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TITLE:

Production of Recombinant PRMT Proteins using the Baculovirus Expression Vector System

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KEYWORDS:

protein arginine methyltransferases, PRMTs, baculovirus expression vector system, BEVS, *Spodoptera frugiperda* insect cells, recombinant protein, transfection reagents, suspension culture of baculovirus-infected insect cells, SCBIIC, 2.8 L Fernbach Culture Flask, 2.5 L Tunair flask, 5 L reagent bottles.

SUMMARY:

The baculovirus expression vector system (BEVS) is a robust platform for expression screening and production of protein arginine methyltransferases (PRMTs) to be used for biochemical, biophysical, and structural studies. Milligram quantities of material can be produced for the majority of PRMTs and other proteins of interest requiring a eukaryotic expression platform.

ABSTRACT:

Protein arginine methyltransferases (PRMTs) methylate arginine residues on a wide variety of proteins that play roles in numerous cellular processes. PRMTs can either mono- or dimethylate arginine guanidino groups symmetrically or asymmetrically. The enzymology of these proteins is a complex and intensely investigated area that requires milligram quantities of high-quality recombinant protein. The baculovirus expression vector system (BEVS) employing *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) and *Spodoptera frugiperda* 9 (Sf9) insect cells has been used for expression screening and production of many PRMTs, including PRMT 1, 2 and 4 through 9. To simultaneously screen for the expression of multiple constructs of these proteins, including domains and truncated fragments as well as the full-length proteins, we have applied scalable methods utilizing adjustable and programmable multichannel pipettes, combined with 24- and 96-well plates and blocks. Overall, these method adjustments enabled a large-scale generation of bacmid DNA, recombinant viruses, and protein expression screening. Using culture vessels with a high-fill volume of Sf9 cell culture suspension helped to overcome space limitations in the production pipeline for single batch large-scale protein production. Here, we describe detailed protocols for the efficient and cost-effective expression of functional PRMTs for biochemical, biophysical, and structural studies.

INTRODUCTION:

Protein arginine methyltransferases (PRMTs) methylate arginine residues in a monomethyl or symmetric/asymmetric dimethyl fashion. The repetitive RG/RGG/GRG sequences are highly preferred by most PRMTs and are found in a wide variety of proteins[1,2]. Arginine methylated proteins such as histones or transcription factors and splicing factors regulate transcription, splicing, and chromatin structure[3,4]. Increasing knowledge of diverse regulation of substrate and cofactor utilization, turnover, and kinetics of PRMTs, as well as generation of selective inhibitors, have shed mechanistic light on these enzymes and their complexes[5,6]. However, not all PRMT family members are studied to the same extent; for example, PRMT9 was only recently discovered to be a member of the PRMT family[1]. Structure and enzyme function studies for these proteins require sufficient, often milligram, quantities of recombinant protein to be available.

The *Escherichia coli* (*E. coli*) prokaryotic expression system is usually the first choice for expression screening utilizing multiple constructs for a given protein[7-9]. However, *E. coli*-based expression does not always result in sufficient quantities of PRMT proteins in their active forms, as we have noted in particular for PRMT5 and PRMT7 (see below). Thus, PRMTs that failed to express in *E. coli* or needed to be produced by the eukaryotic expression machinery were subcloned into vectors appropriate for the expression screening in the alternative baculovirus expression vector system (BEVS). While *E. coli* expressed samples of PRMT1, PRMT3 and PRMT8 have been utilized extensively for *in vitro* assays and crystallography, other PRMTs such as PRMT5, which requires MEP50 binding partner of its dual methyltransferase domain, and PRMTs such as PRMT7 and 9, necessitate insect cell expression to obtain sufficient quantities of active protein. Overall, the standardized medium-throughput methyltransferase assays for PRMT4, 5, 6, 7, and 9 have utilized the BEVS in insect cells[6]. The baculovirus expression vector system (BEVS) is a versatile platform to produce recombinant proteins requiring the eukaryotic expression machinery that enables post-translation modifications essential for biochemical, biophysical, and structural studies[10-12]. Several BEVSs have become commercially available since the first reported use of baculoviruses in 1983 for protein expression[13]. Most of these protocols employ different strategies for the transfer of the expression plasmid into insect cells. These include Bac-to-Bac, flashBAC, BaculoGOLD Bright, BacVector-3000, BacMagic, BacPAK, etc. Our protocol is based on the most commonly used system in BEVS, the Bac-to-Bac system[14], which is designed to transfer the gene/cDNA encoding the protein of interest (POI, here the PRMTs) into the baculovirus genome maintained in a specialized strain of *E. coli* via site-specific transposition[15].

Briefly, the plasmid transfer vector containing the gene of interest was transformed into DH10Bac *E. coli* competent cells to generate recombinant viral bacmid DNA. Adherent Sf9 cells were then transfected with bacmid DNA. Four to five days after transfection, initial recombinant baculoviruses secreted into the cell culture medium were recovered and labeled as the P1 virus. The P1 baculovirus stocks were then used for virus amplification (i.e., generation of P2 baculovirus stocks) and protein expression screening. Based on the expression screening results, P2 viruses for the best expression construct of the protein were identified

and used to generate suspension cultures of baculovirus infected insect cells (SCBIIS) for the large-scale protein production. Here, we describe our detailed protocols and describe the rationale behind our reagent and culture vessel choices to support our strategy of developing a more time-efficient, cost-efficient, and scalable methodology to obtain sufficient quantities of desired recombinant proteins.

PROTOCOL

NOTE: The overview of the BEVS protocol steps is outlined in **Figure 1**.

1. Generation of a recombinant bacmid DNA

1.1 Preparation of LB agar selective plates for DH10Bac transformation

1.1.1 Prepare LB agar plates containing: 50 μg/mL kanamycin, 7 μg/mL gentamicin, 10 μg/mL tetracycline, 200 μg/mL Bluo-gal, and 40 μg/mL IPTG to select for DH10Bac transformants.

1.1.2 Weigh 25 g of premixed LB broth and 13 g of Bacto Agar and put into 2 L flask. Bring the volume to 1 L with distilled water and autoclave for 15-30 min at 121 °C.

1.1.3 Set a water bath at 50 °C and cool the autoclaved agar solution in the water bath for 40-60 min until it is cooled down to 50-55 °C.

1.1.4 To the cooled solution, add gentamicin to a final concentration of 7 μg/mL, kanamycin to a final concentration of 50 μg/mL, tetracycline to a final concentration of 10 μg/mL, Bluo-gal to a final concentration of 200 μg/mL, and IPTG to a final concentration of 40 μg/mL.

1.1.5 Mix the agar solution and aliquot 7-10 mL of the medium to each 60 mm plate using a 50 mL pipette. Let the plates sit at room temperature (2 h) to harden. Invert, wrap and store at 4 °C. Plates containing antibiotics are stable for up to 4 weeks.

1.2 Transformation of the plasmid into DH10Bac E. coli competent cells

1.2.1 Calculate the required volume of competent cells.

1.2.2 Thaw competent cells on ice, gently spin down, and resuspend back by gentle tapping.

1.2.3 Use a 12-channel pipette to dispense 4 μL of the cells into each well of the 96 well PCR plate.

1.2.4 Add ~ 0.3-0.5 μg of recombinant plasmid DNA to the competent cells and mix gently by tapping. Incubate the mixture on ice for 10-15 min.

1.2.5 Heat-shock the mixture in a PCR machine at 42 °C for 45 s. Chill on ice for 2 min.

1.2.6 Dispense 0.5 mL of SOC medium to each well of the 96-well blocks (96-deep well 2.4 mL).

1.2.7 Use a 12-channel pipette to transfer the transformed bacterial suspension into the corresponding well of the block and cover with an airpore sheet.

1.2.8 Place the block in a shaking incubator at 37 ˚C with medium agitation (205 rpm) for 4-5 h.

1.2.9 Evenly spread 30-50 µL of the culture over the surface of the LB agar plate using sterile glass beads. Store the rest of the culture at 4 °C.

1.2.10 Incubate the plates at 37 °C until the color of the blue/white colonies is discernible (40-48 h). Discard the culture when enough white, large colonies are obtained on the plate.

1.2.11 To ensure that white colonies contain only recombinant bacmid DNA, re-streak one isolated white colony onto fresh LB agar plates containing antibiotics, bluo-gal, and IPTG to verify the phenotype.

1.2.12 Incubate for 48 h at 37 °C.

1.2.13 Pick one verified white colony from a re-streaked plate for the extraction of recombinant bacmid DNA.

1.3 Isolating recombinant bacmid DNA

1.3.1 Inoculate a single, isolated white colony into 3 mL of LB medium supplemented with 50 µg/mL kanamycin, 7 µg/mL gentamicin, and 10 µg/mL tetracycline in 24 well blocks (24-well blocks round bottom) and cover with an airpore sheet.

1.3.2 Grow at 37 °C overnight with shaking at 250 rpm.

1.3.3 Centrifuge the 24 well blocks at 2,100 x *g* for 10 min. Decant the supernatant, invert the block, and tap gently on an absorbent tissue paper. Add 250 µL of solution 1 (cell resuspension solution) to each well.

1.3.4 Seal the blocks with tape pads or any other sealing films and place them onto shaking platforms at 75 rpm for 5-10 min. Check each well to ensure proper cell lysis, and resuspend if necessary, using a 1 mL tip.

1.3.5 Add 250 µL of solution 2 (cell lysis solution) to each well, seal the blocks, and place onto shaking platform at 75 rpm for 30 s (to avoid cross contamination of the samples, do not invert 24-well blocks) incubating at room temperature for 4 min.

1.3.6 Add 250 µL of solution 3 (neutralization solution), seal the blocks, and place onto the shaking platform at 75 rpm for 30 s. A thick white precipitate of protein and *E. coli* genomic DNA will form. Place the sample on ice for 10-15 min.

1.3.7 Centrifuge for 60 min at 2,100 x *g* at 4 ˚C to tightly pellet the white precipitate material. During centrifugation, label a new microcentrifuge tube and add 0.8 mL of absolute isopropanol.

1.3.8 Gently transfer the supernatant to the tube containing isopropanol, avoiding any white precipitate material. Mix by gently inverting the tube a few times and then place on ice for 5 to 10 min. At this stage, the sample can be stored at –20 °C overnight.

1.3.9 Centrifuge the sample for 15 min at 14,000 x *g* at 4 ˚C. Remove the supernatant and add 0.5 mL of 70% ethanol to each tube. Invert the tube several times to wash the pellet. Centrifuge for 5 min at 14,000 x *g* at room temperature. (Optional: repeat wash)

1.3.10 Bring samples inside the laminar flow hood to ensure the plasmid preparation's sterility and remove as much of the supernatant as possible.

NOTE: The pellet may become dislodged from the bottom of the tube, so carefully watch the pellet when discarding the supernatant.

1.3.11 Air-dry the pellet inside the laminar flow hood for 15 - 20 min and dissolve the DNA in 50 µL of filtered elution buffer, 10 mM Tris-Cl, pH 8.5 (make sure that pellets are not over-dried).

1.3.12 Since recombinant bacmid DNA is greater than 135 kb in size, to avoid shearing of DNA, dissolve the pellet by gentle tapping.

1.3.13 To verify the presence of the gene of interest in bacmid DNA, set up the PCR reaction mix in a 96-well PCR plate.

1.3.14 Prepare the PCR mix as follows: 1 µL of buffer, 0.2 µL (10 mM each) of dNTPs, 0.1 µL of Taq DNA polymerase, 0.1 µL (25 µM) of forward and reverse primers (BACV2FWD: tattccggattattcataccg; BACV2REV: ctctacaaatgtggtatggc), 1 µL of bacmid DNA and 8 µL of water.

1.3.15 Perform amplification of the target genes with initial denaturation for 2 min at 95 ˚C, followed by 25 cycles of 94 ˚C for 30 s, 55 ˚C for 30 s, and 72 ˚C for 1 min/kb.

1.3.16 Terminate the reaction after a final extension for 7 min at 72 ˚C.

1.3.17 Analyze 10 µL of the amplification product on 1% (w/v) agarose gel containing nucleic acid staining solution by electrophoresis.

1.3.18 Store the verified bacmid DNAs at 4 °C.

2. Generation of recombinant baculovirus stocks

NOTE: Use exponentially growing Sf9 cells with a viability of 95% or greater for any step of the baculovirus expression protocol, including cell transfection for baculovirus generation, baculovirus volume amplification, protein expression screening, and protein production.

2.1 Transfection of Sf9 cells with Bacmid DNA and transfection reagents (T.R.) such as JetPrime or X-tremeGENE 9.

NOTE: Trypan Blue staining and a hemocytometer can be used to determine viable cell counts and % cell viability. Non-viable cells take up the stain and appear blue under the microscope while viable cells remain unstained. To calculate % cell viability, a total cell count is obtained (unstained and stained) and the viable cell count is divided by the total cell count and multiplied by 100.

2.1.1. Dilute the exponentially growing Sf9 cells to a final cell density of 4 x 10^5 cells/mL in serum free insect media and pour into the sterile reagent reservoir.

2.1.2. Use a programmable multichannel pipette to seed 0.5 mL of the diluted Sf9 cells into each well of a 24-well plate.

2.1.3. Label one well of the plate as a control (untransfected) and use it as a control to compare the transfected and non-transfected cells to assess for potential signs of infections.

2.1.4. After seeding the cells into the plates, gently rock the plates back and forth several times to ensure an even monolayer of cells. Do not swirl the plates because the cells will cluster into the center of the well.

2.1.5. Incubate the plates at 27 °C for at least 1 h to allow for cell attachment to the culture plates.

2.1.6. Mix well the transfection reagent vial. For each transfection, add 2 µL of the transfection reagent to 100 µL of the transfection buffer. Any other unsupplemented insect medium can also be used. Deposit the diluted transfection reagent in a sterile reagent reservoir and gently mix for 10 s.

2.1.7. Using a 12-channel pipette, transfer 102 µL of the diluted transfection reagent into a sterile 96-microwell plate.

2.1.8. Transfer 10 µL of a 0.2 µg/µL solution of recombinant bacmid DNA into the corresponding well of a 96-well microwell plate and mix by gently shaking (tapping) the plate from the sides.

2.1.9. Incubate the transfection mixture for 15-20 min to enable complex formation.

2.1.10. Using an adjustable 6-channel pipette designed for transfer between 96- and 24-well plates, add the transfection mix onto the cells dropwise into corresponding wells of transfection plates and incubate for 4-5 h at 27 °C.

2.1.11. Gently rock the plates back and forth several times during the incubation time to ensure the even distribution of the transfection mixture over the cell monolayer.

2.1.12. 4-5 h after transfection, add 1.5 mL of insect serum-free medium supplemented with 10% (v/v) final of heat inactivated fetal bovine serum and antibiotic-antimycotic to 1% (v/v) final volume (100 units/mL of penicillin, 100 µg/mL of streptomycin, and 0.25 µg/mL of amphotericin B).

2.1.13. Incubate the cells in a 27 °C incubator for 72-96 h. Gently rock the transfection plates once a day when possible.

2.1.14. Look for signs of infection (SOI), evident in transfected cells at 72-96 h post-transfection (**Figure 2**). Keep in mind that the transfected cells will start producing the virus and further infect the culture; thus, look for the signs of infection.

NOTE: Signs of infection are structural changes in the insect cells, such as a 25-50% increase in the cell diameter, enlarged cell nuclei, uniformly rounded shape, loss of proliferation and adherence to the culture dish surface, as well as a decrease in cell viability (**Figure 2**. Baculovirus Infected and uninfected Sf9 cells).

3. Small–scale protein expression screening and virus amplification

3.1 Infection of Sf9 cells with P1 baculovirus stocks.

NOTE: 4-5 days after transfection, signs of infection should be evident in the transfected cells when compared to the control (untransfected) cells under an inverted microscope. The initial recombinant baculoviruses secreted into the cell culture medium should be ready to collect.

3.1.1. Seed 2 x 10^5 exponentially growing Sf9 cells in serum-free insect media into each well of the 24-well plates in a total volume of 2 mL for infection with P1 viruses to amplify virus volume (resulting in the generation of the P2 viruses).

3.1.2. After pipetting the cells into the plates, gently rock the plates, using back and forth motion to ensure an even monolayer. Do not swirl the plates because the cells will cluster into the center of the well.

3.1.3. Incubate the plates at 27 ˚C for at least 1 h to allow for cellular adherence to the plate.

3.1.4. Dispense 4 mL of Sf9 cells at a density of 3.5-4 x 10^6 in insect serum-free medium into each well of 24-well blocks to infect with P1 viruses for the protein expression screening.

NOTE: Label one well of the 24-well plates and 24-well blocks as control and use as an uninfected control for comparison of infected and non-infected cells when looking for SOI.

3.1.5. Use a programmable electronic multichannel to allow the simultaneous collection of P1 viruses (step 2.1.13), infection of freshly seeded Sf9 cells (step 3.1.1) and infection of the suspension cells in the 24-well blocks (step 3.1.4) with 150 µL of P1 viruses.

3.1.6. Spin down the rest of the collected P1 viral stocks for 15 min at 17,970 x *g*, transfer into microcentrifuge tubes and store in the dark at 4 ˚C.

3.1.7. Gently rock the 24-well plates (step 3.1.1) on a reciprocating shaker to ensure an even distribution of the added P1 viruses over the cell monolayer; repeat this a few times during the incubation time.

3.1.8. Cover the 24-well blocks with the suspension culture of infected Sf9 cells (step 3.1.4) with an airpore sheet.

3.1.9. Incubate the 24-well blocks at 27 ˚C, shaking at 245 rpm for 72-96 h.

3.1.10. Look for SOI within 72-96 h after infection time in the 24-well plates with P2 viruses (step 3.1.1) and in the 24-well blocks (step 3.1.4) with infected cells for the expression screening.

NOTE: 4-5 days after infection of Sf9 cells with P1 viruses, SOI should be evident in the infected cells when compared to the non-infected control cells under an inverted microscope.

3.1.11. Collect P2 viruses from 24-well plates, centrifuge for 15 min at 17,970 x *g*, transfer into microcentrifuge tubes and store in the dark at 4 ˚C.

3.1.12. At 72-96 h post Sf9 infection, spray over the 24-well blocks with 70% ethanol, bring into the laminar flow hood, and check the cell density and viability in a few wells by Trypan Blue staining.

3.1.13. Proceed to protein purification if cells have signs of infection and viability is close to 70-75% as assessed by Trypan Blue staining.

3.1.14. Pellet the cells by centrifuging the 24-well blocks at 525 x *g* at 4 ˚C for 15 min. Discard the supernatant and thoroughly re-suspend the pellets in 1 mL of lysis buffer comprising 25 mM Tris pH 8.0, 300 mM NaCl, 0.6 % NP-40, 2 mM imidazole, 5% glycerol (v/v) and 1x protease

inhibitor cocktail (100x protease inhibitor cocktail comprises aprotinin 0.25 mg/mL, leupeptin 0.25 mg/mL, pepstatin A 0.25 mg/mL; E-64 0.25 mg/mL).

3.1.15. Store the cell suspension at -80 °C for the subsequent test purification (see 3.2.2).

3.2 Protein purification from frozen cell suspension in 24-well test-expression blocks.

3.2.1 Assembly of the binding block (**Figure 3**).

3.2.1.1 Place 3 overlapping layers of parafilm on the top of the 96-deep well block (96-deep well 2.4 mL).

3.2.1.2 Place a 96-well filter plate (filter microplate, 96-well, polypropylene with 25 µm ultra high molecular weight polyethylene membrane) on the top of the 96-deep well block.

3.2.1.3 Push down the filter plate to secure the tips into the parafilm to seal off the filter plate from the 96-well deep block.

3.2.1.4 Transfer 50 µL of pre-equilibrated 50% Ni-NTA resin slurry into each well of the filter plate.

3.2.2 Test expression procedure

3.2.2.1 Place the frozen cell suspension (step 3.1.15) present in 24-well blocks in a water bath at RT for 5-10 min, then shake at 450 rpm for 20 min.

3.2.2.2 Centrifuge the 24-well blocks at 3,275 x *g* for 15 min.

3.2.2.3 Using a multichannel pipette, transfer the cleared lysates into a filter plate containing 50 µL of pre-equilibrated 50% Ni-NTA resin slurry (step 3.2.1.4) and seal the filter plate with a 96-well cap mat (96-well cap mat, for use with square well, 2 mL).

NOTE: Use a few rubber bands to keep the binding block together during incubation and centrifugation steps.

3.2.2.4 Place the secured binding block for 45-60 min into a rotator in a cold room to incubate cleared lysates with Ni-NTA resin.

3.2.2.5 After incubation, carefully lift the filter plate and remove the parafilm layer from the surface of the 96-deep well block (step 3.2.1.1).

3.2.2.6 Place back the filter plate on top of the 96-deep well block and spin down the secured binding block for 2 min at 235 x *g*.

3.2.2.7 Wash bond Ni-NTA resin 2x with 2 mL of washing buffer comprising 25 mM Tris pH 8.0, 300 mM NaCl, 5% glycerol, and 15 mM imidazole.

3.2.2.8 Spin down the block with washing buffer each time for 5 min at 235 x *g* to ensure complete removal of residual liquid.

3.2.2.9 Transfer the filter plate on the top of the 96-well PCR plate containing 10 µL of 4x loading dye.

3.2.2.10 Add 40 µL of elution buffer (25 mM Tris pH 8.0, 300 mM NaCl, 5% glycerol, 500 mM imidazole) to each well of the filter plate and incubate for 5 min.

3.2.2.11 Spin down the block to elute proteins into the 96-well PCR plate at 235 x *g* for 10 min.

3.2.2.12 Seal the 96-well PCR plate with high temperature resistant tap pad and heat at 98 ˚C for 3 min.

3.2.2.13 Load 15 µL of eluted protein samples in standard Laemmli buffer on 4–20% SDS-PAGE gel next to the protein ladder and run the gel with standard running buffer containing SDS.

3.2.2.14 Stain the gel with Coomassie blue and de-stain with water. Analyze the results of test expression to identify the best expressing constructs for large-scale production.

4. Preparations of the baculovirus-infected insect cells (SCBIIC) for protein production

4.1. 4 days before the scheduled production time, split exponentially growing Sf9 cells to a final cell density of 2 x 10^6 cells/mL into 125 mL / 250 mL / 500 mL Erlenmeyer glass shake flasks with baffles in 50 mL / 100 mL / 200 mL of Insect Serum-Free medium containing 1% (v/v) final antibiotic-antimycotic.

4.2. Add 0.150 mL / 0.300 mL / 0.6 mL of appropriate P2 viruses, incubate infected cells at 165 rpm on an orbital shaker with a one-inch stroke and at a lower temperature of 25 ˚C to slow down the cell division.

4.3. At 4 days post-infection, check cells under a microscope for SOI and proceed to production if the cell viability as verified with Trypan Blue stain is close to 70-75%.

5. Sf9 cell preparations for large-scale protein production

5.1 4 days before the scheduled production time, calculate the required volume of Sf9 cells for large-scale protein production.

5.2 Seed 2 L of exponentially growing Sf9 cells in Insect Serum-Free Medium to a cell density of 1 x 10^6 cells /mL in 2.8 L Fernbach shake flasks.

5.3 Incubate flasks at 27 ˚C with shaking set at 150 rpm.

NOTE: To prevent bacterial contamination in the Sf9 cell culture, use gentamicin to a final concentration of 10 µg/mL or penicillin/streptomycin to 50 U/mL and 50 µg/mL, respectively.

6. Infection of the Sf9s with SCBIIS for the large-scale protein production

6.1 Split 2 L or 4 L of exponentially growing Sf9 cells in Insect Serum-Free Medium to a final cell density of 4 x 10^6 cells/mL in 2.5 L Tunair shake flasks or 5 L reagent bottles.

6.2 Add 10-12 mL/L of the baculovirus infected insect cell (SCBIIC) suspension culture.

6.3 Incubate the infected culture of Sf9 cells on a shaker with 145 rpm at the lower temperature of 25 ˚C (to slow down cell division) for 72-96 h.

6.4 At 72 h post-infection, check cells under a microscope for SOI and assess cell viability.

6.5 Usually, after about 72 h post-infection, the viability of Sf9 cells drops to 70%–75% (measured using Trypan Blue stain). Harvest infected Sf9 cells in a 1 L polypropylene bottle by centrifugation at 900 x *g* for 15 min at 4 ˚C.

6.6 Resuspend the cell pellet collected from 1 L of the production cell culture with 20-25 mL of 1x PBS by gently swirling and transfer into 50 mL conical tubes.

6.7 Spin down the cell suspension at 900 x *g* for 15 min and discard the PBS solution.

6.8 Resuspend the washed cell pellet with 20-25 mL of the suspension buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 5% glycerol, 1x protease inhibitor cocktail) and flash freeze in liquid nitrogen; store at −80 ˚C until purification.

NOTE: Purification procedures for the PRMTs have been described in detail in the SGC published paper[6].

REPRESENTATIVE RESULTS

An overview of the BEVS protocol is outlined in **Figure 1**. Multiple expression constructs of PRMTs, including full-length, domains and truncated fragments, were generated at the Structural Genomics Consortium (SGC, Toronto) according to in-house strategies with an attempt to increase the success rate for identifying soluble and stable proteins with a relatively high expression level[7,9]. Interested readers are encouraged to review the SGC's definitions and methodology of designing a "fragment" as the segment of the gene sequence incorporated into an expression clone, "domain" as a PFAM-annotated structural domain and "construct" as the

fragment cloned in an expression vector, all of which have been described in detail in an earlier publication[7]. Expression constructs of PRMTs presented in this protocol are for the production of the polyhistidine-tagged proteins cloned into the pFBOH-MHL vector, which is a derivative of the pFastBac1 vector. In **Figure 4**, we present SDS-PAGE analysis of the His-tagged soluble constructs of PRMT1, 2, 4-9 purified from pellets collected after 4 mL of production in Sf9 cells (step 3.1.4). Full-length (FL) PRMT1 and PRMT9 are not presented in this gel, since FL PRMT1 has been produced from *E. coli*, and FL PRMT9 produced from BEVS has been purified by Flag-tag[6]. The truncated constructs of PRMT1, FL PRMT4, and all the PRMT8 constructs show a relatively high yield, but protein eluates contain fractions of co-purified contaminants. These constructs require further optimization of the purification protocols. Additional approaches are thus required to improve the purity of these proteins from scale-up productions, such as a reduction in the amount of nickel beads at the stage of the incubation with a clarified lysate; an increase of the imidazole concentrations in the wash buffers; cleavage of the His-tag with TEV protease, followed by application on a Ni-affinity resin; and, additional purification steps such as size-exclusion and ion-exchange chromatography. The constructs of PRMT2 show significantly lower yield compared to other proteins and full-length PRMT2 protein accompanied by a strong contaminant band. Scale-up production and two steps of purifications such as IMAC and size-exclusion confirmed a low expression level for this construct along with the persistent presence of the co-purifying contaminant for the FL protein. Pure proteins have been obtained for the PRMT5 complex produced and purified with its obligate binding partner, MEP50. The truncated construct of PRMT9 has almost two or three-fold lower expression level, close to 1.5 mg/L, as compared to other PRMTs. Nevertheless, the recombinant viral stocks of this construct have been used for scale-up production, diffracting crystals were obtained, and the structure was solved for this protein along with PRMT4, 6 and 7 (**Figure 5**).

For the scale-up productions, the corresponding P2 viruses were used to infect the suspension culture of Sf9 insect cells. This step generates 50/100/200 mL of baculovirus infected cells containing infected cells and P3 viruses in the supernatant. For the large-scale protein production, 2 L of Sf9 cells were cultured in each of 2.8 L Fernbach shake flasks at 150 rpm, 27 ˚C (**Figure 6**). On the day of production, 2 L of Sf9 cells (cell viability > 97%) in 2.5 L Tunair shake flasks or 4 L in 5 L reagent bottles were diluted to a cell density of 4 x 10^6/mL. These cells were infected directly with 10-12 mL/L of suspension culture of baculovirus infected insect cells and incubated at a lowered temperature of 25^{o}C, at 145 rpm. Infection of the production batch directly with a suspension culture of baculovirus-infected insect cells significantly reduced laborious and time-consuming steps in virus volume amplification, excluding the extra handling of the infected cells, and avoided reduction in titer and virus degradation. SF9 cell culture maintenance and scale-up production has been done in the culture vessels with a high fill volume to adopt a large-scale protein production in the one batch (**Figure 6**).

Full length PRMT 4, 5 (in complex with MEP50), 6, 7, and 9 proteins produced from the Baculovirus mediated production platform have been used for kinetic characterization and inhibitor compound screening at the SGC[6]. Crystal structures were solved and deposited into the Protein Data Bank (PDB) for the full length or truncated forms of the proteins PRMT 4, 6, 7, and 9 with various chemical probes and inhibitors. Expression plasmids for these PRMTs were

deposited to the Addgene plasmid repository (Addgene is a distributing partner of the SGC, https://www.addgene.org/) and are available to the research community (**Figure 5**).

FIGURE LEGENDS:

Figure 1: Schematic overview of the steps of the baculovirus expression process

Figure 2: Baculovirus Infected and uninfected Sf9 cells. Signs of infection are structural changes in the insect cells, such as a 25-50% increase in the cell diameter, enlarged cell nuclei, uniformly rounded shape, loss of proliferation, and adherence to the culture dish surface, as well as a decrease in cell viability. White scale bar 200 µm. The signs of infection presented here are the same for the transfected cells using both transfection reagents, JetPrime and X-tremeGene 9. The particular example shown is for the JetPrime transfection reagent. (**A**) Uninfected Sf9 cells as a control. (**B**) Baculovirus-infected Sf9 cells.

Figure 3: Binding plate assembly for quick purification of test expression proteins. Please see text for details, steps 3.2.1-2

Figure 4: Protein expression screening results. Protein expression screening results of the baculovirus mediated protein production in 4 mL of Sf9 suspension culture infected with corresponding P1 recombinant viruses for different PRMTs and the PRMT5-MEP50 complex.

Figure 5: Summary of expression constructs for PRMT4, 6, 7, and 9 used for crystal structure studies at the Structural Genomics Consortium, Toronto (SGC). The crystal structures were solved and deposited into the Protein Data Bank (PDB) for the full length or truncated forms of proteins PRMT 4, 6, 7, and 9 with various chemical probes and inhibitors. Expression plasmids for these PRMTs were deposited to the Addgene plasmid repository and are available to the research community (Addgene is a distributing partner of the SGC, https://www.addgene.org/).

Figure 6: Sf9 insect cell maintenance and protein production in the different culture vessels: (**A**) 2.8 L Fernbach flask for cell maintenance and protein production. Use of the 72% fill volume increases throughput rate by 2.5-fold in one shaking platform. (**B**) Tunair shake flasks (only 9 flasks out of 10 are presented in this picture) and reagent bottles with an 80% fill volume drastically increase the shaking platform's production capacity.

DISCUSSION:

One of the advantages of BEVS in insect cells centers on the capability of the post-translational modification machinery to enable more complex modifications such as phosphorylation, myristoylation, and glycosylation. Together with the highly efficient folding of mammalian proteins, these modifications facilitate high amounts of modified and folded protein suitable for physiologically relevant downstream experiments[16].

Here, we described detailed protocols of the BEVS emphasizing critical elements for successful expression screening of multiple constructs of PRMT proteins and large-scale PRMT protein

production in the Baculovirus expression platform: 1) The use of regular, adjustable, and programmable multichannel pipettes to transfer the biological materials between 24- and 96 - well cell culture plates and blocks at the stages of bacmid DNA and virus generation; collection of the recombinant viruses, amplification of viral volumes of the recombinant viruses and preparation of the protein expression screening blocks. 2) High performance and cost-effective transfection reagents for the generation of recombinant viruses. 3) Suspension culture of baculovirus-infected insect cells (SCBIIC) for large-scale protein production. 4) Utilization of high-fill volume 2.8 L Fernbach shake flasks to maintain Sf9 suspension culture and 2.5 L Tunair shake flasks and 5 L reagent bottles for the large-scale protein production.

Special considerations and rationale for the transformation and transfection steps.

Although a commercial protocol recommends using 100 µL of competent cells for one transformation[14], the transformation efficiency of commercial DH10Bac *E. coli* competent cells is as high as 1 x 10^8 cfu/ µg DNA, so we use only 4 µL. This is enough for each transformant to obtain isolated white recombinant colonies for the bacmid DNA isolation. Adherent Sf9 cells in the 24-well transfection plate were seeded at a cell density of 2 x 10^5 /mL in 0.5 mL of Serum-Free Insect Media. This volume is enough to ensure even coverage of the working surface of the well. At the same time, it does not dilute the transfection mix too much, which enhances transfection efficiency. Transfection reagents are non-toxic to the Sf9 cells, and media exchange is not necessary. Instead of a media change, an additional 1.5 mL of media containing 10% (v/v) FBS is added into the transfection plate at 4-5 hours post-transfection time to facilitate cell growth. The transfection efficiency of both transfection reagents is high. Still, with X-tremeGene 9, the signs of infection in the transfected cells (**Figure 2**) appear 10-12 h earlier than with the JetPrime reagent, so we choose between these reagents depending on the working schedule of the next steps in the protocols, which provides some flexibility in the overall process.

Protein test expression screening can be set up with P2 viruses if the amount of the initial recombinant viruses, collected from the transfection plate and labeled as P1, is a limiting factor to use for the protein expression screening.

Considerations when moving from small to large culture volumes.

Historically, it was believed that optimal cell growth requires a high air space in the suspension culture of Sf9 cell maintenance and scale-up productions. However, in 2014, it was reported that high air space in culture vessels is less critical than previously thought[17]. A culture vessel set up using appropriately adjusted shaking speed to the orbital throw of the shaking platform will provide sufficient oxygen transfer even in the high-fill volume suspension culture by creating and maintaining small air bubbles for a longer time. With this approach, commercially available insect cells can be cultured at a higher shaking speed within a normal range of the cells' doubling time without sacrificing high cell viability.

Thus, 6 years ago, we started to increase the suspension culture volume in a shaking flask during cell maintenance and introduced a different type of culture vessel for protein production while adjusting and monitoring shaking conditions (**Figure 6**). To establish optimal conditions in

these culture vessels, we monitored the culture parameters such as: cell doubling time along with cell viability, uniform distribution of cells in terms of size and shape, clumps or aggregation, and infectability of cells.

For example, for Sf9 cell maintenance, in the 2.8 L Fernbach shake flasks, we culture 2 L instead of 0.8 L of the Sf9 suspension cells shaking at 150 rpm at 27 ˚C and cell viability most of the time is close to 99%, with evenly shaped healthy dividing cells. For scale-up production, we infect 4 L of cells in 5 L reagent bottles shaking at high speed as 145 rpm at a lowered temperature of 25 ˚C. The most commonly used incubators with a built-in shaking platform can hold 6 x 2.8 L shake flasks or 6 x 5 L reagent bottles, or 10 x 2.5 L Tunair shake flasks. Thus, the capacity of the one shaking platform, if we fill Fernbach shake flasks and reagent bottles to 1/3 versus to the high-fill volume of the vessels is 4.8 L versus 12 L using 2.8 L Fernbach shake flasks and 10 L versus 24 L using 5 L reagent bottles (**Figure 6**). Suspension cell culture maintenance and scale-up production in the culture vessels with a high fill volume have helped us overcome limitations of the production volumes and adopt a large-scale platform. Thus, this is highly useful for labs with no access to bioreactors and/or limited space in the production pipelines.

This protocol could be readily adapted for the production and purification of protein constructs with different affinity tags by utilizing appropriate resins and modifying purification buffers as has been described in the SGC published paper[6] for the Flag-tagged full length proteins of PRMT4, 7, 9 and the His-tagged PRMT5-MEP50 complex and PRMT6. Although we describe a BEVS protocol for the PRMT family of proteins, the same approach can be applied to any other protein family.

DISCLOSURES.

The authors declare no conflict of interest.

ACKNOWLEDGMENT:

The authors wish to thank Dalia Barsyte-Lovejoy for taking the time to provide valuable feedback and critical comments on the manuscript and all our SGC colleagues who worked with the PRMT protein family expressed from the Baculovirus Expression Vector System.

The SGC is a registered charity (number 1097737) that receives funds from AbbVie, Bayer AG, Boehringer Ingelheim, Genentech, Genome Canada through Ontario Genomics Institute [OGI-196], the EU and EFPIA through the Innovative Medicines Initiative 2 Joint Undertaking [EUbOPEN grant 875510], Janssen, Merck KGaA (aka EMD in Canada and US), Pfizer, Takeda and the Wellcome Trust [106169/ZZ14/Z].

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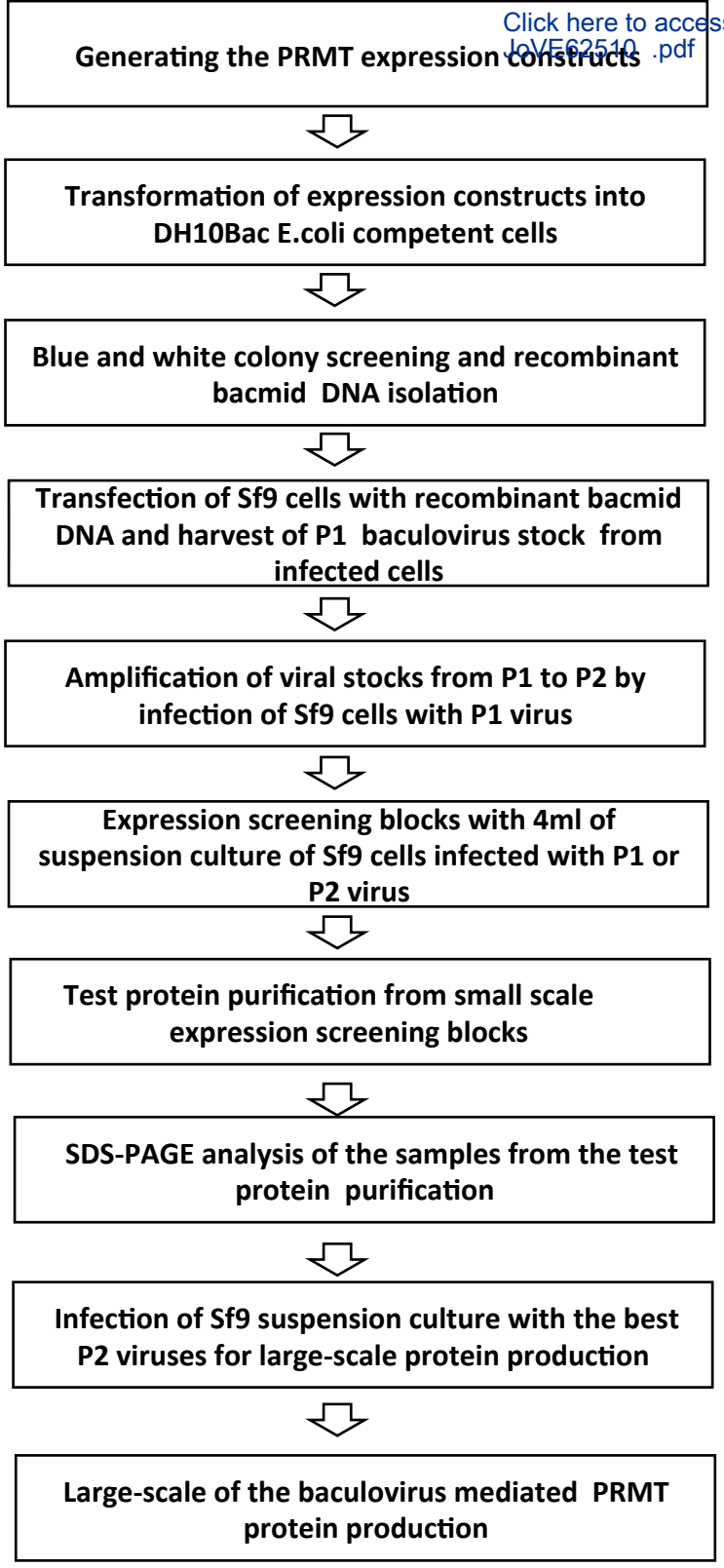


Figure 1

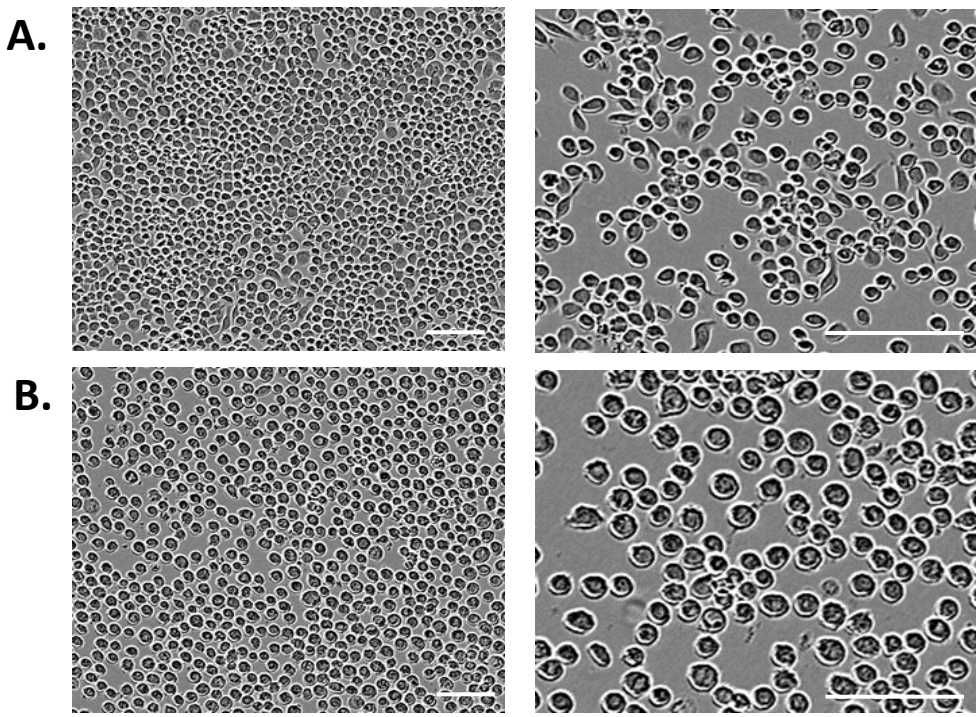


Figure 2

1. Place 3 overlapping layers of parafilm on the top of the 96 deep well block (Masterblock 96 deep well 2.4mL).
2. Place a 96 well filter plate (Filter microplate, 96-well, polypropylene, with 25 µm ultra high molecular weight polyethylene membrane) on the top of the 96 deep well block.
3. Push down the filter plate to secure the tips into the parafilm to seal off the filter plate from the 96 well deep block.
4. Transfer 50µl of pre-equilibrated 50% Ni-NTA resin slurry into each well of the filter plate.
5. Using a multichannel pipette, transfer the cleared lysates into a filter plate containing 50µl of pre-equilibrated 50% Ni-NTA resin slurry.
6. Seal the filter plate with a 96-well cap mat (96-well cap mat, for use with square well, 2 mL).
7. Proceed to step 4 of 3.2.2 section.

Note. Use a few rubber bands to keep the binding block together during incubation and centrifugation steps.

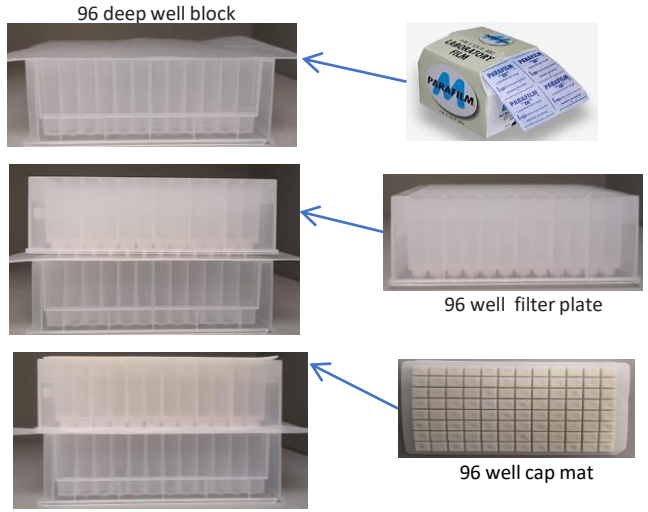
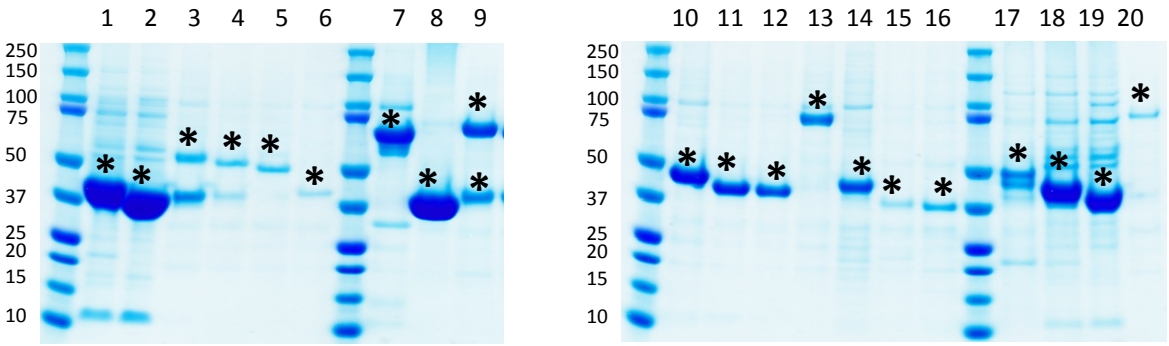
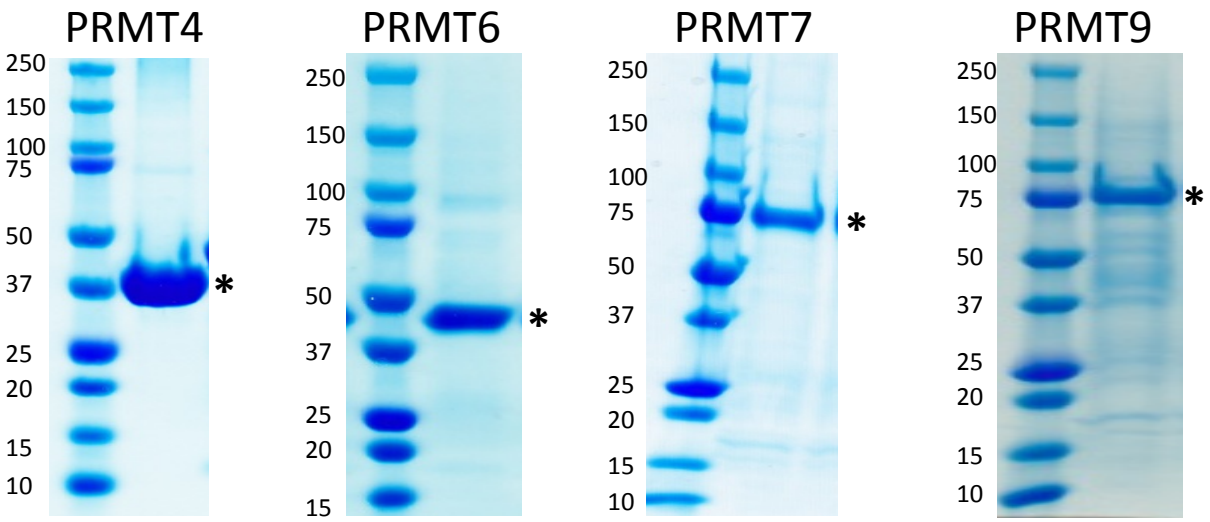


Figure 3



Lane #	Protein name	Construct: start-end (amino acid residues)	Full-length protein	Ref Seq
1	PRMT1	30-371	371	BC109282
2	PRMT1	43-371	371	BC019268
3	PRMT2	1- 433	433	BC000727
4	PRMT2	13 - 433	433	BC000727
5	PRMT2	21 - 433	433	BC000727
6	PRMT2	89 - 433	433	BC000727
7	PRMT4	1 - 608	608	NM_199141
8	PRMT4	140 - 480	608	NM_199141
9	PRMT5 MEP50	1-637 1-342	637 342	BC025979 BC016946
10	PRMT6	1 - 375	375	NM_018137
11	PRMT6	24 - 375	375	NM_018137
12	PRMT6	33 - 375	375	NM_018137
13	PRMT4	1- 692	692	BC000146
14	PRMT7	1 - 356	692	BC000146
15	PRMT7	361 - 692	692	BC000146
16	PRMT7	369 - 692	692	BC000146
17	PRMT8	1 - 394	394	BC022458
18	PRMT8	35 - 394	394	BC022458
19	PRMT8	61 - 394	394	BC022458
20	PRMT9	127-845	845	BC064403

Figure 4



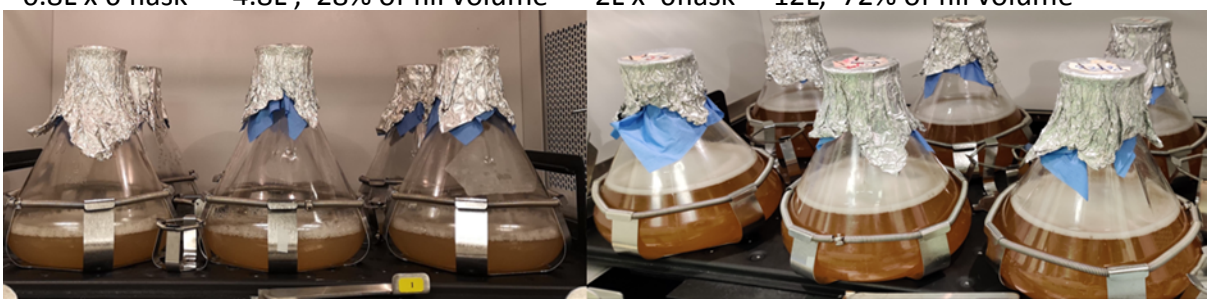
PDB code	Protein name	Addgene Plasmid	Construct start-ends at amino-residues	Number of amino acids of full-length protein
6DVR	PRMT4	Plasmid # 60080	146-489	608
6D2L	PRMT4	Plasmid # 60080	146-489	608
5U4X*	PRMT4	Plasmid # 60080	140-480	608
4IKP*	PRMT4	Plasmid # 60080	140-480	608
4HC4	PRMT6	Plasmid #61964	1-375	375
4QQK	PRMT6	Plasmid #61964	1-375	375
5E8R	PRMT6	Plasmid #61964	1-375	375
5HZM	PRMT6	Plasmid #61964	1-375	375
4QPP	PRMT6	Plasmid #61964	1-375	375
6W6D	PRMT6	Plasmid #61964	1-375	375
6WAD	PRMT6	Plasmid #61964	1-375	375
6P7I	PRMT6	Plasmid #61964	1-375	375
5WCF	PRMT6	Plasmid #61964	1-375	375
6OGN	PRMT7	Plasmid #162260	1-692	692
6PDM	PRMT9	Plasmid #162264	127-845	845

Figure 5

A. Growth of Sf9 insect cells and protein production in 2.8L Nalgene Fernbach Culture Flask

Low throughput with high air space
0.8L x 6 flask = 4.8L , 28% of fill volume

2.5 x higher throughput with a high-fill volume
2L x 6flask = 12L, 72% of fill volume



B. Growth of Sf9 insect cells and protein production in 2.5 Tunair shake flasks and 5L reagent bottles

2L x 9 bottles = 18L, 80 % of fill volume

4L x 6 bottles = 24L , 80% of fill volume

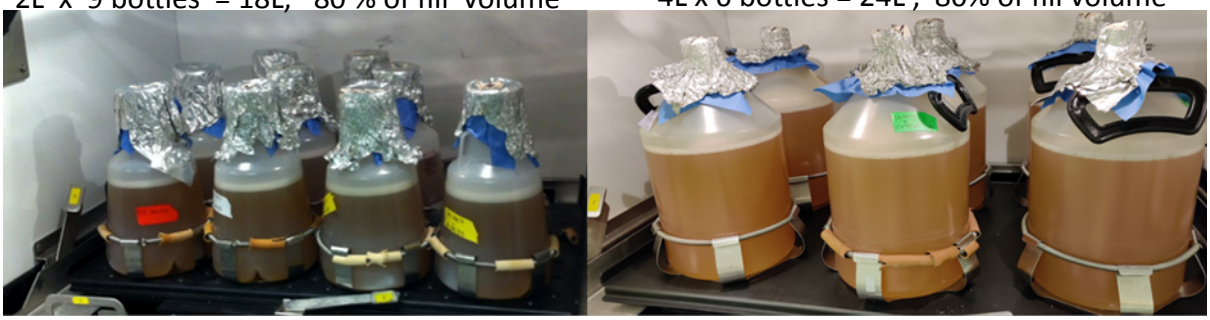


Figure 6

Dear Editor and production team,

Thank you for the helpful suggestions and comments. Please see the implemented changes as outlined below.

Kind regards,
Alma Seitova

Editorial and production comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Done
2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points
Done
3. Please provide an email address for each author.
Done.
Ashley Hutchinson: ashley.hutchinson@utoronto.ca
Almagul Seitova: alma.seitova@utoronto.ca
4. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).
Corrected.
5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Bac-to-Bac (Invitrogen) flashBAC (Oxford Expression Technologies), BaculoGOLD Bright (BD Bioscience), BacVector -3000 (Novagen), BacMagic, EMD Biosciences, BacPAK (Clontech), JetPrime, X-tremeGene 9, DH10Bac™, (I-MAX, Wisent Inc., or HyClone, Cytiva), etc.
Corrected.
6. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, alphabets, or dashes.
Done.
7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever

possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”
Done.
8. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).
Done where it’s applicable.
9. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?
Done.
10. Please include a figure or a table in the Representative Results showing the effectiveness of your technique backed up with data. Please ensure all figure are referenced in order. Please discuss all figures in the Representative Results. However, for figures showing the experimental set-up, please reference them in the Protocol.
The “Representative Results section was revised according to the suggestions and comments.
11. Please ensure the results are described in the context of the presented technique - you performed an experiment; how did it help you to conclude what you wanted to and how is it in line with the title.
Done
12. Please do not make points in the discussion section. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
The discussion section was revised according to suggestions.
13. Please include a Disclosures section, providing information regarding the authors’ competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included.
Disclosures section included.
14. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, (YEAR).] For more than 6 authors, list only the first author then et al.
Done.
15. Please remove the figure legends from the uploaded figures. Please place all the figure legends after the representative result section.
Figure legends from the uploaded figures removed.
16. Please sort the materials table in alphabetical order.

Done.
Changes to be made by the Author(s) regarding the video:
1. Please increase the homogeneity between the video and the written manuscript. Ideally, all figures in the video would appear in the written manuscript and vice versa. The video and the written manuscript should be reflections of each other.
The homogeneity between the video and the written manuscript has been increased where it is applicable.
2. Furthermore, please revise the narration to be more homogenous with the written manuscript. Ideally, the narration is a word for word reading of the written protocol. However, you do not need to show every step in the video. Steps which make a cohesive story can be shown in the video.
The narration revised according to the comments.
3. Please remove the word Dr. from the name of Dr, Alma Seitova in the introduction section to match the JoVE format.
Done
4. Please ensure that the protocol chapter titles are same in the video and in the text.
Done.
5. 2:37, 7:22: Please ensure that the narration matches the video.
Done.
6. Please include a separate representative result section at the end of protocol and before the conclusion section. Please include a figure or a table in the Representative Results showing the effectiveness of your technique backed up with data. Please present the representative result as if you performed an experiment and how did it help you to conclude what you wanted.
Done.
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9. Text and graphics
Done.
• Section title cards need to be centered in frame
10. Video
• Fade up from black at beginning of video. Done.
• Fade to black at end of video. Done.
• 01:40 - Instead of speeding up video, consider cutting out unnecessary portions such as closing medium caps and removing pipette packaging, and use cross dissolves transitions between edits. Done.
11. Audio
• Author statement audio sounds good but is too quiet. Increase audio gain by 6db.
Increased audio by 20%
• VO is low quality and difficult to hear. Strongly consider re-recording at higher

quality with more practiced delivery. Then match volume to the boosted author statement audio. . Done

• 00:03 - Consider editing out mouth clicking sound. Fixed.
• 03:30 - VO speaker stumbles over word "phenyle". It has been said “Finale”
• 04:08 - Try to add a pause between sentences here. Done

Once done please ensure that the revised video is no more than 15 min length. Please upload the revised video at
https://www.dropbox.com/request/LLRTMuJC7SFKSvUTu0Wd?oref=e

We thank the reviewers for insightful comments and suggestions. Please find our responses below and changes tracked in the manuscript. We hope that the edited manuscript addresses all reviewer concerns and is suitable for publication in JoVE.

Reviewers' comments:
Reviewer #1:
Revision of the manuscript JoVE62510: Recombinant PRMT proteins production in Baculovirus expression vector system

Overall comment
The manuscript describes protocols for large-scale PRMT protein production in the Baculovirus expression system.
This is an interesting and well-organized protocol that can be useful for those scientists who want to produce recombinant PRMT proteins for a number of in vitro assays or for small molecules/drug screenings.
The protocol is overall well-presented and it shall be published for this special issue of JoVE. Only minor corrections/amendments are required and outlined below, grouped based on the paper organization in to paragraphs.

Minor corrections

Main Text and Protocol
* 1.1: Remove symbols like TM, or ®
Done.
* RPM is written sometimes in uppercase and sometimes in lowercase throughout the protocol: be homogenous
Done.
* 1.3 "a 1 mL tips": correct tips plural with singular

Corrected.

* Be homogenous with the terms referring to time (sec vs. seconds; min versus minutes) and with the spacing between the number and the term (min/hours/secs)

Done.

* 2.1 first point: the sentence is too long and organize with a complex structure which does not facilitate understanding. Please rephrase, possibly dividing the period into two sentences.

The sentence has been rephrased:

1.Dilute the exponentially growing Sf9 cells to a final cell density of $4x10^5$ /mL in Serum Free Insect Media and pour into the sterile reagent reservoir.

2.Use a programmable multichannel pipette to seed 0.5 mL of the diluted Sf9 cells into each well of a 24 well plate.

* 2.1 towards the end of the paragraph: "after 4-5 hours of post-transfection time" this is a strange sentence: it should be "after 4-5 hours" or 4-5 hours post-infection

The sentence has been corrected according to the review's comments:

12.Four to five hours after transfection, add 1.5 mL of Insect Serum-Free Medium supplemented with 10% (v/v) final of Heat Inactivated Fetal Bovine Serum and antibiotic-antimycotic to 1% (v/v) final (100 units/mL of penicillin, 100 µg/mL of streptomycin, and 0.25 µg/mL of amphotericin B).

* 2.1, first point: "as soon as viruses" I think the sentence misses the verb 'as soon as the viruses are ready.."

Done

* The sentence "Use a programmable electronic multichannel allowing collection of P1 viruses and at the same time the infection of the next batch of prepared adherent SF9 cells with a 150µl of P1 viruses" which is present in both 2.2 and 3.1 paragraphs is not clear: please rephrase

The sentence has been rephrased:

Use a programmable electronic multichannel to allow simultaneous 1) collection of P1 viruses (2.1.13) used for the infection of freshly seeded Sf9 cells (3.1.1) and 2) infection of the suspension cells in the 24 well blocks (3.1.4) with 150 uL of P1 viruses.

* Paragraph 2.2: change the expression "After 72-96 hours of post-infection time" as suggested before.

Done: 14.Look for signs of infection (SOF), evident in transfected cells at 72 -96 hours post-transfection (Figure 2).

* Notes are not listed with homogenous format: sometimes are put in parentheses, sometimes a bullet point: we advise to choose one homogenous format (in parentheses is advisable)

Corrected.

* Paragraph 3.2.2: the SDS-page-based analysis to test expression procedure is not

well described: no information about percentage of acrylamide (which may vary depending on the size of the recombinant fraction., but this could be DISCUSSED), or about the loading buffer used (e.g. is it SDS-based? Is it standard Laemmli buffer?)
The missing information has been added.
Load 15ul of eluted protein samples in standard Laemmli buffer on 4–20% Criterion SDS-PAGE next to the protein ladder and run the gels with standard running buffer containing SDS.

* 4.1 "sighs of": please correct the typo
Corrected.
* 4.3 "Follow the same procedure as described above" I would change the sentence into "follow the same procedure described above" and refer to the exact step numbers
This section has been deleted and more steps after large-scale production added as production harvest procedure.

Discussion
* Very interesting and well-organized, with useful tips and critical considerations.
* However, please, revised carefully the English form, to remove typos and grammar errors.
The section has been revised.

* (c) "High - fill volume of the suspension culture of the SF9 cells in the 2.8L Fernbach shake flasks for maintenance and in the production vessels as 2.5L Tunair shake flasks and 5L reagent bottles" this sentence is unclear, it should be rephrased, possibly splitted in two parts.
The sentence was clarified:
Utilizing high-fill volume 2.8L Fernbach shake flasks for the maintenance of Sf9 suspension culture and 2.5L Tunair shake flasks and 5L reagent bottles for the large scale protein production

* In this session It could be useful to include some comments/considerations about known post-translational modifications regulating the activity of this class of enzymes, which have been shown to be preserved through the Baculovirus expression system. This would highlight the peculiar advantage of this platform over bateria-based ones
We thank the reviewer for this suggestion. Indeed, the role of post-translational modifications in protein expression is worth discussing. We have added the following sentences.
One of the advantages of BEVS in insect cells centers on the capability of the post-translational modification machinery to enable more complex modifications such as phosphorylation, myristoylation and glycosylation. Together with the highly efficient

folding of mammalian proteins, these modifications facilitate high amounts of modified and folded protein suitable for physiologically relevant downstream experiments[17].

Figures and figure legends
* Figure format is overall not paper-like; figures seem directly modified from PPT slides rather than professional figure for a manuscript.
The figures have been reformatted.
* The ordering of the figures does not reflect their order of appearance in the protocol: I suggest renumbering, with Figure 4 being anticipated before Figure 2 and 3 as it is introduced in the paragraph Representative Results, while Figure 2 and 3 are discussed in the Discussion session.
* The Format of figure legends is not homogenous (especially figures 3,4 ,5 etc.)
The figure legends has been reformatted and renumbered accordingly.
* Figure 5 legends is not clear: what does it mean exactly that are available for the research community? Should be a link or website indicated, if feasible based on the journal guidelines?
The sentence has been revised and the link to the Addgene website included: Expression plasmids for these PRMTs were deposited to the Addgene plasmid repository (Addgene is a distributing partner of the SGC, https://www.addgene.org/) and are available to the research community (Figure 5).

Reviewer #2:
Summary
In the manuscript by Hutchinson A. and Seitova A., authors describe a medium-throughput protocol to screen/produce recombinant PRMT proteins using Baculovirus expression system. This protocol could be potentially applied to other recombinant protein production. Author explain not only how to combine 24- and 96 well plates to make the pipeline into a throughput fashion, but also provide some time/money saving tips, such as cheaper transfection reagents (JetPrime), using suspension culture of infected insect cells and much larger cell culture by high - fill volume. This type of manuscript fits into the JoVE.

Major Concerns:
1. Authors demonstrated the ability to produce most recombinant PRMT proteins by Baculovirus system. In the introduction, they mentioned that However, this platform has limited use in the expression of some PRMTs requiring eukaryotic expression machinery to obtain a protein in a soluble and active form. Thus, PRMTs that failed to express in a soluble form in E. coli or needed to be produced by eukaryotic expression machinery for some applications, were subcloned into vectors for the expression screening in the alternative system; baculovirus expression vector system

(BEVS). "
This description "imply" many PRMTs can be produced by E. coli
However, most of PRMTs that have been studied and structurally determined were produced by E. coli system.
For the proper comparison, can author tell us among 21 PRMT's constructs in this manuscript, how many of them were not able to be produced by E. coli and how many of them produced by E. coil were not active.
We thank the reviewer for this comment and pointing out the need for clarification. We have added the following sentences to clarify:
While E. coli expressed samples of PRMT1, PRMT3 and PRMT8 have been utilized extensively for in vitro assays and crystallography, other PRMTs such as PRMT5 that require MEP50 binding partner of dual methyltransferase domain, PRMTs such as PRMT7 and 9 necessitated insect cell expression to obtain sufficient quantities of active protein. Overall, the standardized medium throughput methyltransferase assays for PRMT4, 5, 6, 7, and 9 have all utilized the BEVS in insect cells [6].

2. For protocol 2.1/2.2: Look for signs of infection in 72-96 hours post infection time. Although authors briefly describe sign of infection and show figure 2 (after 96-120 hours).. Can author describe how to determine the time for virus harvest in detail? Since this is most confusing part for beginners.
We have introduced in the sections 2 and 3 these two notes to address this comment:

1. Look for signs of infection (SOF), evident in transfected cells at 72 -96 hours post-transfection (Figure 2). Keep in mind that the transfected cells will start producing the virus and further infect the culture; thus, we are looking for infection signs.

Note: Signs of infection are structural changes in the insect cells, such as a 25-50% increase in the cell diameter, enlarged cell nuclei, uniformly rounded shape, loss of proliferation and adherence to the culture dish surface, as well as a decrease in cell viability (Figure 2. Baculovirus Infected and uninfected Sf9 cells).

Minor Concerns:
Step 1.2 - LB agar plating seems the lest throughput style. From 96 wall transformed cell, is there any way to plate on 24 or 96 well block?
Yes, it is very time-consuming process, but it has to be done individually for the each of transformants. Definitely, we should think over how to make this step less-time consuming.

Reviewer #3:
Manuscript Summary:
In the present manuscript Hutchinson and Seitova describe a protocol for the large-scale production of PRMTs using the baculovirus expression vector system. In order to screen simultaneously multiple PRMT-containing constructs the authors apply a

"medium-throughput" system to their protocol.
There are interesting technical improvements presented by the authors, in particular for an audience dedicated to perform large screening of baculoviral constructs. However, the manuscript, which was obviously not proofread and requires improvement of the English, is badly structured, full of inconsistencies and imprecisions, and lacks important information. Some figures and figure legends are incomplete or misleading.
Therefore, I would consider the manuscript of Hutchinson and Seitova insufficient for publication in its present form. Numerous major and minor points (detailed below) should definitely be addressed before recommending it for publication in JoVE.

Major Concerns:
Throughout the text
- The text is full of errors of different kinds (syntax, grammar, punctuation, SI rules, and spelling) that renders its reading pretty laborious. The authors are advised to thoroughly proofread their text (even if the final format of the protocol is aimed to be a video).
- The protocol would gain in clarity if the authors replaced the bullet points of their different chapters with numbers.
- The correct abbreviation of Spodoptera frugiperda 9 cell is "Sf9", not "SF9".
We thank the reviewer for such a thorough feedback on the manuscript. The changes below were implemented to address the issues.
Title
- I would suggest to rephrase the title as follows: "Production of recombinant PRMT proteins using the baculovirus expression vector system".
The title has been rephrased as per reviewer suggestion:
Production of recombinant PRMT proteins using the baculovirus expression vector system.

Abstract
- Sentence 2: "This methylation regulates the function of proteins involved in the cell cycle, transcription, splicing, and epigenetics". This statement is misleading: the substrates of PRMTs are involved in multiple biological processes such as… However, cell cycle might be regulated on the level of transcription and splicing, and epigenetic mechanisms regulate transcription. Therefore, the here chosen enumeration of biological processes is not equivalent and not logical. The authors shall rephrase their words.
We thank the reviewer for bringing this inaccuracy to attention. The sentence was corrected.
Protein arginine methyltransferases (PRMTs) methylate arginine residues on a wide variety of proteins that play roles in numerous cellular processes.

- Sentences 4 and 5: "The catalytic activity of most PRMT depends on their homodimerization. An exception is PRMT7/9 with two catalytic domains". This point has no relevance here. The authors shall either move these 2 sentences to the Introduction part and add detailed and referenced explanations or delete these sentences.
The sentences were removed

- Sentence 6: an "a" shall be added to the sentence as follows: "The enzymology of these proteins is a complex and currently intensely investigated area that requires sufficient quantities of recombinant protein".
We thank the reviewer for the clarification, the sentence was amended.

-Sentence 8: "Multiple constructs of each PRMT were generated at the Structural Genomics Consortium (SGC, Toronto) to improve the success rate for obtaining soluble proteins with a high expression level". The authors shall better explain their thoughts why this strategy can improve the success rate, e.g. the use of plasmids encoding full length versus truncated PRMT proteins. Can they as well mention already in the abstract the type of tag they used? This information (His-tag) is given only in the Section 3.1 of the protocol, which is definitely too late for the reader´s understanding.
-Sentence 9: "To screen the expression of many proteins, a medium-throughput system was applied in our protocols". Do the authors mean "to screen simultaneously the expression of many constructs"? Please rephrase. Can the authors give their definition of a "medium-throughput" in comparison to "high-throughput"?
We thank the reviewer for this comment and pointing out the need for clarification.
We have deleted sentence 8 and 9 and edited this section as follow:
To simultaneously screen for the expression of multiple constructs of these proteins, including domains and truncated fragments as well as the full-length proteins, we have applied scalable methods utilizing adjustable and programmable multichannel pipettes, combined with 24- and 96-well plates and blocks.
We have included more information as follow in the description of the Figure 4:
Expression constructs of PRMTs presented in this protocol are for the production of the polyhistidine-tagged proteins cloned into pFBOH-MHL vector, which is a derivative of the pFastBac1 vector (Invitrogen).

- Sentence 11: "Utilizing culture vessels in high - fill volume helped overcome the challenge of space limitations in the production pipeline for a large scale of protein production in one batch". What are "vessels in high - fill volume"? Do the authors mean vessels with/containing high-fill volume of Sf9 culture? Please properly rephrase the sentence (and with a "to" after "helped").
The sentence has been rephrased:

Using culture vessels with a high-fill volume of Sf9 cell culture suspension helped to overcome space limitations in the production pipeline for single batch large-scale protein production.

-Sentence 12: "In addition, PRMT5 has also been co-expressed with its obligate binding partner MEP50 by co-infection of the corresponding recombinant viruses". Why is there an emphasis made for PRMT5? In sentence 7, the authors mentioned that PRMT4-9 have been expressed. This implies PRMT5. The fact that PRMT5 has been successfully expressed together with MEP50 can be mentioned in the Result part.
Sentence deleted from this section.

Introduction
- Sentence 3: "Arginine methylated proteins are involved in the cell cycle, transcription, splicing, and epigenetics (3,4)". See comment of Abstract/sentence 2
The sentence was corrected to:
Arginine methylated proteins such as histones or transcription and splicing factors regulate transcription, splicing, and the chromatin structure

- Sentence 4. "The catalytic activity of most PRMT depends on their homodimerization, except PRMT7 and PRMT9 that have two catalytic domains (4)". See comment of Abstract/sentence 4 and 5.
The sentence was taken out.

- Sentence 6: "However, not all PRMT family members are studied to the same extent". Can the authors further develop their statement and provide with examples of PRMTs studied to different extents?
We have further elaborated on the most recent member of PRMTs.
"However, not all PRMT family members are studied to the same extent, for example, PRMT9 was only recently discovered to be a member of the PRMT family [1]."

- Sentence 8: "Here we provide robust eukaryotic expression methods for several PRMTs to obtain large active protein quantities for further functional and structural investigations". The authors shall reword their sentence: "quantities" can not be active, but enzymes.
Sentence was changed to:
Here we provide robust eukaryotic expression methods for several PRMTs to obtain large quantities of active proteins for further functional and structural investigations.

- Sentence 10: "However, this platform has limited use in the expression of some PRMTs requiring eukaryotic expression machinery to obtain a protein in a soluble and active form". What do the authors mean? Which PRMTs are the authors referring

to? Did the authors compare bacterial expressed and in Sf9 cells expressed recombinant PRMT preparations (except PRMT5) with regard to their activity in enzymatic assays?
We thank the reviewer for bringing this point and we have clarified the sentence. However, E. coli-based expression does not always result in sufficient quantities of PRMT proteins in active forms, as we have noted in particular for PRMT5 and PRMT7 (see below).

- Sentence 11: "Thus, PRMTs that failed to express in a soluble form in E. coli or needed to be produced by eukaryotic expression machinery for some applications…". Which PRMTs are the authors referring to? They shall provide examples.
Please see the above and additional address to the reviewer 2 point.
While E. coli expressed samples of PRMT1, PRMT3 and PRMT8 have been utilized extensively for in vitro assays and crystallography, other PRMTs such as PRMT5 that require MEP50 binding partner of dual methyltransferase domain, PRMTs such as PRMT7 and 9 necessitated insect cell expression to obtain sufficient quantities of active protein. Overall, the standardized medium throughput methyltransferase assays for PRMT4, 5, 6, 7, and 9 have all utilized the BEVS in insect cells [6].

The authors shall modify the second part of the sentence "…were subcloned into vectors for the expression screening in the alternative system; baculovirus expression vector system (BEVS)" as follows "…were subcloned into vectors appropriate for the expression screening in the alternative baculovirus expression vector system (BEVS)."
The sentence has been modified.
were subcloned into vectors appropriate for the expression screening in the alternative baculovirus expression vector system (BEVS).
- Sentence 12: please correct the sentence as follows "The baculovirus expression vector system (BEVS) is a versatile platform to produce recombinant proteins requiring eukaryotic expression machinery that enables post-translational modifications…".
The sentence corrected accordingly to the review's comment.
- Sentence 13: "Several BEVSs developed have become commercially available since the first use of baculoviruses for protein expression has been reported in 1983 (13)". I recommend to delete "developed".
Deleted. Several BEVSs have become commercially available since the first reported use of baculoviruses for protein expression in 1983 [13].

- Sentence 15: "Our protocol is based on the most commonly used system in BEVS, the Bac-to-Bac system (14), which is designed to transfer the expression plasmid into the baculovirus genome maintained in a specialized strain of E. coli via site-specific transposition [15]". This is incorrect and requires rephrasing. The Bac-to-Bac system is designed to transfer the gene/cDNA encoding for the protein of interest (POI, here

the PRMTs) into the baculovirus genome. The authors shall be more accurate and correct this misleading phrasing, which appears many times throughout the text.
The sentence corrected.
Our protocol is based on the most commonly used system in BEVS, the Bac-to-Bac system [14], which is designed to transfer the gene/cDNA encoding for the protein of interest (POI, here the PRMTs) into the baculovirus genome maintained in a specialized strain of E. coli via site-specific transposition [15].

- Sentence 17: to improve the logical flow of the text, please write "Adherent Sf9 cells (Invitrogen) were then transfected with bacmid DNA".
Sentence corrected.
Adherent Sf9 cells (Invitrogen) were then transfected with bacmid DNA.

- Sentence 18: "After 4-5 days of the post- transfection time,...". This clause, repeated very often throughout the text, makes no sense. It shall be replaced by the authors with "...transfected with bacmid DNA. Four to five days after transfection / post-transfection, ..." or "...transfected with bacmid DNA. Four to five days later, ...". Moreover, the sentence "...initial recombinant baculoviruses were secreted into the cell culture medium, were recovered, and labeled as the P1 virus" shall be replaced by "...initial recombinant baculoviruses secreted into the cell culture medium, were recovered, and labeled as the P1 virus".
This section has been corrected.
Four to five days after transfection time, initial recombinant baculoviruses secreted into the cell culture medium, were recovered, and labeled as the P1 virus.

- Sentence 19: "The recombinant virus titer amplified by infecting adherent culture of SF9 cells with P1 virus and resulting P2 viruses collected and used for the protein expression screening". This sentence makes no sense in its current form. The authors shall rephrase it using the passive form as follows "The recombinant virus titer was amplified by infecting adherent culture of SF9 cells with P1 virus and the resulting P2 viruses were collected and used for the protein expression screening."
This section has been corrected as follow: The P1 baculovirus stocks were then used for virus amplification (i.e. generation of P2 baculovirus stocks) and protein expression screening.

- Sentence 20: "Based on the expression screening results, corresponding P2 viruses for the best expression construct of the protein used to generate the suspension culture of baculovirus-infected insect cells (SCBIIS) for large- scale protein production". The authors shall reword their sentence and use the passive form when needed.
The sentence has been revised.

Based on the expression screening results, P2 viruses for the best expression construct of the protein were identified and used to generate suspension cultures of baculovirus infected insect cells (SCBIIS) for large-scale protein production.

Protocol
1.1
step 2:
"LB broth (MILLER)" is mentioned in the main text but does not appear in the Table where instead "Lb Broth (Lennox)" stands. Can the authors rectify?
LB broth source has been corrected.
Weigh 25 g of premixed LB broth (Lennox) and 13 g of Bacto Agar.

1.2
step 2:
- The 96 well PCR plates are not listed in the Table. Please correct.
Done
- "a" shall be added in "Use a 12-channel pipette...".
Corrected.
step 3:
- Which type of pFastBac donor plasmid containing the different PRMT constructs did the authors use for the transformation? This information is missing in the whole manuscript and shall be added by the authors.
Missing information has been added.
Expression constructs of PRMTs presented in this protocol are for the expression of the polyhistidine-tagged proteins cloned into pFBOH-MHL vector, which is a derivative of the pFastBac1 vector (Invitrogen).

- Is the distinction between a "high-throughput cloning" and a "regular mini-prep" relevant here? What is a "high-throughput cloning"? The authors shall explicitly mention the amount (in µg) of plasmid DNA used for the transformation?
This section has been modified.
Add ~ 0.3-0.5µg of plasmid DNA to the competent cells and mix by gently tapping. Incubate the mixture on ice for 10-15 minutes

step 4:
- How is the heat shock performed? In a PCR machine (since the authors use a 96 well PCR plate), or in a water bath? For sake of clarity, please indicate this in the text.
This section has been modified
Heat-shock the mixture in a PCR machine at 42°C for 45 seconds. Chill on ice for 2 minutes.

- A new bullet point or number shall be created for the "Dispense 0.5 ml of S.O.C. medium..."
Done

- The authors add 0.5 ml of SOC or LB medium to the transformed bacteria for the recovery. Can the authors comment on when is recommended to use one medium instead of the other (SOC or LB)?
LB deleted

- "a" shall be added in "Use a 12-channel pipette..."
Done

- Several protocols, including the Bac-to-Bac protocol from Invitrogen advise to re-streak white colonies onto fresh LB agar plates to confirm the phenotype of the colonies. Can the authors comment of this point? In addition, to verify the presence of the gene of interest (GOI) in the recombinant bacmid, a PCR is recommended to be performed with as template, either the purified recombinant bacmid DNA or directly a picked colony. The proper control and verification of the integration of the GOI into the bacmid DNA is missing in the authors´ protocol, but is an important prerequisite for the production of recombinant proteins and shall be described in details by the authors.
We thank the reviewer for bringing these points and we have included these two-steps into protocol.

1. To ensure that white colonies contain only recombinant bacmid DNA, re-streak one isolated white colony onto fresh LB-agar plates containing antibiotics, bluo-gal and IPTG to verify the phenotype.
2. Incubate for 48 hours at 37 °C.
3. Pick one verified white colony from re-streaked plate for the extraction of recombinant bacmid DNA.
4. To verify the presence of the gene of interest in bacmid DNA set up the PCR reaction mix in 96-well PCR plate.

1.3
steps 2-5:
- The centrifugation speeds shall be expressed in "g". This stands for the whole manuscript. Done
- Solution 1, Solution 2, and Solution 3 are neither described in the text nor listed in the table under these terms. For the sake of clarity, the authors shall correct this discrepancy.
Done. Description of the solutions written in the protocol and in the table.

Solution 1 (Cell Resuspension Solution), Solution 2 (Cell Lysis Solution) (Neutralization Solution)

- At which speed is the 24 well block shaked on the shaking platform? Standard protocols performed with reaction tubes do not recommend during the lysis and neutralization steps to shake the bacterial suspension but to cautiously invert it to prevent the shearing of the bacmid DNA (as pointed out by the authors at step 12) and the bacterial chromosome. Can the authors provide clarification to this technical point?

Yes, we are agree with this comment, it has to be cautiously inverted. But, since we do use 24 well blocks covered with tape pads, to avoid cross contaminations of the samples by inverting,
we gently shake them on the shacking platform at 75 rpm as specified in the text.

step 6:

- The centrifugation is, compared to standard protocols, exceptionally long (one hour) and at low speed (3000 rpm). Is it due to the use of 24 well blocks, whose maximum centrifugation speed is limited? Can the authors clarify this point?

Yes, it is correct. One hours of the centrifugation has been used in this protocol to pellet white precipitate due of the speed limitations for the 24 well blocks.

step 11:

- Which elution buffer is used by the authors? H2O? TE? The authors shall clarify this point.

We've clarified this question and wrote the composition of the elution buffer.
11. Air-dry the pellet inside of the laminar flow hood for 15 - 20 minutes and dissolve the DNA in 50 µl of filtered elution buffer, 10mM Tris-Cl, pH 8.5 (make sure that pellets are not over- dried).

2.1

step 1:

- "Seed exponentially growing in Serum Free Insect Media (I-MAX, Wisent Inc., or HyClone, Cytiva) suspension culture of SF9 cells at a cell density 4x105 cells/ml into each well of four 24 well plate, total 0.5ml". This sentence is syntactically incorrect. The authors shall rephrase it as follows "Seed 2x105 of exponentially growing Sf9 suspension cells into a well of a 24 well plate containing 0.5ml of Serum Free Insect Media (I-MAX, Wisent Inc., or HyClone, Cytiva)."

The sentence corrected as follow:

2. Dilute the exponentially growing Sf9 cells to a final cell density of $4x10^5$ /mL in Serum Free Insect Media and pour into the sterile reagent reservoir.

3. Use a programmable multichannel pipette to seed 0.5 mL of the diluted Sf9 cells into each well of a 24 well plate.

step 2:
- What does "side-to-side and back-and forth pattern" mean, in particular "side-to-side"? Do the authors recommend the use of a reciprocating shaker?
We have corrected this sentence.
After pipetting the cells into the plates gently rock the plates back and forth several times to ensure an even monolayer of the cells. Do not swirl the plates because the cells will cluster into the center of the well.
Yes, reciprocating shaker on the low speed could be used at this step.

step 3:
- The rationale of incubating the cells at 27°C for one hour is to let the cells attach to the culture vessel. Can the authors make this technical point clear in their protocol?
Sentence corrected as follow:
4. Incubate the plates at 27 °C for at least one hour to allow for cell attachment to the culture plates.

step 4:
- The authors use Grace´s insect medium for the dilution of the transfection reagents but I-MAX medium for the seeding of the cells (step 2). After transfection, any Insect Serum Free Medium is added to the cells (step 10). For the sake of clarity, can the authors either keep the same medium throughout their protocol (and correct the Table) or explain the reason for the use of one medium or the other.
The sentence above corrected:
Mix well the transfection reagent vial. For each transfection, add 2 µL of JetPrime or X-tremeGENE 9 to 100 µL of JetPrime Buffer (supplied with the JetPrime transfection kit) or any other unsupplemented insect medium can be substituted. Deposit the diluted transfection reagent in a sterile reagent reservoir and gently mix for 10 seconds.

step 5:
- Please correct: "Using a 12-channel pipette transfer 102 µl of the diluted transfection reagent into a sterile 96-microwell plate".
Done.
Using a 12-channel pipette transfer 102 µl of the diluted transfection reagent into a sterile 96-microwell plate

step 6:
- Please correct: "Transfer 10 µl of a 0.2µg/µl recombinant bacmid DNA into ...".
Done.

Transfer 10 µl of a 0.2 µg/µl of recombinant bacmid DNA into corresponding well of a 96-well microwell plate and mix by gently shaking (tapping) the plate from the sides.

step 8:
- Please correct." Using an adjustable 6-channel pipette designed for the transfer between 96- and 24 well plates, add dropwise the transfection mix onto the cells ..."
Done. Using an adjustable 6-channel pipette designed for the transfer between 96- and 24 well plate overlay the transfection mix onto the cells dropwise, in corresponding wells of transfection plates and incubate for 4 hours at 27°C.

step 9:
- See comment of step 2.
- "an incubation time"? Please rephrase as follows "To ensure even distribution of the transfection mixture over the cell monolayer gently rock the plates back and forth several times during the incubation time.
Done.
To ensure even distribution of the transfection mixture over the cells monolayer gently rock the plates back and forth several times during the incubation time

step 10:
- Same comment as for Introduction sentence 18: "After 4-5 hours of post-transfection time,..." is not proper written English. The authors shall write instead "Four to five hours after transfection...".
Corrected

- Please correct: "10% final of Heat Inactivated Fetal Bovine Serum (FBSHI)".
Done.

step 12:
- What are the signs of infection? The description of these signs shall be given at this step, not in the Discussion part.
- Importantly, for the sake of clarity, the authors shall clearly state at the end of chapter 2.1 that the medium supernatant containing the baculoviral particles is referred to as P1 viral stock.
Descriptions of the signs of infection has been introduced right after the step 12: Look for signs of infection (SOI), evident in transfected cells at 72 -96 hours post-transfection (Figure 2). Keep in mind that the transfected cells will start producing the virus and further infect the culture; thus, we are looking for infection signs.

Note: Signs of infection are structural changes in the insect cells, such as a 25-50% increase in the cell diameter, enlarged cell nuclei, uniformly rounded shape, loss of

proliferation and adherence to the culture dish surface, as well as a decrease in cell viability (Figure 2. Baculovirus Infected and uninfected Sf9 cells).

2.2
step 1:
- "As soon as P1 viruses ready to collect...": what does this mean on a technical point of view? After 72 hours? 96 hours? When the signs of infection are evident? The authors shall clarify and rephrase their sentence.
We have included this note before P1 virus stocks collection from transfected cells.
Note: Four to five days after transfection, signs of infection should be evident in the transfected cells when compared to the control (untransfected) cells under an inverted microscope. The initial recombinant baculoviruses secreted into the cell culture medium should be ready to collect.

- What does a "cell confluence of 80-85% into 24 well plate" represent in terms of cell number? As the authors did for the former and following chapters, they shall provide a cell concentration or number.
- In which medium are the cells seeded? Any insect cell medium?
- In "24 well plates" or "a 24 well plate".
The sentence corrected.

5. Dilute the exponentially growing Sf9 cells to a final cell density of $4x10^5$/mL in Serum Free Insect Media and pour into the sterile reagent reservoir.
6. Use a programmable multichannel pipette to seed 0.5 mL of the diluted Sf9 cells into each well of a 24 well plate.

step 4:
- To improve the logic and flow of the protocol, the sentence shall be modified as follows "Use a programmable electronic multichannel to allow at the same time the collection of P1 viruses (2.1.12) and the infection of freshly seeded Sf9 cells (2.2.1) with 150µl of P1 viruses".
The sentence corrected;
Use a programmable electronic multichannel to allow simultaneous the collection of P1 viruses (2.1.13), the infection of freshly seeded Sf9 cells (3.1.1) and the infection of the suspension cells in the 24 well blocks (3.1.4) with 150 uL of P1 viruses

- The collection of P1 viral supernatants (and of P2 viral supernatants at step 3.1) is very briefly described by the authors. Aren´t the supernatants centrifuged and aliquoted as recommended by various protocols? The authors shall be more explicit in their explanations.
Missing information added.
Spin down the rest of collected P1 viral stocks for 15 minutes at 17970 x g, transfer into microcentrifuge tubes and store in the dark at 4°C.

step 5:
- "Store the rest of collected P1 viral stock in dark at 4°C".
Done

step 6:
- Gently rock the plates on a reciprocating shaker to ensure even distribution of the added viruses over THE cell monolayer; repeat this a few times during THE incubation time."
Done.
Gently rock the plates on a reciprocating shaker to ensure even distribution of the added viruses over the cell monolayer of the cells; repeat this a few times during the incubation time.

step 7:
- "Look for signs of infection (SIF) within 72 - 96 hours after infection time".
Corrected.

3.1
step 1:
- Does "mln/ml" stand for "106"? the authors shall write in a consistent manner abbreviations, system units,...
Corrected: Dispense 4ml of Sf9 cells at a density of 3.5-4x10^6 in SFX-Insect serum free medium into each well of 24-well blocks to infect with P1 viruses for the protein expression screening.

- After using I-MAX medium, Grace´s medium, and any insect medium in the former steps of the protocol, the authors employ for their small-scale expression screening SFX-Insect medium. Is there a particular reason or is the choice of medium random? The authors shall clarify this point.
Corrected. We have taken out any specific name of the insect cells medium in the corresponding sections:
Mix well the transfection reagent vial. For each transfection, add 2 µL of JetPrime or X-tremeGENE 9 to 100 µL of JetPrime Buffer (supplied with the JetPrime transfection kit) or any other unsupplemented insect medium can be substituted. Deposit the diluted transfection reagent in a sterile reagent reservoir and gently mix for 10 seconds.
Dispense 4ml of Sf9 cells at a density of 3.5-4x10^6 in SFX-Insect serum free medium into each well of 24-well blocks to infect with P1 viruses for the protein expression screening.

step 2:
- "Use programmable electronic multichannel pipette allowing the collection of P2 viruses from a well-infected SF9 cell…". What does "well-infected" mean for the authors? Can the authors rephrase using more appropriate terms?
- In addition, correct the sentence as follows: "Use a programmable electronic multichannel pipette to allow simultaneously the collection of P2 viruses from infected Sf9 cells (2.2.7) and the infection of freshly seeded suspension cultures of Sf9 cells (3.1.1) with P2 viruses for the test expression".
The sentence was deleted.

step 4:
- Same comment as in step 4 of chapter 2.2.
Corrected.

step 5:
- "After 72-96 hours of post-infection time" shall be corrected (as explained in above comments).
Corrected.

- How do the authors monitor and more importantly estimate the viability of the P2-infected cells? No indication of the use of Trypan Blue solution appears in the text. Can the authors please clarify this important point?
Missing information has been added:
After 72-96 hours after infection of Sf9 cells, spray over the 24 well blocks with 70% ethanol, bring into the laminar flow hood, and check the cells density and viability in a few wells by using Trypan Blue stain.

These two notes have been introduced earlier in the protocol, in the section 2:

Note. Use an exponentially growing Sf9 cells with a viability of 95% or greater at any steps of the baculovirus expression protocol, including cell transfection for the baculovirus generation and titer amplification, protein expression screening and protein production.
Note: Trypan Blue staining and a hemocytometer can be used to determine viable cell counts and % cell viability. Non-viable cells take up the stain and appear blue under the microscope while viable cells remain unstained. To calculate % cell viability, a total cell count is obtained (unstained and stained) and the viable cell count is divided by the total cell count and multiplied by 100.

step 6:
- "Proceed to protein purification if cells look well infected and viability is close to 70-75%". Again, how is this percentage technically obtained? And what do, according

to the authors, "well infected" cells look like? The authors shall rephrase their sentence using appropriate scientific terms (they can refer to the signs of infection described in the former chapter).
Corrected.
13. Proceed to protein purification if cells have signs of infections and viability is close to 70-75% as assessed by Trypan Blue staining.

step 7:
- What is the intention of the authors with the note given in brackets? Some pieces of information (type of flag, type of expression) should have been given before (in the Introduction or/and as soon as the plasmid is mentioned in the protocol), others should be given later, in the results (which of the screening results are shown). Finally, others are simply nonsense: what do Table 1 (the Material and Methods table presumably) and Figure 3 (photo of Sf9 cells in flasks) refer to here?
The sentence has been corrected and the note in brackets has been deleted.
Pellet the cells by centrifuging the 24-well blocks at 525 x g at 4°C for 15 minutes

step 8:
- "Discard THE supernatant and thoroughly suspend the pellets in 1ml of lysis buffer comprising 25mM Tris pH 8.0, 300 mM NaCl, 0.6 % NP-40, 2 mM imidazole, 5% glycerol (v/v) and 1X protease inhibitor cocktail (100x protease inhibitor cocktail comprises aprotinin 0.25mg/ml, leupeptin 0.25mg/ml, pepstatin A 0.25mg/ml; E-64 0.25mg/ml). and Store THE frozen lysate at -80°C for the further test purification". Please add articles where needed.
Corrected.
14. Pellet the cells by centrifuging the 24-well blocks at 525 x g at 4oC for 15 minutes.

Discard the supernatant and thoroughly re-suspend the pellets in 1 mL of lysis buffer comprising 25 mM Tris pH 8.0, 300 mM NaCl, 0.6 % NP-40, 2 mM imidazole, 5% glycerol (v/v) and 1X protease inhibitor cocktail (100x protease inhibitor cocktail comprises aprotinin 0.25 mg/mL, leupeptin 0.25 mg/mL, pepstatin A 0.25 mg/mL; E-64 0.25 mg/mL).

3.2
- The procedures described in 3.2.1 and 3.2.2 represent one of the technical novelties provided by the authors in the manuscript, but unfortunately are very confusing and barely understandable. To improve the comprehension by a large audience the authors are advised to accompany their text (rephrased in proper English) with a schematic workflow in which each of the steps is clearly explained. Addition of photos or schematic drawings of the assembly of the binding plate and the first steps of the test expression procedure shall be considered.

Additional Figure 3 has been added: “Assembly of the binding plate”.
The procedures described in the sections 3.2.1 and 3.2.2 has been rephrased as follow:

3.2 Protein purification from frozen cell suspension in 24 well test-expression blocks.

3.2.1 Assembly of Binding block (Figure 3).

1. Place 3 overlapping layers of parafilm on the top of the 96 deep well block (Masterblock 96 deep well 2.4 mL).
2. Place a 96 well filter plate (Filter microplate, 96-well, polypropylene with 25 µm ultra high molecular weight polyethylene membrane) on the top of the 96 deep well block.
3. Push down the filter plate to secure the tips into sealed with parafilm surface of the 96 well deep block.
4. Transfer 50 µL of pre-equilibrated 50% Ni-NTA resin slurry into each well of the filter plate.

3.2.2 Test expression procedure

1. Place the frozen in 24 well blocks cells suspension (3.1.14) in a water bath at R.T. for 5-10 minutes, then shake at 450 rpm for 20 minutes.
2. Centrifuge the 24 well blocks at 3275 x g for 15 minutes.
3. Using a multichannel pipette, transfer the cleared lysates into a filter plate containing 50 µL of pre-equilibrated 50% Ni-NTA resin slurry (3.2.1.4) and seal filter plate with a 96 well cap mat (96-well cap mat, for use with square well, 2 mL).

   Note: Use a few rubber bands to keep the binding block together during incubation and centrifugation steps.
4. Place the secured binding block for 45-60 minutes into a rotator in a cold room to incubate cleared lysates with Ni-NTA resin.
5. After incubation time, carefully lift the filter plate and remove the parafilm layer from the surface of the 96 deep well block (3.2.1.1).
6. Place back the filter plate on top of the 96 deep well block and spin down the secured binding block for 2 minutes at 235 x g.
7. Wash bond Ni-NTA resin 2x with 2 mL of washing buffer comprising 25 mM Tris pH 8.0, 300 mM NaCl, 5% glycerol, and 15 mM imidazole.
8. Spin down the block with washing buffer each time for 5 minutes at 235 x g to ensure complete removal of residual liquid.
9. Transfer the filter plate on the top of the 96 well PCR plate containing 10 uL of 4x loading dye.

10. Add 40 uL of elution buffer (25 mM Tris pH 8.0, 300 mM NaCl, 5% glycerol, 500 mM imidazole) to each well of the filter plate and incubate for 5 minutes.
11. Spin down the block to elute proteins into the 96 well PCR plate at 235 x g for 10 minutes.
12. Seal the 96 well PCR plate with high temperature resistant tap pad and heat at 98°C for 3 minutes.
    3.2.2

step 8:
-"Commassie" is wrongly spelt.
Corrected.

- "Analyze the protein expression and solubility checking corresponding to the expected molecular weight most prominent band to identify the protein's best expression constructs for large-scale production". This sentence makes no sense and shall be rewritten by the authors. Can the authors develop on how is the solubility of PRMTs "analyzed" by Coomassie staining? The expected molecular weight (MW) of most if not all the proteins expressed by the 21 different constructs (Figure 4) is smaller than the apparent molecular weight of the most prominent Coomassie-stained protein bands (even after addition of 1 kDa for the His-tag present in the constructs). Can the authors comment on this? Based on this observation, the comparison of apparent MW observed on Coomassie-stained gels with expected MW can certainly not be the only criteria used by the authors to validate whether or not a protein of interest is expressed. A more elaborate and thorough quality control shall be conducted for the produced recombinant proteins. Western blotting could be performed using the eluates obtained in 3.2.2 together with antibodies recognizing PRMTs or the His-tag to verify the specificity. Additionally, could an enzymatic assay (in vitro methyltransferase assay) be envisaged with some of the proteins whose yield is high enough? The authors shall comment on these points and develop them in their text.
The sentence has been edited and additional information describing the results of the expression screening presented on the Figure 4 has been introduced in the representative results. The MW sizes of proteins have been clarified.
13. Stain the gel with Coommassie blue and de-stain with water. Analyze the results of test expression to identify the best expressing constructs for large-scale production.

4.1
- "Preparations of the suspension culture of the baculovirus-infected insect cells (SCBIIC) for protein production" could be simplified as follows "Preparation of baculovirus-infected insect cells (SCBIIC) for protein production". The fact that the cells are infected while in suspension can be given later in the text.

The sentence has been rephrased.
4.1 Preparations of baculovirus-infected insect cells for protein production.

step 1:
- Please modify as follows: "Four days before scheduled production time, start infecting suspension Sf9 cells with the appropriate P2 viruses".
Done.

step 2 and 3:
- What is the meaning of "healthy dividing cell" here? The use of "healthy" cells shall be an implicit requirement for each step of the protocol. Do the authors mean "exponentially growing"?
The sentence corrected.
...exponentially growing in Serum Free Insect Media Sf9

- In which type of culture vessels are the Sf9 cells split for the production of SCBIIC? This information is missing in the text.
Missing information included:
Four days before scheduled production time split exponentially growing Sf9 cell at a final cell density of 2 x 10^6 /mL into 125 mL / 250 mL / 500 mL Erlenmeyer glass shake flasks with baffles in 50 / 100 / 200 mL of Insect Serum-Free Medium containing 1% (v/v) final antibiotic-antimycotic.

- Why is the incubation temperature lowered to 26°C?
Add 0.150ml/0.300ml/0.6ml of appropriate P2 viruses and incubate infected cells at lower temperature of 26^0C to slow down cell division. The incubation is done at 165 rpm on an orbital shaker with a one-inch stroke.

- The detailed composition of the antibiotic-antimycotic has been given before (2.1). It is therefore not needed here.
Corrected.

- For the sake of clarity the authors are advised to combine step 2 ("Split...") and step 3 ("Depending...") and rewrite their sentence as follows: "Split exponentially growing Sf9 cells at the final cell density of 2 x 106 /ml into XX vessels in 50/100/200ml of Insect Serum Free Medium containing 1% antibiotic-antimycotic". "Add 0.150ml/0.300ml/0.6ml of P2 viruses and incubate the cultures at 26°C at 165 RPM on an orbital shaker with a one-inch stroke".
Step 2 and 3 combined.
Four days before scheduled production time split exponentially growing Sf9 cell at a final cell density of 2 x 10^6 /mL into 125 mL / 250 mL / 500 mL Erlenmeyer glass

shake flasks with baffles in 50 / 100 / 200 mL of Insect Serum-Free Medium containing 1% (v/v) final antibiotic-antimycotic.
Add 0.150 mL / 0.300 mL / 0.6 mL of appropriate P2 viruses and incubate infected cells at a lower temperature of 26°C to slow down cell division. The incubation is done at 165 rpm on an orbital shaker with a one-inch stroke.

step 4:
-"After 4 days of post-infection time" shall be corrected as explained in above comments and the number 4 be written in letters.
Corrected.
3. After four days post-infection, check cells under a microscope for signs of infection and proceed to the production if the cell viability as verified with Trypan Blue stain is close to 70-75%.

4.2
step 1:
- The authors shall rewrite their sentence according to the comments given for 4.1/step 2 and 3.
Done.

- Why is only gentamicin used at this step? And why this particular antibiotic and not for instance penicillin or streptomycin?
We have corrected this sentence and included the following note:
Note: To prevent bacterial contamination in the Sf9 cell culture, we recommend using gentamicin to the final concentrations of 10 µg/ml or penicillin/streptomycin to the final concentration of 50 U/ml and 50 µg/ml, respectively.

step 3:
- "Incubate the infected culture of Sf9 cells…"
- For which volume and vessel are the 135 and 145 RPM speeds recommended?
Thank you for bringing this to our attention. To avoid confusion we've deleted 135rpm used for the half-filled production vessels, since they haven't been described in the Figure 5.
Incubate the infected culture of Sf9 cells on a shaker with 145 rpm at the lower temperature of 25 °C (to slow down cell division) for 72-96 hours

- Why is the incubation temperature lowered to 25°C?
…lowered temperature as 25^0C to slow a cells division.

step 4:
- Please rephrase the sentence using articles and correct the "after 72 hours of post-infection time" according to the former recommendations.

- What does this sentence exactly mean? Where is the "baculovirus mediated protein production"? in the supernatant of infected cells or in the cells? In the present form, this sentence is simply not understandable and must be rewritten using exact scientific terms required for full comprehension. In addition, the authors must indicate how the cells shall be harvested (speed, time, and temperature of the centrifugation), and the cell pellets stored (as batch or aliquots, at which temperature).
- As mentioned before, the cell viability could be more rigorously estimated with the use of Trypan Blue solution.
The sentence has been clarified and missing information added.
After 72 hours post-infection, check cells under a microscope for signs of infection and assess cell viability.
Usually, after about 72 hours post-infection, the viability of Sf9 cells drops to 70%–75% (measured using Trypan Blue stain). Harvest infected Sf9 cells in the 1 L polypropylene bottle by centrifugation at 900 x g (Beckman JLA 8.1000) for 15 minutes at 4oC.

4.3
- This paragraph describing in two bullet points the infection, production, and purification of biotinylated proteins is not acceptable in its present form. What is in fact the rationale of this paragraph here? Why not the production of Flag-tagged PRMT protein for instance? If the authors find pertinent to include such protocol, it shall be in an accurate, and scientific manner detailed.
This paragraph has been deleted to facilitate the protocol flow as requested.

- Instead of providing such a protocol, it would be more helpful for the readers to get to know how the authors perform the large-scale His-tag PRMT purification of 12 litres (and more) of Sf9 cultures: in which vessel(s)?, with which volume of lysis buffer (per how many cells or volume of culture?), volume of resin, etc… Which yield of purified recombinant PRMT protein do the authors gain from 12 litres (this could be exemplified for a few PRMT constructs)?
Additional information has been included into this section of the protocol according to the reviewer's comments and missing information on the expression yield of purified proteins have been included into representative results.
5.Usually, after about 72 hours post-infection, the viability of Sf9 cells drops to 70%–75% (measured using Trypan Blue stain). Harvest infected Sf9 cells in the 1 L polypropylene bottle by centrifugation at 900 x g (Beckman JLA 8.1000) for 15 minutes at 4oC.
6. Resuspend the cell pellet collected from 1 L of the production cell culture with 20-25 mL of 1xPBS by gently swirling and transfer into 50 mL conical tubes.
7. Spin down the cell suspension at 900 x g for 15 minutes and discard the PBS solution.

8. Resuspend the washed cell pellet with 20-25 mL of the suspension buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 5% glycerol, 1x protease inhibitor cocktail) and flash freeze in the liquid nitrogen; store at −80oC until purification.
Note: Purification procedures for the PRMTs have been described in the details in the SGC published paper[6].

Representative results
- The first paragraph of this section is the simple listing of sentences (written in bad English) collected from the different sections of the protocol and does not provide with any new information. It is accompanied by a workflow of the whole procedure (Figure 1). Both paragraph and Figure should have been certainly more useful not at the end of the protocol but at its beginning to present the reader the key steps of the procedure.
The authors are strongly advised to modify their text accordingly.
The first paragraph of this section has been deleted from representative results and moved to the Introduction section as follow:
Briefly, the plasmid transfer vector containing the gene of interest was transformed into DH10Bac E. coli competent cells to generate recombinant viral bacmid DNA. Adherent Sf9 cells (Invitrogen) were then transfected with bacmid DNA. Four to five days after transfection, initial recombinant baculoviruses secreted into the cell culture medium, were recovered, and labeled as the P1 virus. The P1 baculovirus stocks were then used for virus amplification (i.e. generation of P2 baculovirus stocks) and protein expression screening. Based on the expression screening results, P2 viruses for the best expression construct of the protein were identified and used to generate suspension cultures of baculovirus infected insect cells (SCBIIS) for large-scale protein production.

First section of the Representative results have been modified as:
An overview of the BEVS protocol is outlined in Figure 1. Multiple expression constructs of PRMTs, including full-length, domains and truncated fragments, were generated at the Structural Genomics Consortium (SGC, Toronto) according to the SGC strategies with an attempt to increase the success rate for identifying soluble and stable proteins with a relatively high expression level[7, 9]. Interested readers are encouraged to review the SGC's definitions and methodology of designing "fragment" as the segment of the gene sequence incorporated into an expression clone, "domains" as PFAM-annotated structural domains and "construct" as the fragment cloned in an expression vector, all of which have been described in detail in an earlier publication[7].

- The order of the figure numbering shall be set in a logical order, meaning in the order of appearance in the text. Figures 1 and 2 remain as they are but Figure 3, Figure 4, and Figure 5 shall be labelled Figure 5, Figure 3, and Figure 4, respectively.
The numbering of the Figures has been corrected.

- "In Fig. 4 we present the expression-screening results for the soluble constructs of PRMT1, 2, 4-9 with a different expression level, purity, and stabilities".
The simple presentation in Figure 4 (renamed 3) of Coomassie Blue-stained gels containing protein bands of various intensities, molecular weights, and purities, without any further interpretation or conclusion in the text is insufficient and useless.
We have included additional information regarding description of the test-expression screening results presented in the Figure 4 as follow:
Expression constructs of PRMTs presented in this protocol are for the production of the polyhistidine-tagged proteins cloned into pFBOH-MHL vector, which is a derivative of the pFastBac1 vector (Invitrogen). In Figure 4, we present SDS-PAGE analysis of the His-tagged soluble constructs of PRMT1, 2, 4-9 purified from pellets collected using 4 mL of production in Sf9 cells (3.1.4).

Firstly, the gels are not properly labeled: for most of the constructs presented here, the expected molecular weight does not correspond to the apparent molecular weight and (example lanes 1 and 2 for PRMT1) and contaminants can be present in the preparation (example for PRMT2 and PRMT8). Therefore, the designation of the PRMTs by the authors would have been of great help. Secondly, what is the rationale of comparing different truncated versions (either N terminal-, C-terminal-, or both) of the same PRMT with each other? Based on which theoretical or experimental background does this study stand? Actually, all the truncated versions of PRMTs are compared to their corresponding full-length version, with the exception of PRMT1 and PRMT9. Can the authors explain why? How, for instance, would have full-length PRMT1 appeared on the same Coomassie stained gel? And finally, what can the authors conclude for their comparative analysis? Can they make any correlation between PRMT sequence and expression/stability? Can they provide an estimation of the yield (mg/ml) of purified recombinant PRMT proteins?
Unfortunately, it is difficult to draw parallels between the primary amino acid residue sequence of PRMTs and expression/stability as many other parameters are at play such as localization, processing, and binding partners. Thus, we feel that a larger study would be needed to draw reliable conclusions.
In the meantime, we have included missing information as follow in this section describing results of the expression screening:
Full-length (FL) PRMT1 and PRMT9 are not presented in this gel, since FL PRMT1 has been produced from E. coli, and FL of PRMT9 produced from BEVS has been purified by Flag-tag6. The truncated constructs of PRMT1, FL of PRMT4 and all of the PRMT8 constructs show a relatively high yield, but protein eluates contain fractions of co-

purified contaminants. These constructs require further optimization of the purifications protocols. Further approaches are thus required to improve the purity of these proteins from scale-up productions such as: a reduction in the amount of nickel beads at the stage of the incubation with a clarified lysate; an increase of the imidazole concentrations in the wash buffers; cleavage of the His-tag with TEV protease, followed by application on a Ni-affinity resin; and, additional purification steps such as size-exclusion and ion exchange chromatography. The constructs of PRMT2 show significantly lower yield compared to other proteins and full-length of PRMT2 protein accompanied by a strong contaminant band. Scale-up production and two steps of purifications such as IMAC and size-exclusion confirmed a low expression level for this construct along with the persistent presence of the co-purifying contaminant for the F protein. Pure proteins have been obtained for the PRMT5 complex produced and purified with its obligate binding partner MEP50. The truncated construct of PRMT9 has almost two or three fold lower expression level, close to 1.5 mg/L, as compared to other PRMTs. Nevertheless, the recombinant viral stocks of this construct have been used for scale-up production, diffracting crystals were obtained, and the structure was solved for this protein along with PRMT4, 6 and 7 (Figure 5).

- The fraction of contaminants co-purified with the PRMTs vary a lot from PRMT to PRMT and for a PRMT from truncated from to truncated form: for instance, the three preparations of PRMT6 (lanes 11 to 13) are very pure whereas PRMT1 (lanes 1 and 2) and PRMT8 (lanes 18 to 20) eluates contain a lot of non-specific products. Full-length PRMT2 (lane 3) has a huge contaminant whereas shorter versions of PRMT2 not. Can the authors elaborate on this observation?
We have included additional information as have shown above.

- "PRMT5 presented with its obligate binding partner MEP50 by co-infection of the corresponding recombinant viruses". Again, why comparing tagged PRMT5 to non-tagged PRMT5 and what is the conclusion the authors can make? Please correct "PRMT5 is presented with its obligate…
To avoid confusion, we have deleted one of the lines on the gel for the PRMT5 complex.

- "Expression plasmids for these PRMTs were deposited to Addgene collection by SGC and are available to the research community (Figure 5)". As recommended before, Figure 5 shall be renamed Figure 4.
Sentence has been corrected.

Discussion
- Please correct: "Here, we described detailed protocols emphasizing critical elements

for successful expression screening of multiple constructs of PRMT proteins and large-scale PRMT protein production in the baculovirus expression platform:
Corrected.
Here, we described detailed protocols emphasizing critical elements for successful expression screening of multiple constructs of PRMT proteins and large-scale PRMT protein production in the Baculovirus expression platform:

- Please correct: "viral titers and volumes" and "preparation of the protein expression screening blocks.
Corrected.
…collection of the recombinant viruses, amplification of viral volumes of the recombinant viruses and preparation of the protein expression screening blocks.

- "High - fill volume of the Sf9 suspension culture in 2.8L Fernbach shake flasks for maintenance and in 2.5L Tunair shake flasks and 5L reagent bottles for the production.

Corrected
High – fill volume of the Sf9 suspension culture in 2.8L Fernbach shake flasks for maintenance and in 2.5L Tunair shake flasks and 5L reagent bottles for the production.

Special considerations and rationale for cell culture and transfection
- "Although a commercial protocol recommends …". Please name the manufacturer in question.
Although a commercial protocol (Invitrogen) recommends using 100ul of the competent cells for one transformation [14], the transformation efficiency of commercial DH10Bac E. coli competent cells is as high as 1 x 10^8 cfu/ µg DNA, so we use only 4µl.

- "Adherent Sf9 cells in the 24-well transfection plate seeded at cells confluence of close to 80-85%, total 0.5ml". A cell concentration is given in section 2.1, not a percentage. Can the authors be consistent in their writing/
Corrected.
Seed 2x105 exponentially growing Sf9 cells in Serum-Free Insect Media into each well of the 24 well plates in a total volume of 2 mL for infection with P1 viruses to amplify virus volume (resulting in the generation of the P2 viruses).

- "… ;additional 1.5ml of media containing FBS X? added directly…" . Add a passive form to give a meaning to the sentence. "…into the transfection plate after 4 hours of post-transfection time.". Correct according to previous recommendations.
Corrected.
Transfection reagents (JetPrime and X-tremeGene 9) are non-toxic to the SF9 cells, and the exchange of media is not necessary. To facilitate cell growth, instead of media

change an additional 1.5ml of media containing 10% (v/v) FBS were added into the transfection plate at four hours post-transfection time.

- In section 2.1 the authors describe the transfection protocol using JetPrime and refer to Figure 2 to show the signs of infection implying that the cells shown in Figure 2 were transfected with JetPrime. In the discussion, while mentioning transfection with Xtreme Gene9 the authors refer again to Figure 2. Can the authors be consistent and more accurate in their description?
Descriptions have been corrected and missing information has been included into Figure 2 legend.
The signs of infections presented here are the same for the transfected cells using both transfection reagents, JetPrime and XtremeGene9. The P1 viruses are collected at the same stage of the SIF developments for both transfection reagents. The particular example shown is for the JetPrime transfection reagent. A) Uninfected Sf9 cells as a control. B) Baculovirus-infected Sf9 cells

- As recommended before, the signs of infection shall be described at the appropriate step, namely after the first infection and not here, in the discussion.
Descriptions of the signs of infection have been moved to the appropriate section as a last section of the transfection procedure.
Look for signs of infection (SOI), evident in transfected cells at 72 -96 hours post-transfection (Figure 2). Keep in mind that the transfected cells will start producing the virus and further infect the culture; thus, we are looking for infection signs.
Note: Signs of infection are structural changes in the insect cells, such as a 25-50% increase in the cell diameter, enlarged cell nuclei, uniformly rounded shape, loss of proliferation and adherence to the culture dish surface, as well as a decrease in cell viability (Figure 2. Baculovirus Infected and uninfected Sf9 cells).

Considerations and alternatives for collection of virus stocks and infections
-" The P1 viruses are collected from the transfection plate, followed by amplification of the viral titer and volume from the stage of P1 to P2 facilitated by using the programmable multichannel pipette". This sentence does make no sense unless proper correction. Please rephrase.
- "To be able to do so,...". What do the authors intend to refer to with "so"?
- "...advance preparations in the laminar hood are needed: 1) adherent SF9 cells in a labeled 24 well plate for infections with P1 viruses to generate P2 viruses; 2) labeled 2ml sterile eppendorf tubes for collections of the P1 viruses; 3) 4ml of the suspension SF9 cells culture at the cell density of 3mln/ml in the sterile 24 well blocks if the protein expression screening will be scheduled with P1 viruses to expedite a screening process". Why would these three "advance preparations" be particularly crucial, relevant or needed to be taken in consideration for the use of a programmable multichannel pipette? The authors shall explain.

- Why would a protein expression screening be done with P1 instead of P2? Sf9 cells are not limiting and the set-up of an infection with P1 is not very challenging. Why did the authors elaborate on this point then?
- Is the use of adherent Sf9 cells for the production of P1 and P2 viruses only justified by the trivial fact that adherent cells can be better observed under the microscope?
- Please correct: " Collected P1 and P2 viral stocks are stored at 4°C protected from light".
- " P1 contains 7.5% of Fetal Bovine Serum (FBS), serum proteins in FBS act as substrates for proteases". The sentence has no meaning as a whole. Can the authors rephrase it and make it clearer: does the presence of serum affect the storage of P1 viruses?
- "For long-term storage, viral stock can be placed into -80°C for later reamplification since repeated freeze/thaw cycles can result in a 10- to 100-fold decrease in virus titer (14)". Is a piece of information missing here? Do the authors mean that the storage at -80°C shall be preferred to the one at 4°C? at -20°C? Or do they mean the storage in aliquots? Please explain.
- "However, this is not feasible in our medium-throughput protocol developed for generating recombinant viruses on the scale of screening 100 or more protein constructs at once". What is not feasible? The storage at -80°C? The storage of aliquots? Please explain.
- In the present protocol, the authors use fresh P1 and P2 viruses to infect Sf9 cells. The rest of the stocks is stored at 4°C. Have the authors tested side by side the infection of cells with fresh, short-termed stored, and long-termed stored P1 and P2 viral supernatants?

Yes, we are agreeing that this whole section "Considerations and alternatives for collection of virus stocks and infections" was very confusing and unnecessary. It has been deleted.

Considerations when moving from small to large culture volumes
-"This step will generate 50/100/200ml of suspension culture of the baculovirus-infected insect cells (SCBIIS), including: a) suspension culture of the infected insect cells; b.) P3 viruses in the supernatant for the infection of the new cells in the production batch". Please simplify the sentence and rewrite it as follows "This step will generate 50/100/200ml of baculovirus-infected insect cells (SCBIIS) containing infected cells and P3 viruses in the supernatant."

Done.

This step will generate 50/100/200ml of baculovirus infected cells (SCBIIC) containing infected cells and P3 viruses in the supernatant.

- Change "in the each of X" to "in X" and add passive forms when needed.

Done

On the production day, 2L of Sf9 cells in 2.5L Tunair shake flasks or 4L in 5L reagent bottles at the cell viability > 97%

- Figure 3 shall be renamed Figure 5
Done

- Please correct: "were diluted to the cell density of 4mln/ml, infected directly with 10-12ml/L of baculovirus-infected suspension insect cells (SCBIIC) and incubated at a lowered temperature of 25°C, at 145RPM."
Done.
...were diluted to the cell density of $4x10^6$/ml, infected directly with 10-12ml/L of baculovirus infected suspension insect cells (SCBIIC) and incubated at a lowered temperature of 25^0C, at 145rpm.

- How did the authors come to the use of 10 to 12 ml of SCBIIC? Have they made beforehand a titration of the SCBIIC with Sf9 cells?
Yes, titration was performed.

- "Large volume of insect cells for the protein production can be infected with a freshly thawed and diluted in the insect medium a frozen vial of baculovirus infected insect cells (BIIC) as described in the paper published in 2006 (18).". Can the authors rephrase their sentence in a proper English and explain their point in more details: can one make freeze downs of the SCBIIC and use them, instead of P3 viruses, to infect Sf9 cells for protein production?
We have not tried to use frozen SCBIIS for the scale-up production and we didn't compare SCBIIC and "traditional" P3 viruses with thawed BIIC viruses. Definitely, it could have been interesting comparative analysis. We have deleted this section, since we do not have a full comparative data.

Have the authors compared the infection of Sf9 cells with fresh SCBIIC versus thawed one and the infection with SCBIIC versus "traditional" P3 viruses?
We compared only fresh SCBIIC and "traditional "P3 viruses. We found that the infection rate of the Sf9 cells with SCBIIC is more consistent from batch to batch and the increase in the cell diameters of the infected cells is more homogeneous compared to "traditional" P3 viruses. Overall, we have described the advantages of infection of Sf9 cells with fresh SCBIIC as follow:
"Infection of the production batch directly with a suspension culture of baculovirus-infected insect cells (SCBIIC) significantly reduced laborious and time-consuming steps in virus titer and volume amplification, excluding the extra handling of the infected cells, and avoided reduction in titer and virus degradation".

- "...while adjusting and monitoring shaking conditions (Figure 4)". This is Figure 5.
- References (18) and (19) appear in the text before reference (17). The authors shall reformat their references.
The error has been fixed.

- There is a discrepancy between the text and the legend of Figure 3 (renamed Figure 5) regarding the total volume of the vessels: "fill volume of the vessels is 4.8L vs. 12L and 10L vs. 24L" in the text versus "2L x 10 bottles = 20L" and "4L x 6 bottles = 24L". Can the authors clarify this discrepancy and correct?
We've made changes in the Figure 6 legend. "2L x 9 bottles = 18L" saying that only 9 out of 10 shown in the incubator.

References
- The formatting varies from reference to reference (the date). Please format the references consistently.
Done

- Reference (17) has no journal name.
The journal name included

Table of reagents
- Please order the reagents in a logical manner, such as alphabetically or according to the order they appear in the main text.
The reagents rearranged in the alphabetical order

- Please homogenize the font, size, spelling, and capitalization of all the reagents´or equipment´s in the main text and the table.
Done.
- The Cell Lysis, Cell Resuspension, and Neutralization Solutions (Millipore) are not used "For protein purification in expression screening" as stated by the authors but for plasmid and bacmid purification. Please rectify the mistake.
We thank the reviewer for bringing this mistake to our attention, it has been corrected.

- Why are two different types of 6 well plates are used for cell culture?
This has been corrected, only the 24 well plates in the material list.

Figure 1
- Please correct "Sf9"
Done
- The crucial step consisting of generating the PRMT constructs is missing in the workflow and could be added at first.

Done
- Please correct "Transformation of expression constructs into…" and "E. coli".
- "… recombinant bacmid…"
- "…and harvest of P1 baculovirus stock…".
- "… by infection of Sf9 cells…" (why "new"?).
- "Test protein purification…"
- "SDS-PAGE analysis of the results of a test purification" shall be corrected as follows "SDS-PAGE analysis of the samples from the test protein purification".
- A new box "Identification/selection of the best P2 viruses" could be created and a clear definition of "best P2 viruses" could be added in the main text (Representative results).
- "Titer and volume amplification of the best P2 viruses" instead of "Viral titer and volume amplification for the best expression construct".
- "Infection of Sf9 suspension cultures with P2 viruses for…".
- "Large-scale purification of the baculovirus-mediated recombinant protein production". This step is not described in the main text.
All of the errors and mistypes in the Figure 1 have been edited.
Figure 2
- Please correct "Sf9" and "Baculovirus-infected Sf9 cells".
Corrected

- Which transfection reagent is used? This shall be mentioned in the legend and be consistent with what is stated in the main text (see comment of the Discussion section)
Corrections have been done in the legend of Figure 2.

- Again, is Trypan Blue used by the authors to accurately monitor the viability of their cells after infection? no use is mentioned in the main text or in the legend. The authors should clarify this point and provide color photos of Trypan Blue-stained cells.
Yes, we have used Trypan Blue used to accurately monitor the viability of Sf9 cells after infection and have made corresponding changes in the main text and in the legend

Figure 3
- Rename it Figure 5
Done.
- in B: "volume" is missing after "80% of fill"
Corrected.
- Only 9 bottles are visible, not 10: Can the authors clarify this "loss" of one bottle?
The pictures were taken when only 9 bottles out of 10 have been incubated at the time of this production.

We've made changes in the Figure 5 legend. "2L x 9 bottles = 18L" saying that only 9 out of 10 shown in the incubator.

Figure 4
- Rename it Figure 3.
Done.

- For the sake of clarity, can the main bands corresponding to the PRMT be designated by a "<" or a "*"?
Done. Corresponding main bands of PRMTs have been designated by "*"

Figure 5
- Rename it Figure 4.
Corrected.

Minor Concerns:
- Please follow the SI rules and homogenize the units accordingly in the manuscript (X µl or Xµl? X°C or X °C? rpm, RPM, or g?...)
- "X°C" not "X0C"
- "His-Tag" instead of "Histag".
- The different bullet points of section 4 are written in different sizes. Please homogenize them.
All of the above mistypes and errors have been fixed.
