGAACGACGCAG -3´
pDHsp -IAP3-BamHI-R	5´- GCGGATCCCGGATGTAGGAACACCTTGA -3´
iap3-BIR-BamHI-r	5´- CGGGATCCCGGCAGATCGCCGCCGCGGA -3´
iap3-RING-HindIII-F	5´- GGAAGCTTACCGAGCTCATAAAAAGGCCCGT-3´
pDhsp70-F2	5´- CTGCAACTACTGAAATCAACCAAG-3´
Op-IE2R	5´- GACAATACAAACTAAGATTTAGTCAG-3´

Table 1: The primer sets used for PCR-based cloning method. *The underlined DNA base pairs indicated the restriction enzyme sites. The modified primer list reproduced with permission of Springer[11].

Discussion

The concept of heat shock-based pDHsp/V5-His/sf9 cell system was first described by Clem *et al.* in 1994[8]. Comparison of the baculoviral gene promoter (*IE1*) and *Drosophila hsp70* showed that *hsp70* had a higher efficiency in mosquito cells[10]. Furthermore, due to the heat shock induction, the timing of protein expression could be controlled precisely after heat shock treatment. This system was then applied for protein functional assays of shrimp and nucleopolyhedrovirus (NPV) IAP proteins[9,11]. In this protocol, a total of 6 plasmid constructions were used: three (pDHsp/V5-His, pDHsp/Ac-p35/V5-His, and pDHsp/D-rpr/FLAG-His) were provided by Jian-Horng Leu[9], and the other three constructions (pDHsp-iap3/V5-His, pDHsp-iap3-BIR/V5-His, and pDHsp-iap3-RING/V5-His) were constructed by Nai *et al*., 2016[11]. It seems that this promoter in insect cells works more efficiently than baculoviral gene promoters. However, during manipulation of this protocol, there are several points that need to be considered. Before plasmid transfection, checking the insertion of the DNA sequence is important for gene expression; thus, it should be fused with the V5 epitope to form a V5-tagged fusion protein at the end of the target sequence. Moreover, different fusion tags (*i.e.*, FLAG epitope) could also be designed in the pDHsp-based vector for an *in vitro* protein binding assay. This extension procedure would help further investigate protein-protein interactions[9]. Therefore, the commercial V5 polyclonal antibody could be used for the detection of proteins. The transfection ratio in different insect cells should be tested before the experiment. Lower transfection rates might result in non-detectable of protein expression; thus, a pDHsp vector containing a suitable report gene (*i.e.*, green fluoresce gene) could be transfected into insect cells to evaluate transfection efficiency.

The major limitation of this heat shock expression platform is the protein expression level. In some cases, a low protein expression level was found. According to a pilot study, there was no significant dose-dependent effect that occurred when more plasmid DNA was transfected into sf9 cells (data not shown). Thus, this effect may be caused by the ubiquitin proteasome pathway (UPP) or other unknown mechanisms[11]. Researchers should examine the protein expression in the presence of the proteasome inhibitor (*i.e.*, MG-132) to clarify and recolve this issue[11]. The other limitation is that sustainable production of protein associated with the recombinant viruses could not be achieved. Thus, the stabilization and amount of protein expression are also concerns regarding this system. Therefore, a time course of protein production should also be tested by Western blot assay before the functional assay. In this protocol, we compared two time points (1 and 5 h after heat shock) and observed the accumulation of target protein production from 1 h to 5 h. An increase in time points is suggested in order to determine the best timing conditions for the protein functional assay.

For the protein functional assay, the anti-apoptotic protein Ac-P35 (positive control) and an apoptosis-inducer protein (D-RPR) were also expressed using this heat shock transient expression system. These two proteins are described as functional antagonists[9,11,15]. Therefore, the vector/D-RPR and Ac-P35/D-RPR could serve as a negative control and positive control, respectively. Using this comparison, the anti-apoptotic activity of target proteins could be determined.

The presented protocol could provide a platform to express foreign proteins more quickly or could be further applied to evaluate protein anti-apoptotic activity in insect cells. Once researchers obtain the preliminary results of protein function, this system could be used for further studies.

Disclosures

The authors declare that they have no competing financial interests.

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