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Chapter 17

Insect Cells-Baculovirus system for the production of difficult to express proteins: from expression screening for soluble constructs to protein quality control

Simon Pichard¹, Nathalie Troffer-Charlier¹, Isabelle Kolb-Cheynel¹, Pierre Poussin-Courmontagne¹, Wassim Abdulrahman², Catherine Birck¹, Vincent Cura¹ & Arnaud Poterszman^{1*}

¹ Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), Center for Integrated Structural Biology (CBI), Centre National de la Recherche Scientifique (CNRS), UMR7104, Institut National de la Santé et de la Recherche Médicale (INSERM), U1258, Université de Strasbourg, BP 163, 67404 Illkirch Cedex, France

² leadXpro AG, PARK innovAARE, 5234 Villigen, Switzerland

* Correspondance : Arnaud.POTERSZMAN@igbmc.fr

Abstract

Rapid preparation of proteins for functional and structural analysis is a major challenge both in academia and industry. The number potential targets continuously increases and many are difficult to express proteins which, when produced in bacteria, result in insoluble and/or misfolded recombinant proteins, protein aggregates or unusable low protein yield. We focus here on the baculovirus expression vector system which is now commonly used for heterologous production of human targets. This chapter describes simple and cost-effective protocols that enable iterative cycles of construct design, expression screening and optimization of protein production. We detail time and cost-effective methods for generation of baculoviruses by homologous recombination and titer evaluation. Handling of insect cell cultures and preparation of bacmid for co-transfection are also presented.

Key words: Baculovirus, Insect cells, Homologous recombination, Protein expression and production, quality control.

1 Introduction

In the last decade the landscape of structure determination methods has considerably evolved, with cryo-electron microscopy being able to reach near-atomic resolution [1], serial crystallography overcoming the sub-micrometer crystals bottle-neck [2] or free-electron laser tackling time resolved approaches [3]. However these biophysical progresses were not accompanied by major biochemical progresses leading to the current situation where resolution in many cases is still limited by the yield or the sample homogeneity of human targets. This is particularly the case for multi-subunits protein complexes or membrane proteins. In addition, raising of binders such as nanobodies or antibodies as biologics or to be used as crystallization chaperones [4, 5] requires high yields of homogenous and thermostabilized antigen protein samples for animals immunization or synthetic libraries screening approaches.

While *Escherichia coli* is a robust and inexpensive expression host, there are serious limitations for production of eukaryotic proteins [6]. In particular, bacteria are unable to accomplish post-translational modification and folding events often required for the synthesis of functional eukaryotic proteins. Eukaryotic proteins expressed in bacteria are often improperly folded and accumulate as insoluble polypeptides/inclusion bodies that are very difficult to recover without harsh denaturants and subsequent cumbersome protein-refolding procedures.

In contrast to *E. coli*, the mammalian and baculovirus/insect cell systems promote good protein folding and post-translational modifications which are in most of the cases identical to those observed in the original expression system [7].

Baculoviruses are rod-shaped, double-stranded, DNA viruses which infect and kill a large number of different invertebrate species especially insects [8]. The most common baculovirus used for expression studies is *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) [9, 10], which infects the lepidopteran species *Spodoptera frugiperda* as host insects. AcMNPV particles are surrounded with a protective matrix consisting of the protein polyhedrin [11], which permits survival in the environment and efficient dissemination to new hosts. Under the control of the *pPolh* promoter, polyhedrin is expressed at considerable levels and can represent up to 50% of total cellular proteins. As the polyhedrin coat is not essential for virus propagation in vitro and the naturally occurring polyhedrin gene within the wild-type baculovirus genome can be eliminated or replaced by a recombinant cDNA which will be expressed instead of polyhedrin [9, 12]. Insect cells infected

by recombinant baculoviruses can overexpress target proteins with yields which typically reach several milligrams of protein from one liter of culture. In this regard insect cells expression with BVEVS offers an excellent compromise between optimal molecular fidelity level which could be reached with human protein targets expressed in mammalian cells and very high yields which could be reached in bacterial expression systems. In the past expression in insect cells with the BVEVS suffered from being cost and time consuming. Advances in vector design and process optimization for BVEVS have resulted in overall improvement of the technologies for expression screening and large/medium scale production [13] [7, 14-16].

Two strategies are commonly used to insert foreign genes into the baculoviral genome and generate recombinant viruses. The first strategy, based on the Tn7-mediated transposition in bacteria of an expression cassette containing the target gene into the bacmid [17] was patented by Invitrogen as the bac-to-bac system. This entry system allows to rigorously characterize bacmids before transfection in insect cells but the selection suffers from a number of drawbacks which limits the through-put. Selection is carried out in a specialized *E. coli* strain via blue/white selection followed by PCR screening to eliminate false positives that result from non-productive transposition events between the transfer plasmid and either the *E. coli*/chromosome or the bacmid.

The second strategy to generate recombinant viruses is based on homologous recombination in insect cells. As an engineered baculoviral DNA/bacmid lacking a gene essential for viral replication (orf1629) is used for co-transfection, the knock out will fail to initiate an infection unless rescued by recombination with a transfer vector that will repair the defect. This obviates the need for purification and further selection (neither in bacteria nor in insect cells) [18, 19] which allows to rapidly screen expression constructs for further structural analysis and/or generate variants for functional investigations.

In this chapter, we provide simple and efficient protocols for expression screening as well as to optimize and scale up production. We describe the procedure, starting from the cloning of a gene of interest into shuttle vector and generation of the recombinant virus by homologous recombination, evaluation of protein expression, protein production/purification and quality control (Fig. 1). We detail handling of insect cell culture as well as preparation of bacmid for co-transfection and virus titration. These protocols do not require extensive background in cell biology and could apply in a lab equipped for basic cell culture and biochemical work. These protocols were implemented in our laboratory to generate constructs for the reconstitution and characterization of human proteins and multi-protein complexes that include the CAK and pTefb kinase complexes as well as the ten subunit transcription/DNA repair factor TFIIH (see for example [20-23]).

2 Materials

2.1 Working environment and instruments

1. Standard eukaryotic cell culture lab equipped with an incubator that can accommodate a shaking tray or a temperature controlled shaker (125 mm orbital), an inverted phase-contrast microscope/fluorescence microscope and optionally an automated cell counter (Cellometer AutoT4 (Nexcelom BioScience) or equivalent). Also a 6x 1L centrifuge with adaptors for 15, 50 and 250 ml tubes as well as for plates and access to a liquid nitrogen tank. For virus titration, access to a standard flow cytometer will be required (Fortessa X20 (BD Biosciences) or equivalent).
2. Standard molecular biology and biochemistry lab with a thermocycler, a refrigerated bench top centrifuge, a sonicator (3 mm probe), centrifuges and devices for DNA and protein gel electrophoresis and Western-blot transfer system (Bio-Rad or equivalent). For protein purification, access to a chromatographic system and a set of adapted columns are needed.
3. Dynamic light scattering experiments will require an instrument such as the DynaPro Nanostar system (Wyatt Technology) with either 1 µl quartz cuvettes (Wyatt Technology) or a 50µl disposable cuvettes (UVette from Eppendorf). Differential scanning fluorimetry experiments (NanoDSF), will rely on the Prometheus NT.48 system (Nanotemper). Standard or High Sensitivity Capillaries for protein concentrations < 0.2 mg/mL (Nanotemper) will be required.

2.2 Reagents for molecular biology: preparation of transfer vector and bacmid purification

1. MB grade Ethanol 100%, 2-propanol 100%, Na/Acetate 3 M, pH 5.2.
2. LB agar, LB medium DH5α competent cells, antibiotic stock solutions (Ampicillin, Kanamycin, Chloramphenicol).
3. pAC8 or pAC8_Dsred vectors (Table 2 and Fig. 2A).
4. BA10:KO₁₆₂₉ [18] or derivatives [24] in *E. coli* DH10B strain.
5. DNA purification kit (MN, NucleoSpin Plasmid Ref. 740588)
6. DNA maxiprep kit (MN, NucleoBond Xtra Maxi Ref. 740414)

7. PCR reaction kit with High-fidelity DNA polymerase and PCR clean-up kit (MN, NucleoSpin Gel and PCR clean-up Ref. 740609).
8. dNTPs mix. Prepare and aliquot 10 mM dNTPs stock solutions in ultrapure water.
9. DpnI (10 u/μl) restriction enzyme and corresponding buffer.
10. NdeI (NEB R0111S), BamHI HF (NEB R3136T), Bsu36I (NEB R0524S)
11. Shrimp Alkaline Phosphatase (SAP) (Fermentas) or Calf Intestinal Phosphatase (CIP) (NEB M0525S)
12. Pro-Ligation free cloning master mix (abm®, E086/E087), equivalent kit for homology based cloning or T4 DNA polymerase

2.3 Reagents for insect cell culture and baculovirus expression.

2.3.1 Cell culture dishes

1. 6- and 24 well plates, flat bottom, low evaporation lid (BD, B3668-1)
2. T25, T75, T175 tissue culture flasks (Falcon, 353014, 353024, 353028)
3. 250ml and 2L Glass or Disposable (Greiner Bio-one cat. 676050) Erlenmeyer flasks
4. Plate sealer, breathable, gas permeable, 80 x 150 mm (Greiner bio one 676 050)
5. Disposable sterile conical tubes for 15, 50 and 250 ml

2.3.2 Cell lines and media

1. Sf9 adapted in Grace Medium, 10% FBS
2. Sf9 and Sf21 adapted in Sf-900™ II SFM medium
3. ExpiSf9 cells (Gibco A35243)
4. High Five™ (Gibco, B85502) adapted in Insect Xpress™
5. Sf-900™ II SFM medium (Gibco, 10902)
6. Grace's Insect Medium (Sigma-Aldrich® G8142)
7. Fetal Bovine Serum (Fisher Scientific® 10270-106)
8. Insect- (Lonza, BELN12-730Q)
9. ExpiSf CD Medium (Gibco A3767801)

2.3.3 Maintenance, transfection and titration

1. Sterile Dulbecco's phosphate-buffered saline solution PBS (1X)
2. Trypan blue solution (Bio-Rad 145-0021)
3. Cell culture grade DMSO
4. Sterile cryogenic tubes, 1.5 ml (Nalgene, Ref. 5000-1020)
5. Controlled Rate Freezer System for 12 tubes, (PANASONIC Biomedical, BCS-136)
6. Sterile 1,5mL Eppendorf tubes
7. Lipofectamine™ 2000 (Fisher Scientific® 11668019)
8. HEPES Transfection Buffer: 25 mM Hepes pH 7.1 +/- 0.05, 125 mM CaCl₂, 140 mM NaCl.
9. Allophycocyanin (APC) conjugated anti-pg64 monoclonal antibody (Baculovirus Envelope gp64 Monoclonal Antibody (AcV1), (eBioscience™). Stock solution at 0.2 mg/mL

2.4 Reagents for protein purification.

2.4.1 General stock solutions

1. Dulbecco's phosphate-buffered saline solution (PBS 10x), 87% glycerol
2. Protease inhibitor cocktail (EDTA-free protease inhibitor cocktail tablet (Complete, Roche))
3. 1 M HEPES, pH 7.5; 1M Tris/HCl pH 7.5
4. 3M KCl, 5M NaCl
5. 10% detergent solutions (Tween 20, Triton-X100, CHAPS, NP-40)
6. 1 M (Dithiothreitol) DTT, 0.5 M TCEP (Tris[2-carboxyethyl] phosphine hydrochloride) (Fluorochem, BV1300)

2.4.2 Chromatography

1. Affinity resins: Ni-NTA agarose (Qiagen or equivalent); Glutathione sepharose 4B (GE Healthcare or equivalent); Strep-Tactin sepharose (IBA or equivalent); Anti-Flag

M2 affinity gel (Sigma-Aldrich). Ni-NTA and GST resins are available in spin column and 96-well spin plate format.

2. Concentrated ligands for elution by competition: Imidazole 10X (2M, pH 7.8), L-Glutathione reduced 10X (100 mM); D-Desthiobiotin 10X (50 mM), Flag-peptide (DYKDDDDKA) 10X (1000µg/ml) all buffered at pH 7.5.
3. Protease solutions for elution by on-column cleavage: 1 mg/ml stock solutions of TEV or 3C purified protease (Note 24A)
4. Preparative size exclusion column such as superdex s75 or s200 HiLoad 10/30 or 16/60, GE.

3 Methods

3.1 Design and preparation of the transfer vector

Variable yields and poor solubility are major impediments to streamlined production of many recombinant proteins, in particular for proteins classified as difficult-to-express including membrane proteins or large multi-domain proteins, which often do not function as isolated entities but in complex with other macromolecules. As solubility and expression level of constructs cannot be predicted, most strategies to optimize production of recombinant proteins rely on systematic screening.

When a full-length protein fails to express in soluble form, a common strategy for improving production is to design truncated constructs encompassing single- or multi-domain fragments. Expression constructs should correspond to structural units, and unless needed for functional reasons, any flexible, non-structured tails should be removed. In absence of direct structural information on domain boundaries from closely related proteins, design of expression constructs rely on educated guesses from analysis of multiple sequence alignments, from predictions of secondary structure and disordered regions as well as from algorithms that look domains.

Meta-analysis servers which collect and display information from prediction algorithms and databases searches help selection of domain boundaries (Table 1). Widely accepted guidelines for a priori soluble proteins are (i) to respect the boundaries of predicted globular domains as well as predicted secondary structural elements and (ii) to avoid inclusion of low-complexity regions, hydrophobic residues at the termini as well predicted membrane spanning regions. Ideally, the approximate boundaries of the region of interest might be identified using a functional assay, scanning deletion mutagenesis as well as limited proteolysis combined with mass spectrometry analysis. When reliable 3D models are available, *in silico* design methods can be used to propose guide lines for protein optimization [25].

Foreign cDNAs cannot be directly inserted into the baculoviral genome. cDNAs are cloned into transfer plasmids (also known as donor plasmids) which are used to manipulate the viral genome. Several technologies are available to drive recombinant protein expression. The protocol described here is based on homologous recombination between an engineered baculovirus DNA and a transfer vector harbouring an expression cassette for expression of the target gene under the control of a strong promoter. Most use the polyhedrin or the p10

promoters and allow expression of single gene. Dual, triple and quadruple expression vectors for co-expression of multiple cDNAs with a single virus also exist.

Screening not only includes optimization of expression conditions and constructs variants, as described above, but also of fusion tags which can have a positive impact on the yield, solubility and even the folding of their fusion partners [26].

We detail the insertion of a cDNA into pAC8 [27] or their pAC8_DsRed derivatives which harbor a p10 driven expression cassette for co-expressing the mCherry infection marker with the protein of interest. These vectors contain a polyhedrin promoter, the sequence coding for fusion protein/affinity tag including Protein A, FLAG, GST, Strep, and His6, followed by a protease 3C cleavage site and a poly-linker (NdeI, PmeI, and BamHI) (Table 2 and Fig. 2A).

cDNAs can be inserted into the transfer vector using restriction/ligation cloning or homology-based technologies such as Sequence and Ligation Independent Cloning (SLIC) [28], Gibbons-assembly [29] or In-Fusion cloning. The latter technology is based on the use of overlapping PCR products treated by the poxvirus DNA polymerase whose 3'–5' exonuclease activity chews back one strand of each fragment, allowing complementary base pairs of the vector and insert to join and anneal in the expected orientation. These overhangs are annealed in vitro and the recombinant circular construct is rescued in *E. coli*.

1. Design and order PCR primers to amplify the target gene (Note 31A). They should be composed of a 5' part corresponding to the cloning junction (which provides a homology to the vector sequence for the homology based cloning reaction) followed by a 3' part complementary to the insert target (which provides a priming region for the PCR reaction) (Fig. 2B). The forward primers (Gene_FW) include the first 15-21 nt of the cDNA, preceded by a sequence complementary to that of the oligonucleotide used to amplify the promoter region. The reverse primers (Gene_RV) include the last 15-21 nt of the gene, followed by a STOP codon and the reverse complement of the primers used for the backbone amplification (Table 3).
2. Amplify the cDNAs using your favorite DNA polymerase. Set up 50 µl PCR reactions using the conditions suggested by the polymerase manufacturer with 1-50 ng of template DNA if you amplify your fragments from a plasmid or 100-200 ng of template if you amplify your genes from cDNA. We typically denature the template DNA at 98°C for 30s, perform 25 cycles of amplification (denaturation at 98°C for 10s, annealing at the calculated T_m for 15s and elongation at 72°C for 30s in the case of a 1 kb gene) followed by a final incubation at 72°C for 5 mins.

3. Load 1/10 of the PCR reaction volume on a 1% agarose gel in parallel with a molecular weight marker to verify that the PCR amplification resulted in a single band of the expected size.
4. If the fragment was amplified from a plasmid, treat the amplified fragment with 10 units of the DpnI restriction enzyme at 37°C for 1 h to digest methylated template.
5. Purify the PCR reactions on PCR clean-up columns following the instruction manual, elute bound DNA in 30 µl and determine the concentration of the extracted DNA spectrophotometrically. We usually prepare between 1 and 2 µg of each fragment.

As pAC8 transfer vectors are relatively large plasmids (around 7 000 bp), the backbone DNA is prepared by NdeI/BamHI restriction. Inverse-PCR is an alternative to enzymatic digestion when smaller plasmids are used or when the plasmid backbone has to be modified:

6. Digest 3-5 µg of plasmid in a total volume of 30 µl for 3 hours at 37°C with 10 units of NdeI and BamHI HF in CutSmart Buffer (New England Biolabs) and carefully check that digestion is complete and resulted in the expected products. We typically load 1/10 of the reaction volume on a 1% agarose gel
7. Purify the linearized plasmid using clean-up columns following the instruction manual, elute bound DNA in 30 µl and measure the DNA concentration spectrophotometrically.
8. For Infusion cloning, prepare an annealing mix containing 100 ng of the vector backbone (23 fmoles), the cDNA in a 1:3 ratio, 10 µl of 2X Pro-Ligation free cloning master mix (abm®, E086/E087) in a total volume of 20 µl. Incubate the annealing mix at 50°C for 15 minutes and leave on ice until transformation or store at -20°C. SLIC is a cost-effective alternative to Infusion cloning that relies on the exonuclease activity of T4 DNA polymerase (Note 31B).
9. Transform the Infusion reaction into 50 µl of *E. coli* competent cells, plate the transformation mixture on LB agar plates containing the appropriate antibiotic and incubate overnight at 37°C. If a control experiment lacking one of the fragments was included, significantly more colonies should be obtained in the presence of all inserts than in the negative control.
10. Pick-up 2-8 colonies, grow 100 µl pre-cultures for 3-4 hours and screen for positive colonies by PCR using the PH_FW primer and an oligonucleotide which hybridizes on the reverse strand of the cDNA. Select two positive colonies, grow overnight cultures

to purify DNA for purification and sequence. We use the PH_FW and PH_RV primers (Table 3) plus internal primers if required to sequence.

11. Mini- or Midi-prep a sequence validated plasmid and precipitate 10µg DNA with 300 mM final Na/Acetate pH 5.2 (final concentration) and 3 volumes of Ethanol 100%. Place at -80°C for more than 1 hour and centrifuge at 14 000 rpm for 15 min. Carefully remove the supernatant, add 1 ml of cold 70% Ethanol and centrifuge again.

From this point manipulate under a laminar flow hood!

12. Remove Ethanol and air-dry the precipitated DNA under the sterile hood. Re-suspend DNA in 50µl sterile ultrapure water. Take an aliquot to measure the DNA concentration and store at -20°C.

3.2 Generation and amplification of recombinant baculovirus

To generate recombinant baculoviruses suitable for homologous recombination, transfer vector are recombined with linearized viral DNA in insect cells (Fig. 3A). As discussed in Section 3.6, linearized viral DNA is commercially available from a number of sources or can be prepared from a bacmid that propagates in *E. coli*. In this section, we detail the co-transfection of the transfer plasmid with a linearized viral DNA. This allows integration of the expression cassette into the viral genome by homologous recombination in insect cells, producing a recombinant baculovirus which will replicate. After several infection cycles, typically 5-7 days the virus enriched culture medium is collected. This initial stock (P0) can be used for initial evaluation of protein expression and will be amplified to obtain the viral stock (P1) required for large scale expression.

Handling of insect cells is described in Section 3.5 where procedures for thawing, maintenance and freezing are detailed. All waste cells, viruses, used media and plastic-ware are to be treated overnight with bleach or autoclaved before discarding.

For efficient transfection, virus amplification and protein production healthy cells are absolutely required. Cells should be maintained in exponential growth phase, should not be overgrown and passages should be limited. A doubling time of 18–24 h and a continuous viability >95% are prerequisites for successful work. We advise to perform control experiments with known cDNA and viruses to monitor infectivity and expression levels (Note 32A).

3.2.1 Co-transfection of transfer plasmid and viral DNA with Lipofectamin

For co-transfections, we use semi-adherent Sf9 cells adapted to a serum-containing medium (Table 4) and a linearized viral DNA prepared from BAC10:KO1629 (see Section 3.6). The protocol described below was optimized to maximize the number of infected cells 5 to 7 days after transfection. The transfection reagent used is Lipofectamin 2000 but similar efficiencies can be obtained with lipid or PEI based agents.

1. Seed a 6 well plate using 0.75×10^6 Sf9 cells per well in 1.6 ml insect cell culture medium (Grace Medium + 10%FBS) (cell density of 0.08×10^6 Sf9 cells/cm²) and incubate the plate at 27°C for 20 min.
2. For each well, dilute 3 µl of Lipofectamine 2000 transfection reagent in 185 µL of Grace's Insect Medium without serum (Note 32B). Incubate 5 min at room temperature. To the Lipofectamine solution, add 750 ng of transfer vector DNA and 250 ng of linearized bacmid (ie use 3 µL of Lipofectamin per µg of DNA), and incubate at room temperature for 20 min.
3. Check that cells adhere, eliminate the medium and wash with 3mL of Grace's Insect Medium not supplemented with serum. Respect recommended incubation time as extended incubation may lead to formation of large and difficult to transfect DNA/lipofectin complexes.
4. Add the DNA/Lipofectamine solution *drop wise* to the cells, homogenize by shaking the plate gently and incubate at 27 °C. Four hours after the co-transfection, eliminate the DNA/Lipofectamine solution. Add 3 ml of insect cell medium to the cell layer and return to the incubator for at least 5-7 days.
5. Observe cells daily for signs of infection under an inverted microscope and for expression of the fluorescent control. If a fluorescent reporter was used, fluorescent cells shown be visible 2 to 3 days after transfection. Confluent growth should be observed in the negative control wells, whereas infected cells should swell, stop dividing and appear uniformly rounded with enlarged nuclei. At this stage, if only a very low proportion of cells are infected, signs of infection are not always easy to detect.
6. After 5-7 days incubation collect the culture, centrifuge at 1000 g and store the supernatant at 4°C, protected from light. More than 80% of cells in the positive control should express the fluorescent protein.

This is the passage 0 (P0) virus stock, which should be amplified to obtain the passage 1 (P1) virus stock for protein production. It can be used directly to test the expression of the desired proteins in small scale experiments before amplification.

Include a negative control without a transfer plasmid and positive control with a transfer vector expressing a fluorescent protein (pAC8-GFP).

3.2.2 Co-transfection of transfer plasmid and viral DNA with Calcium Phosphate

Calcium phosphate co-precipitation, a popular transfection method which only requires easily available and inexpensive reagents can be used as an alternative to commercially available reagents. An HEPES-buffered saline solution containing sodium phosphate is slowly mixed with a CaCl_2 solution containing the DNA. Phosphate, calcium and DNA form a co-precipitate, which adheres to the cell surface and is taken up by the cell, presumably by endocytosis.

1. Seed 2.0×10^6 Sf9 cells in 6mL insect cell culture medium (Grace Medium + 10%FBS) (cell density of 0.08×10^6 Sf9 cells/cm²) in a T25 Flask and incubate at 27°C.
2. Meanwhile dilute 2µg of transfer vector and 0.5µg of Bacmid in 750µL of HEPES transfection buffer composed of 25 mM HEPES pH 7.1, 125 mM CaCl_2 , 140 mM NaCl (Solution A) and prepare a 14mL Falcon tube containing 750 µL of Grace's Insect Medium, 10% Fetal Calf Serum (Solution B).
3. Add the DNA solution (Solution A) drop wise in the Grace's Insect Medium, 10% Fetal Calf Serum (Solution B) and incubate the precipitate 30min under the hood. Calcium phosphate precipitate forms due CaCl_2 in the transfection buffer and the phosphate in the medium. It is important to gently agitate Solution B while adding drops of Solution A drop wise – (typically 1 drop/s).
4. Verify that cells have attached to the flask, eliminate the medium and add the precipitate to the cells and Incubate cells with the precipitate during 4h at 27°C.
5. Eliminate the precipitate and add 5mL of Grace Medium supplemented with 10% FCS and incubate the flask at 27°C for at least 5-7 days.
6. Follow steps 5 and 6 from Section 3.2.1

3.2.3 Evaluation of protein expression

The P0 virus can be used for an initial screening to determine if the protein(s) of interest are expressed using western blot analysis and for small scale purification. The protocol detailed below describes batch affinity purification of intracellular proteins produced in 6-well plates (3×10^6 cells and 25 μ l affinity resin). It can be adapted to spin-column purification if the expression level of the expressed protein is sufficient or if proteins are expressed from larger cultures (25×10^6 cells and 100 μ l affinity resin). The use of magnetic beads instead of standard affinity resins is also an alternative.

1. Seed a 6 well plate with Sf9 insect cell in the appropriate culture medium, add 150 μ l of P0 and incubate at 27°C. We usually plate 3×10^6 Sf9 cells per well (1.5 ml of Sf9 cells at 2.0×10^6 Sf9 cells/ml) in 3.0 ml of (Grace Medium + 10%FBS), leading a cell density of 0.3-0.4 10^6 Sf9 cells/cm². Include a non-infected control.
2. After 72h incubation, observe the plate using an inverted microscope. Infected cells should have stopped dividing and swelled –not easy to detect by eye-. If the bacmid or the transfer plasmid encodes a fluorescent reporter protein check for its expression.
3. Re-suspend cells by gently pipetting up and down and analyze an aliquot with a cell counter. Cells should have transfer into 15 ml tubes and centrifuge at 1000 g for 10 min at 20°C. Infected insect cells are very sensitive and should be re-suspended gently to minimize cell lysis at this step.
4. Wash cell pellet by re-suspension with 1ml of PBS glycerol 10% followed by spinning at 1000 g for 10 min. Discard the supernatant and store the pellet at -20°C or proceed immediately. From this step always work on ice with cold buffer or manipulate at 4° C.
5. Re-suspend cells in 1.2 ml of lysis buffer [50 mM HEPES pH 7.5, 250 mM KCl, 5% glycerol] supplemented with EDTA-free protease inhibitor cocktail (Complete, Roche) at recommended concentration and sonicate for 4 x 30 sec with 3 mm probe with 30% intensity. Alternatively use a lysis buffer containing 1% Tween 20 and incubate 15 mins at room temperature with agitation.
6. Save 15 μ l aliquots to analyze the total proteins (total extract).
7. Centrifuge at 20 000 g for 20 min at 4°C. Save 15 μ l aliquots of the supernatant to analyze soluble proteins (soluble extract) and transfer the remaining into a 1.5 ml tubes containing 25 μ l of affinity resin, previously equilibrated in the binding buffer as recommended (Note 32C).

8. Incubate under gentle agitation at 4°C for typically 1 hour in the case of an IMAC resin and between 3 and 12 hours in the case of GST or Flag affinity resin.
9. Spin 1 min at 500g at 4°C, remove the supernatant and re-suspend in 1 ml of lysis buffer.
10. Repeat step 6 twice and carefully remove the liquid phase above the beads.
11. Bound proteins are eluted by adding 35 µl of lysis buffer supplemented with 250 mM Imidazole for IMAC purification, with 10mM GSH for GST affinity purification, or with 1 mg/ml FLAG peptide (Note 32D).
12. Incubate 1 hour in the case of an IMAC resin and between 3 and 12 hours in the case of GST or Flag affinity resin before spinning 1 min at 500 g at 4°C and collecting the eluate.
13. Analyze the different samples using SDS PAGE with Western Blotting (typically 5-10 µl of the eluate) and/or Coomassie staining (typically 20 µl of the eluate).

An example of expression screening where truncations of the PRMT2 target protein fused to an N-terminal GST tag are evaluated is shown Fig. 4A.

Note that secreted proteins are purified from the culture medium. In this case, we usually harvest the culture 96-120 h post infection, and after centrifugation, the conditioned culture medium is incubated with affinity beads which are processed as described above (Steps 7-12).

3.2.4 Virus amplification

As high titer baculovirus stocks are required for infection of insect cells for optimum protein production (see Section 3.3.3), the P0 stock needs to be amplified. Virus stocks are obtained by infecting insect cells at a low multiplicity of infection (MOI) and harvested 5-7 days post infection (Note 32E).

The protocol below is for amplifying viruses from P0 using from a 100 ml suspension culture using Sf9 Oxford 3 cells in SF900-II or Insect-XPRESS (Table 4). For non-experienced users, we recommend to start with viruses that in addition the target protein, express a fluorescent infection marker to monitor amplification.

1. Add 100 µl of P0 to an Erlenmeyer flask containing 100 ml of Sf9 cells at 1×10^6 cells/ml in exponential growth phase and incubate at 27°C with agitation (100 rpm). Assuming a titer of 10^8 viruses per ml, these conditions correspond to an MOI of 0.1 (1 virus for 10 cells). Ideally, different volumes of P0 can be tested.

2. Optional. Observe cells daily for signs of infection under an inverted microscope (or with fluorescent microscope if appropriate). Confluent growth of cells will be seen in the negative control wells, whereas infected cells should swell, stop dividing and appear uniformly rounded with enlarged nuclei. During infection cell size can increase up to 20-30%.
3. Incubate at 27°C during at least 5-7 days and harvest virus when 50% of cells or more collapse. Centrifuge at 1000 g to remove cell debris for 10 min add 5-10% serum and store the supernatant at 4°C, protected from light. This is the P1 virus stock (Note 32F).

Ideally the virus titer of the P1 stock should be measured but this is not done on a routine basis. As virus titers of freshly amplified viruses do not vary significantly from one virus to the other, we generally assume a concentration of a 10^8 infectious particles per ml and determine experimentally the optimum volume of virus to be used if optimization is required.

In case titration is required, we use a protocol based on the detection of the baculovirus envelope protein gp64 displayed on the surface of infected cells (see Section 3.6.2).

3.3 Optimization, large scale expression and purification

Production in quantity and quality of recombinant proteins is affected by numerous factors. We usually test protein production using suspension cultures at the 25 or 50 ml scale before large scale expression. Small scale purifications with two lysis/purification buffers will provide first information on protein solubility and expression yield.

3.3.1 Infection parameters and first optimization

For each virus, optimization of growth and infection require careful characterization. Key parameters are the amount of virus and the time of infection: (i) When infecting cells for protein production, the objective is to infect all cells synchronously and therefore use more viral particles than cells. As we usually do not titer amplifications, we determine the best virus to cell ration empirically by testing different conditions: 5, 20 and 50 μ l of P1 virus per million of cells to infect. Assuming an average titer of 10^8 infectious particles per ml, these conditions correspond to multiplicities of infections (MOIs) of 0.5, 2 and 5. (ii) The best time to harvest depends on the nature of the target protein. For intracellular proteins, cells are usually

analyzed 48 and 72h post-infection. Some stable proteins might accumulate to high levels 72 h post-infection while others, sensitive to degradation, will need to be collected after 24 or most commonly 48 hours.

Protein expression may also be affected by the cell line and expression with Sf9, Sf21 or High five can be compared (Table 4). For cell line optimization, we usually test SuperSF9-3™ (OET) which offer a high general expression for a variety of recombinant proteins, ExpiSf9™ which can be infected at high cellular density and Sf21 have short doubling time.

The optimal MOIs and time to harvest are usually optimized. We test increasing amounts of virus (3 conditions) and at least two infection time (2 conditions).

1. Seed 3 pairs of 100 ml Erlenmeyers with 25 ml cells at the recommended cell density.
2. Use the P1 stock to infect the first pair with 5 µl of virus solution for 10^6 cells (MOI # 0.5), the second with 20 µl of virus solution for 10^6 cells (MOI # 2) and the third with 50 µl of virus solution for 10^6 cells (MOI # 5), close the flask with an air pore sheet or with a vented cap and incubate at 27°C.
3. After 48h, harvest one flask of each pair of suspension. Infected cells should have stopped dividing, swelled – the mean diameter typically increases by 15-30% - (Fig. 3B). If the virus encodes a fluorescent reporter protein, observe cells using fluorescence microscopy to verify expression of the control protein.
4. Centrifuge cells at 1000 g for 15 min at 20°C, re-suspend the cells in PBS containing 10% glycerol, centrifuge again and snap-freeze.
5. Repeat steps 3 and 4 after 72 h.
6. Lyse and purify cell pellets as described Section 3.2.2, steps 5 4 to 10
7. Compare the level of expression in the different pellets

This will allow to choose the optimal culture conditions (time to harvest and MOI). In most cases, optimum expression is achieved 48 h post infection with an MOI of 2.

3.3.2 Medium/large scale production

Once the optimal culture conditions have been determined, production of infected cells can be scaled up to purify large amounts of the target protein. At this stage, it can be worth screening for an optimal lysis buffer. Optimal conditions should maximize solubility and

stability of the target protein while minimizing oxidation, unwanted proteolysis and aggregation (Note 33A).

Suspension culture experiments are usually performed with 2 L Erlenmeyers containing 500 ml and up to 8 flasks are used in parallel. For larger volumes, devices suitable for production at 5-20 L scale such as wave bags as well as further optimization and standardization are required. This includes for example setup of procedures for virus stock preservation and infection of large volumes of culture [30].

1. Seed 2L Erlenmeyer flasks with 500 ml medium and infect using parameters (cell type, density, MOI) determined above Section 3.3.1.
2. After 48 or 72 h, take an aliquot to verify that cells have been infected using a cell counter: They should have stopped dividing and swelled –the mean diameter typically increases by 15-30%. If the virus encodes a fluorescent reporter protein, observe cells using fluorescence microscopy to verify expression of the control protein (Fig. 3B).
3. Harvest cells by centrifugation at 1000 g for 15 min at 20°C and re-suspend the cells in PBS containing 10% glycerol (Note 33B).
4. Aliquot into 50 ml Flacon tubes, centrifuge again, snap-freeze and store the pellet at -80°C. We usually prepare aliquots corresponding of 250, 500 or 1000 ml.

3.3.3 Protein purification

We detail here the production and the purification by affinity chromatography of a protein harboring an amino-terminal tag followed by a protease cleavage site such as a Tobacco Etch Virus (TEV). Both buffer composition (ionic strength, pH, presence of reducing agents, detergents,...) and elution protocols (completion or on column cleavage) should be optimized on a case-by-case basis. Here, the first purification step includes the specific elution of the protein of interest either by cleavage of the fusion or by competition with a specific ligand. A further purification step consisting in a size exclusion chromatography removes aggregates and the protease.

1. Re-suspend a pellet from $0.8 \cdot 10^9$ cells (1.0 L of Sf21 cells infected at $0.8 \cdot 10^6$ cells/ml with 16 ml of P1 viral stock) by gently pipetting up and down in 100 ml of lysis buffer

[50 mM Tris-HCl pH 8.0, 250 mM NaCl, 5% glycerol, 5 mM TCEP] supplemented with 0.01% NP40 and one EDTA-free protease inhibitor cocktail tablet (Complete, Roche).

2. Sonicate the cell suspension for 2 x 2 min 50% intensity and centrifuge at 40000 x g for 30 min at 4 °C to remove the cellular debris.
3. Mix the crude extract with 3 ml of affinity resin equilibrated in lysis buffer and incubate 3 h at 4 °C under gentle agitation (Note 33C).
4. Centrifuge at 500g for 5 min, discard the supernatant and pour the resin in an Econo-column (Bio-Rad).
5. Wash the resin twice with 10 ml lysis buffer.
6. Plug the column and apply 3 ml of lysis buffer supplemented with the adapted protease (TEV or 3C) to the column and incubate for 4 hours at 30 °C with gentle mixing (Note 33D).
7. Unplug the column and elute the protein. Wash the resin three times with 2 ml buffer A supplemented with competitor.
8. Concentrate the digests using an Amicon Ultra 10K (Millipore) to a final volume of 1 ml.
9. Inject the concentrate protein on a gel-filtration column (HiLoad 16/60 Superdex S200, GE Healthcare) and elute at 1 ml.min⁻¹ with SEC buffer [20 mM Tris-HCl pH 8.0, 100 mM NaCl and 5 mM TCEP], using an ÄKTA Explorer device (GE Healthcare).
10. Analyze the fractions by SDS PAGE and select the fractions containing the protein of interest.

Pooled fractions are usually concentrated using concentrator, aliquoted and snapped frozen in the presence of 20% glycerol. The purification of the mPRMT2 protein is illustrated Figs. 4B and Fig.4C. A yield of 2 mg of purified protein from 0.8 10⁹ infected cells is usually obtained.

3.4 Protein quality control

Successful downstream application of a recombinant protein depends on its quality which must be evaluated as carefully as possible to validate the production/purification process and assess reproducibility. SDS-gel electrophoresis and UV spectrum analysis can provide first indication on sample purity of the protein sample while mass spectrometry is the method of

choice to validate the identity of the protein sample. To characterize protein homogeneity, size-exclusion chromatography and light scattering techniques are widely used.

3.4.1 Dynamic Light scattering

Dynamic Light Scattering (DLS) is an easy-to-use and non-invasive technique that allows to estimate the size distribution profile of particles in solution [31]. A laser beam irradiates a small volume of protein sample contained in a cuvette or a well plate and the light scattered at 90° is measured by a fast photon detector. A correlator measures the time-dependent fluctuations in the scattering intensity to construct the time autocorrelation function and determine the diffusion coefficient which, by assuming a basic shape, can be converted to a hydrodynamic radius (RH) using the Stokes-Einstein equation.

Measurable sample concentrations mainly depend on the size of the molecule to be analyzed and concentrations that range between 0.5-2 mg/mL usually provide a good starting point. Before starting carefully read the instrument's specification and complete a training session with the person in charge of the instrument (Note 34A). The protocol detailed below describes measurement using the DynaPro Nanostar DLS (Wyatt) instrument:

1. Centrifuge the sample at 20 000 g for 10 min (or filter using a 0.1 µm filters) to remove dust and insoluble aggregates.
2. Transfer supernatant with a dust-free tip into a clean microcentrifuge tube or pipet the supernatant directly into the DLS cuvette. Remember, only the top portion of the sample is dust free after centrifugation.
3. Wait 1 min to allow for automatic settings of the laser and sample to rest.
4. Start the measurement, usually 10 acquisitions of 5 sec.
5. Perform the analysis without and with regularization to calculate the percentage of polydispersity, RH and estimated molar mass (Note 34B).

Well-behaving protein samples should be monodisperse with polydispersity around 20% - ratio between the polydispersity index and the Z-average-, while sample containing impurities or aggregates will show higher polydispersity and/or will contain large aggregates. It is generally admitted that solutions exhibiting a polydispersity below 15% are very likely to crystallize. Monodisperse samples with a polydispersity below 30% are still likely to crystallize but when this value exceeds 30% chances to obtain crystals are negligible and the construct and/or its storage condition should be reconsidered (for additional information see [32-34]). In the example presented Fig. 4A, DLS was used to optimize the homogeneity of *Drosophila melanogaster* PRMT2 (drPRMT2) in view of structural analysis. The particle

size distribution of the purified protein measured at pH 6.5 indicates a monomodal monodisperse solution with a polydispersity above 30%, suggesting that these conditions are unfavorable for crystallization. In contrast at pH 8.5 the polydispersity of 23% is more favorable and the protein is more likely to crystallize (which turned out to be the case).

3.4.2 Nanoscale Differential Scanning Fluorimetry

Nanoscale Differential Scanning Fluorimetry (nanoDSF) is a robust and low volume technology to analyze protein thermal unfolding and determine an apparent melting temperature (T_m) at which half of the protein population is unfolded. The thermal stability of a given protein is typically described by its T_m . In contrast to a Thermofluor assay (also known as Thermal Shift Assay) employing extrinsic environmentally-sensitive fluorophores [35], nanoDSF monitors the changes in the intrinsic protein fluorescence resulting from alterations of the 3D-structure of proteins as a function of the temperature [36]. The indole group of tryptophan is the dominant fluorophore in proteins and its fluorescence is highly sensitive to changes in its immediate environment. The fluorescence signal from tyrosine or phenylalanine residues can also be detected and, although this might require a higher concentration of the protein sample and/or the use of high sensitivity capillaries to increase the fluorescence signal, nanoDSF analysis can also be performed in the absence of tryptophan residues. NanoDSF monitors these fluorescence changes with high time-resolution and can reveal even multiple unfolding transitions. Due to the small volume requirements, robust detection method, fast analysis pace and simplicity of use, nanoDSF has numerous applications including batch-to-batch characterization, buffer and formulation screening as well as screening of small molecule compound libraries for influence on protein stability (refs). In favorable cases, ΔT_m of 0.4°C can be considered significant with experiments performed in triplicates.

The protocol detailed below describes measurement using the Prometheus NT.48 instrument by NanoTemper Technologies to monitor and analyze thermal unfolding. The instrument design allows for the parallel detection of up to 48 samples loaded in high-precision capillaries.

To perform a nanoDSF experiment:

1. Check the fluorescence signal of the protein sample. Usually 0.1 to 1 mg/ml protein solutions will give enough signal for measurements.
2. Prepare the different mixtures to analyze in 10 μl total volume
3. Clean the mirror capillary tray surface prior to each experiment

4. Load the capillaries by capillary forces and place them onto the capillary tray.
5. Open the PR control software, choose the LED excitation power to get optimal fluorescence signal (range 8000-18000 fluorescence units) and define the temperature ramp. A gradient of 1°C/min from 20°C to 95°C is used by default but for quicker analysis the temperature ramp can be increased to value can be increased to 7°C/min.
6. Start the thermal unfolding experiment
7. Fill the sample ID in the result table
8. Once the final temperature is reached, T_m values are calculated and unfolding curves with their first derivative curves are also displayed.

The example presented Fig. 5B, illustrates the impact of ligand binding on of drPRMT2 stability monitored by nanoDSF. The apo enzyme exhibits a clear F350/F330 transition at 51.9°C, suggesting that the sample is homogenous. Incubation with S-Adenosyl-homocysteine (SAH), a natural PRMT2 ligand, leads to a significant shift of the transition temperature ($\Delta T_m = +4.1^\circ$) providing evidence that the ligand binds to drPRMT2 and stabilizes the protein. Furthermore, protein incubation with a synthetic SAH analogue showed a similar stabilization ($\Delta T_m = +4.2^\circ$), revealing a new ligand for the protein.

3.5 Handling insect cell cultures

Successful expression drastically depends on healthy insect cell cultures that should be carefully maintained and tested on a regular basis. The most common cell lines used for BEVS applications are derived from *Spodoptera frugiperda* (Sf9, Sf21) or *Trichoplusia ni*, (High-Five™). Lepidopteran cells can be cultured between 25 and 30°C with an optimum at 27 C in phosphate buffered media, which means that CO₂ incubators are not required. Insect cell and Baculovirus work requires a basic familiarity with insect cell physiology [37] and general cell culture methods (Note 35A). Invertebrate cell cultures are sensitive to environmental factors and we recommend careful control of growth characteristics to determine the accurate cell density range where mid-log phase of growth falls based on actual culture conditions (i.e., incubator, equipment, technicians, etc.) (Note 35B).

3.5.1 Thawing cells

The following protocol describes initiation of cultures from frozen vials.

1. Pre-warm and equilibrate the appropriate growth medium.
2. Select a cryovial from liquid nitrogen storage and thaw in a 37 °C water bath until only a small ice crystal remains.
3. Wipe or spray the cryovial with 70% ethanol and transfer the vial under the hood.

Transfer cells into a T75 flask containing 15 ml of medium or into a 100ml Erlenmeyer with 20 ml as appropriate (Table 4). The initial viable cell density in the culture should be at least $2 \cdot 10^4$ cells/cm² for adherent cells and the $3\text{-}5 \cdot 10^6$ cells/ml.

4. The following information should be recorded in a cell notebook: cell line and batch, medium, passage number and date, density to which the culture has been split (dilution factor or cell density).
5. Place them into the incubator or shaker and proceed as indicated below. Cells should be dividing within two days. Passage the cells at least two times before using in other applications.

3.5.2 Maintenance of cell cultures

Insect cells can be grown as monolayers or as suspension cultures with Erlenmeyer flasks (Table 4). We use semi-adherent Sf9 cells adapted to a serum-containing medium for generation of viruses and initial evaluation of protein expression. Sf9 cells adapted to serum-free medium are used for virus amplification and production (Note 35C). Cells are passaged every 2 to 3 days.

For adherent cells:

1. Observe cells using an inverted microscope to verify that they look healthy (no visible contamination, limited number of floaters,...) and that the monolayer has reached 90% confluence.
2. Detach cells by tapping the flask or by sloughing (streaming medium over the monolayer with a pipette to dislodge cells).
3. Seed new flask at a density of $2 \cdot 10^4$ cells/cm². This corresponds to a 1:3 dilution ie addition 5 ml of the cell suspension from one T75 flask to 10 ml of fresh medium (Grace Medium + 10% FBS). Once cells have attached, verify that initial cell density

is close to 30%. Depending on needs, nearly confluent monolayer cells can be split at any ratio between 1:2 and 1:20.

For cells grown in suspension:

Take an aliquot of the culture, count cells and determine their viability. If the cell density is below 2×10^6 cells/ml, continue to grow the cells.

1. When the cell density reaches 2×10^6 cells/mL, passage the cells at the recommended seeding density (Table 4) into the desired flask and scale up accordingly.
2. Incubate at 27°C for 48 hours with appropriate agitation.

3.5.3 Freezing cells

Once a culture is fully adapted to the culture conditions and growth medium, it is essential to establish a frozen master cell seed stock that should be prepared with the lowest possible passage.

1. Prepare appropriate freezing medium for the cell line and cryogenic storage vials (i.e., cryovials) by labeling each vial appropriately with the cell name, density, and date.
2. Measure the cell density and viability of the stock cells culture to cryopreserve, transfer cells from the culture flask into sterile 50-mL conical tubes, and centrifuge at 50 g to pellet.
3. Remove spent medium and re-suspend the cells in an appropriate volume of cold freezing medium to achieve the desired cell density to add to each cryovial (Table 4)
4. Dispense aliquots of the cell suspension into labeled cryovials. Frequently and gently mix the cell suspension to maintain a homogeneous solution.
5. Place the ampoules in a small CoolCell® LX box and place it in a –80°C ultralow freezer overnight. Store the cells vials in liquid nitrogen.
6. Qualify frozen cells by thawing one vial and testing the thawed cells for bacterial, fungal, and mycoplasma contamination, normal cell morphology, robust growth and capacity to express recombinant protein.

3.6 Linearized viral DNA for co-transfection and virus titration (929)

3.6.1 Preparation of linearized virus

Homologous recombination of a transfer vector with linearized baculoviral DNA is the simplest system to generate recombinant virus but the use of this technology was limited by the fact that isolation of recombinant from parental virus required. Inactivation the essential viral gene (ORF1629) lying adjacent to the PH locus used for recombination resulted in a renewal of this technology, which is now labour and time-effective.

Ready-to-use and genetically optimized linearized baculovirus DNA can be purchased from a number of sources (BaculoGold™ (BD Pharmingen), BacMagic™ and BacVector™ (Novagen), BacPAK6™ (Clontech), flashBAC™ (OET), Sapphire™ (Allele Biotechnology), ...). A number of these contain modifications of viral genome, such as knock-out of viral encoded genes that code for non-essential genes that might interfere with expression of the recombinant proteins or knock-in of helper modules for expression of molecular chaperones that can enhance folding of target proteins in the endoplasmic reticulum (Calreticulin and protein disulfide isomerase), and/or of reporter proteins to monitor propagation, optimization of cultivation conditions or titration by end-dilution methods. The price of the linearized baculovirus DNA required for one co-transfection reaction ranges from 30 to 100 euros and when multiple constructs are needed costs associated with the generation of recombinant virus become an issue.

For standard applications and/or initial construct evaluation, we mainly use the bacmid BAC10:KO1629 [18] and its derivatives [38] as source of viral DNA. It consists of the wild-type AcMNPV genome with a low copy bacterial replicon flanked by 2 Bsu36I restriction sites. These elements as well as Kanamycin and Chloramphenicol resistance markers are inserted at the polyhedrin locus (Fig. 3A). The bacmid is produced in *E. coli*, purified using a plasmid/bacmid isolation kit and linearized to enhance recombination efficiency. A 400 ml culture typically yields 50 µg of purified bacmid, which is sufficient for 50 transfections in 6 well plate or 200 in 24 well format.

1. Inoculate 10 ml of LB medium containing 50µg/ml kanamycin and 34 µg/ml chloramphenicol with a single colony of the relevant bacmid strain (BAC10:KO1629 or derivatives) from a freshly peaked plate or directly with 50 µl of a glycerol stock and incubate at 37°C for 4 to 8 hours to prepare a fresh pre-culture.
2. Seed 4 ml of the pre-culture into a 1L Erlenmeyer containing 200 ml 2LB with the appropriate combination of antibiotics and incubate at 37°C for 12-16 hours. The culture should be grown to an OD 600 of 2.0-3.0 (Note 36A).

3. Extract bacmid DNA using a commercially available kit that is suitable for purification of a 135 kbp DNA such as NucleoBond™ Xtra Midi (Macherey Naegel) and precipitate the DNA with ethanol or isopropanol as suggested by the kit manual.
4. After centrifugation of the precipitated DNA, wash the pellet with 70% ethanol to eliminate traces of salts, carefully remove traces of alcohol from the tube, and air-dry the pellet under a sterile hood for 2 to 4 hours (Note 36B)
5. Re-suspend the dried bacmid pellet with 200 µl of sterile ultrapure water and incubate overnight at 4 C°. Do not vortex bacmids. Once an aliquot was thawed, do re-freeze.
6. Check the homogeneity of the bacmid solution by pipetting up and down slowly and if the solution is too viscous add sterile water until an homogenous solution is obtained. Take an aliquot for UV quantification and store aliquots of 25µg at -20°C in sterile tubes.
7. For quality control, we verify that the A260/A280 ratio is close to 1.8 and analyze the bacmid before and after restriction with Bsu36I and both Bsu36I and BamHI on a 0.8% agarose gel. We typically digest 300 ng of DNA in 10µl using 10 U of restriction enzyme per µg DNA during 2-4 hours at 37 C°. Restriction by Bsu36I should convert the super-coiled and open circle forms of the undigested bacmid into a single linear form, digestion with BamHI should create a ladder. Neither high nor low molecular weight nucleic acid should be visible.
8. As homologous recombination is more efficient with a linear than with circular DNA [39], the purified bacmid is finally linearized using Bsu36I at preparative scale using the same experimental conditions as for step 7. Ideally, use buffers and enzymes manipulated under sterile conditions.

For a preparative digestion, one bacmid aliquot (25 µg) is mixed with 20 µl of 10X NEB3 buffer and 12.5 µl Bsu36I (NEB) (10 U/µl) in a final volume of 200 µl. After 12 hours incubation at 37°C, an aliquot is analyzed by gel electrophoresis to control digestion (Fig. 3A) before heat inactivation of Bus36I (20 min at 72°C). If digestion is not complete add 12.5 µl Bsu36I (NEB) (10 U/µl) and re-incubate for 5 hours.

9. The linearized bacmid can be stored at 4°C for 1-2 month. One batch of digested bacmid allows to perform 100 transfections in standard conditions.

When a new batch of bacmid is digested do not omit to perform a co-transfection control and optionally re-optimize conditions by varying the amount of DNA and the ratio of transfection reagents to DNA.

3.6.2 Virus titration

A wide panel of systems including the original plaque assay, end point dilution assay, antibody-based assays, flow cytometry assays, quantitative real-time PCR, and assays utilizing dye-substrates for metabolic enzymes (e.g., AlamarBlue®) can be used for quantitating baculoviruses. We detail a rapid flow cytometric titering assay based on the detection of the baculovirus envelope protein gp64 displayed on the surface of infected cells using an APC-conjugated anti-gp64 antibody. We use

Cells are infected in the late afternoon of day 1 and analyzed by FACS on day 2 (14 – 16 hours later).

1. Prepare five serial dilutions (dilution range: 10^{-1} to $3.91 \cdot 10^{-6}$) of the virus to be tittered in fresh Sf-900 II medium according to Table 5.
2. Dilute Sf9 cells (Sf9 Oxford 3) to 1.25×10^6 viable cells/mL in Sf-900 II medium. Prepare 2.5 ml per virus to be titrated.
3. For each virus, dispense 1 mL of dilutions 3, 4, 5, and 6 from Step 1 in separate wells of a 24-well plate. Do not forget to include a negative control with medium instead of a virus dilution.
4. To each well add 800 μ L of the diluted cell suspension in each well (ie $1 \cdot 10^6$ cells) and incubate the plate overnight at 27°C on a shaking platform with a 19-mm diameter orbital set to 130 rpm.

The next day prepare per sample: 100 μ L of a 0.15 μ g/mL anti-baculovirus envelope gp64 APC antibody solution in PBS containing 2% FBS, 1 mL of PBS and 0.5 mL of PBS in 2% FBS.

5. Gently re-suspend the cells from each well, transfer into 1.5 ml tubes, centrifuge at 300 g for 10 minutes and carefully aspirate the supernatants.
6. Resuspend each cell pellet in 100 μ L diluted antibody, mix by briefly vortexing for 3–5 seconds and incubate tubes at room temperature for 30 minutes.
7. Wash samples by adding 1 mL PBS, centrifugation at 300 g for 10 minutes, carefully aspirate supernatants and resuspend each cell pellet in 0.5 mL Dilution Buffer
8. Analyze samples using a flow cytometer (Red Laser - Excitation: 633–647 nm; Emission: 660 nm). Record percent positive gp64-expressing cells for viral dilution

and negative control samples. Refer to the instrument manual for setup of acquisition parameters.

9. Finally, select samples containing between 2 and 10 % percent gp64-positive cells and compute the titer (number of infectious viral particles per mL) from the number of cells analyzed (1×10^6 in this example), the percent gp64-positive cells in that respective dilution sample and the dilution factor using the equation:

$$\text{Viral Titer} = \left(\frac{\text{cell density} \times \text{percent gp64 positive cells}}{\text{dilution factor of virus stock}} \right) \times 0.01$$

For high titer virus stocks, values between 0.5×10^8 and 5×10^8 pfu/ml are expected. An example of a P1 titration where the virus solution was diluted $1:4 \times 10^3$ fold is shown Fig. 7. The percentage of gp64 infected cells was 3.04% which corresponds to a virus titer of 1.22×10^8 infectious particles per ml.

4 Notes

Note 24A: Plasmids for bacterial expression of TEV or 3C proteases are available from Addgene. We prepare protease aliquots at a concentration of 1 mg/ml which are stored at -80°C.

Note 31A: We advise to carefully verify the sequences of the vector and insert before starting and the use of a molecular cloning simulator for construct design and primers selection.

Note 31B: A detailed SLIC protocol is described in [40]. Briefly, treat the digested plasmid and insert with T4 DNA Polymerase at room temperature (Use 0.5 units of polymerase in 30 μ L of 1X NEB 2.1 buffer for 1 μ g of DNA), and after 30 min incubation stop the exonuclease reaction by adding 3 μ L of a 10 mM dCTP solution (1/10 of the reaction volume) and leave on ice. Prepare a 20 μ L annealing mix containing the polymerase-treated vector backbone and the cDNA in a 1:3 ratio using 125 ng vector (0.03 pmoles of a 7 kbp plasmid) in your favorite ligation buffer, incubate the annealing mix at 37 C for 30 min and leave on ice until transformation or store at -20 C.

Note 32A: Co-transfection is a critical step in the expression pipeline but which fortunately can easily be optimized using a fluorescent reporter protein. Don't hesitate to run an optimization plate where the cell seeding density, the amount of linearized viral DNA and of transfer vector and the DNA/transfection agent vary. Additionally, we found that use of an engineered bacmid or of a transfer vector which, in addition to the target protein, expresses a fluorescent protein can be extremely usefully, not only as a positive transfection control but also for protocol optimization.

Note 32B: Many transfection protocols require serum-free conditions for optimal performance as serum can interfere with transfection reagents and negatively impact transfection efficiency [41]. Note that this is however not always the case and that some transfection agents can be used in the presence of serum.

Note 32C: Carefully check buffer compatibilities of the resins and adapt the composition of the lysis buffer and adapt the composition of the lysis buffer. For IMAC affinity purification, the lysis buffer can be supplemented with limited imidazole concentration (between 5 and 20mM depending of the resin) to limit a-specific binding. High urea concentration (up to 6 M) can be used to solubilize most proteins and purify under denaturing conditions to evaluate total protein expression.

Note 32D: As an alternative, to elution in native conditions bound proteins can be eluted by adding 25 µl of 4X SDS loading dye. In this case, proteins that are a-specifically retained on the resin or that have precipitated on the beads will also be detected.

Note 32E: The Multiplicity Of Infection is the ratio of agents (here baculoviruses) to infection targets (here insect cells). For efficient amplification one should infect cells using a low MOI (typically 0.05 – 0.2) which ensures that only a few cells are infected initially and limits accumulation of defective interfering particles (DIPs), ie partial genomes packaged by complementation from intact genomes co-infected in the same cell [42]. As the number of mutant virus is also increased by serial passage, we usually perform a single round of amplification. As transfection is a simple procedure, we repeat the transfection to produce more P0 rather than performing several rounds of amplification. For protein production, cells are infected at high MOIs, usually above 1.0 and up to 10.0.

Note 32F: As we use a serum-free medium for virus amplification, FBS is be added to stabilize the virus stocks. Protected from light, viruses can be stored at 4° for 6-12 months or longer but after more than 3-4 months it is recommended to recalibrate experiments before use or to re-amplify.

Note 33A: In absence of a-priori knowledge, we would evaluate different salt concentrations (low, medium and high salt), evaluate a few detergents (ionic and non-ionic detergent) and test different pHs,. High ionic strength enhances solubility of many proteins but is not compatible for a number of applications (ion exchange chromatography, native mass spectrometry, in vitro DNA binding assays,...). Indeed, a substantial number of proteins precipitate when the salt concentration is reduced to physiological levels. For intracellular proteins, care should be taken to maintain a reducing environment. Tris(2-carboxyethyl)phosphine (TCEP), unlike dithiothreitol (DTT), is compatible with all known IMAC matrices. Addition of glycerol and/or arginine Inclusion of glycerol (10%) and/or addition of arginine (in the range of 50–500 mM) can enhance the solubility and stability of many proteins.

Note 33B: If the cDNA contains a secretion signal, the protein will be excreted and should be purified from the culture medium.

Note 33C. In the case of IMAC chromatography, 2h incubation is generally enough. In the case of FLAG or glutathione Sepharose resin (GE Healthcare), an incubation time of 4 or 12 h is recommended.

Note 33D: The amount of required protease is determined empirically by testing conditions (a good starting point is 1/40 mass ratio – 1 mg of protease for 40 mg of fusion protein). As

alternative to elution by protease cleavage, proteins can be eluted by competition with a specific ligand (250 mM Imidazole, 15 mM GSH, 0.5 mg/ml FLAG peptide).

Note 34A: Dust particles which scatter light that may affect the DLS measurements and should be avoided. If a quartz cuvette is used, rinse with ethanol and with water and blow out residual liquid by pressured nitrogen or air. The minimum concentration depends on the particle size (it can be estimated using $[1.44 / MW(kDa)]$ mg/mL). As a rule of thumb, use approximately a concentration > 0.6 mg/mL for a 10 kDa protein and > 0.06 mg/mL for a 100 kDa protein. The data quality generally increases with concentration (in the absence of aggregation). Before measuring the sample, you can perform a measurement with buffer or water to ensure that the cuvette is clean (no dust particles). The count rate should be comparable to that reported in the logbook of the instrument for similar measurements. If you suspect a problem, don't hesitate to purchase and test a BSA standard (Pierce™ Bovine Serum Albumin Standard Ampules, 2 mg/mL for example).

Note 34B: When interpreting the DLS carefully read the instrument manual. First study the measurements statistics table to evaluate the quality of your data and filter out outliers. Use the baseline parameter (quality of the fit of the data to a given autocorrelation function) to judge if your sample is monomodal, bimodal, or multimodal: (i) Monomodal distributions are usually defined by a baseline ranging from 0.997 to 1.002, (ii) bimodal distributions have a baseline range of 1.003 to 1.005 and (iii) baselines greater than 1.005 are from multimodal samples, dust, or noise. The uniformity of sizes are determined through a monomodal (single particle size with a Gaussian distribution) curve fit analysis. The quality of the data is represented in the sum of squares (SOS) error statistic reported for each sample acquisition (a single correlation curve): (i) $1.000 < SOS < 5.000$ Low noise, negligible error, (ii) $5.000 < SOS < 20.000$ Background error because of noise, low protein concentration or a small amount of polydispersity, (iii) $SOS > 20.000$ High noise/error owing to high polydispersity in size distribution (aggregation).

Note 35A: Insect cells and viruses are handled in laminar flow hood under aseptic conditions without antibiotic as these can mask low levels of contamination. Addition of penicillin (5-25 U/ml) and streptomycin (5 µg/ml) or gentamycin (5 µg/ml) however can help punctually to face contamination, if needed. Material taken inside the hood should be treated with 70% EthOH and taken out properly decontaminated (autoclave or beach). Do not use soap when cleaning vessels as residue always remains and may cause cell mortality. We recommend washing with 10% acetic acid or other commercially available cleaners and wash intensively with miliQ water prior autoclaving.

Note 35B: Cell viability can be evaluated with trypan blue. Mix one volume of cells with one volume of a 0.1 % stock solution of trypan blue (in PBS or other isotonic salt solution). Nonviable cells will take up Trypan blue. Healthy, log-phase cultures should contain more than 97 % unstained viable cells.

Note 35C: We regularly perform control experiments to evaluate and optimize the expression pipeline. For co-transfections we compared different cell lines and obtained highest titers for the P0 stock with semi-adherent Sf9 cells grown in grace medium supplemented with 10% FCS. For amplification and production, cells adapted to suspension culture in serum-free media are favored. Supplementation of media with serum promotes cell growth, provides shear force protection and prolongs stability of virus stocks but has a non-negligible cost and leads to excessive foaming with subsequent cell damage. Serum batches can in addition exhibit significant lot to lot variability and should be carefully tested before use.

Note 36A: As a rule of thumb, 1 liter of E. coli culture with an OD600 of 1 consists of 1×10^{12} cells and yields about 1.5 g cell wet weight -. We usually grow several cultures in parallel and prepare cell pellets of 1.5 g which are either processed immediately (in this case linearized bacmid is aliquoted) or stored at -20°C.

Note 36B: To concentrate DNA, do not use membrane based tools such as Nucleobond Finalizer™ (Macherey Naeglel) which are not recommended for constructs larger than 50 kbp.

5 Acknowledgments

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6 Figures and tables

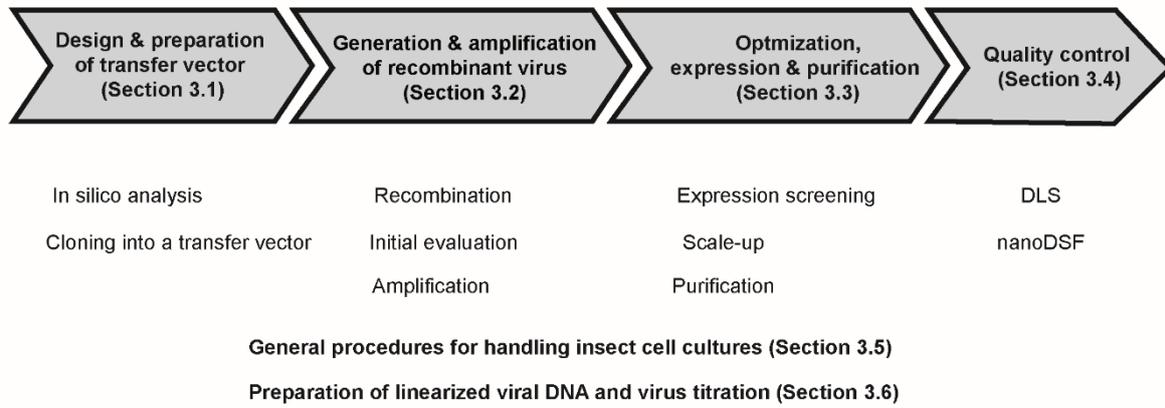


Figure 1. From gene to purified protein.

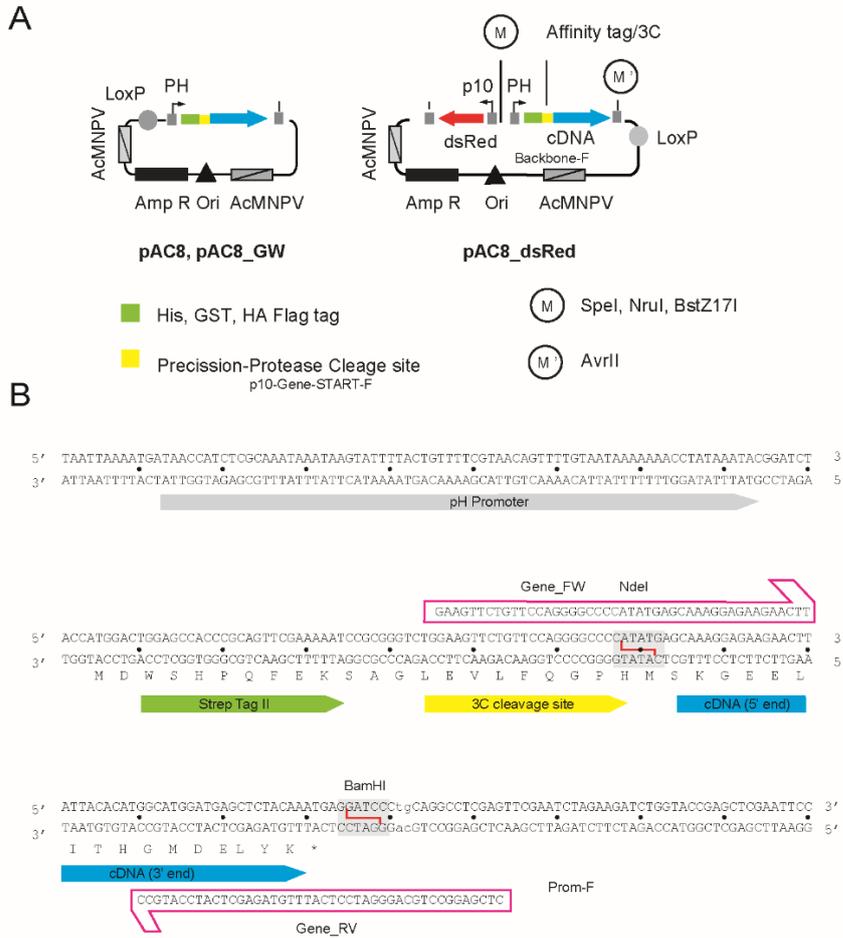


Figure 2. pAC8 vectors. (A). Schematic representation of the transfer vectors pAC8, pAC8_GW and pAC8_dsRed. Expression of target gene in fusion with an amino-terminal affinity tag and a Precision-Protease Cleavage is driven by a PH promoter. The AcMNPV sequences flanking the expression cassette, the replication unit (ColE1) and antibiotic resistance gene (Amp^R) are also indicated. The expression cassettes of pAC8_dsRed derivatives are flanked by multiplication modules (M) and (M') that can be used to assemble co-expression constructs. These plasmids also contain a LoxP site that enables Cre-mediated plasmid fusion. **(B)** Sequence of pAC8-Strep plasmid containing a protein coding sequence inserted into the NdeI and BamHI sites of the vector. The recommended forward (Gene_FW) and reverse (Gene_RV) primers for PCR amplification of the target gene are indicated. The amino acid sequence recognized by the Precision protease (3C) is LEVLFQ!GP.

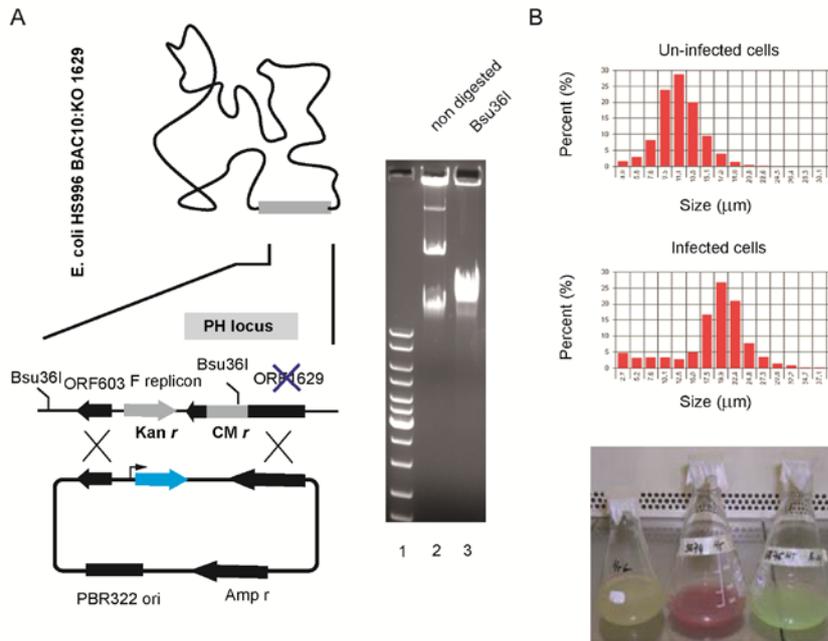


Figure 3. Generation of recombinant viruses and protein production. (A) Linearized viral DNA (purified BAC10:KO1629) is co-transfected with a transfer vector containing the cDNA (in cyan) to be inserted into the viral genome. Homologous recombination replaces the F replicon by the expression cassette for the gene of interest and restores the essential ORF1629, allowing viral replication and expression of the desired protein. Purified BAC10:KO1629 analyzed on a 0.8 % agarose gel before (lane 2) and after digestion with Bsu36I (lane 3) is also shown on the right. **(B)** For production of intracellular proteins, Sf9 cells grown in Erlenmeyer flasks are infected with a recombinant virus expressing the gene of interest, incubated for 48-72 h and harvested. Carefully verify that baculovirus infected cells have stopped dividing and have swelled –the mean diameter typically increases by 15-30%. If, in addition to the target protein, the virus encodes a fluorescent reporter, fluorescence can be used to monitor infection.

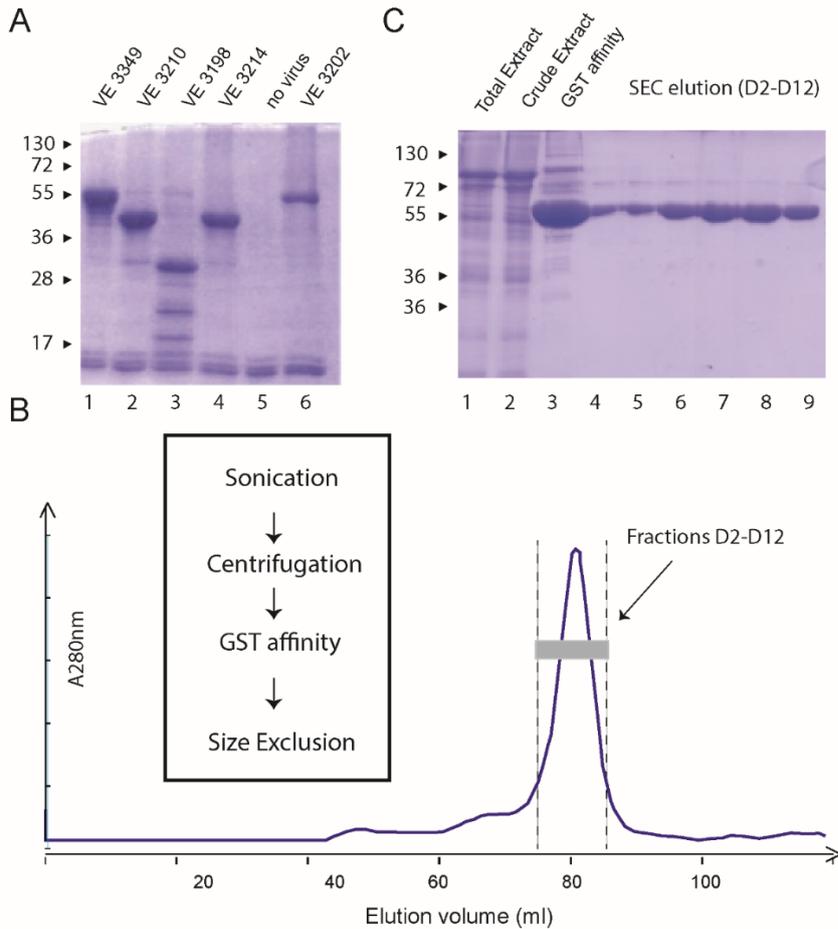


Figure 4. Expression screening and purification. **(A)** PRMT2 deletion constructs were expressed in fusion with an N-terminal GST tag, immobilized on GSH sepharose resin and the bound proteins were analyzed by SDS-PAGE in a 12.5% gel. **(B)** Workflow of mPRMT2 purification and chromatogram of the final size-exclusion chromatography (HiLoad 26/600 Superdex). Fractions between the vertical dashed lines (D2-D12) were pooled and concentrated. **(C)** Analysis mPRMT2 purification by SDS-PAGE in a 12.5% gel. The total extract, the crude extract and the eluate from the Glutathione sepharose column were loaded in lanes 1, 2 and 3. Fractions D2, D4, D6, D8, D10 and D12 from the size exclusion column were analyzed in lanes 4 to 9. The expected mass of the protein is ~50 kDa.

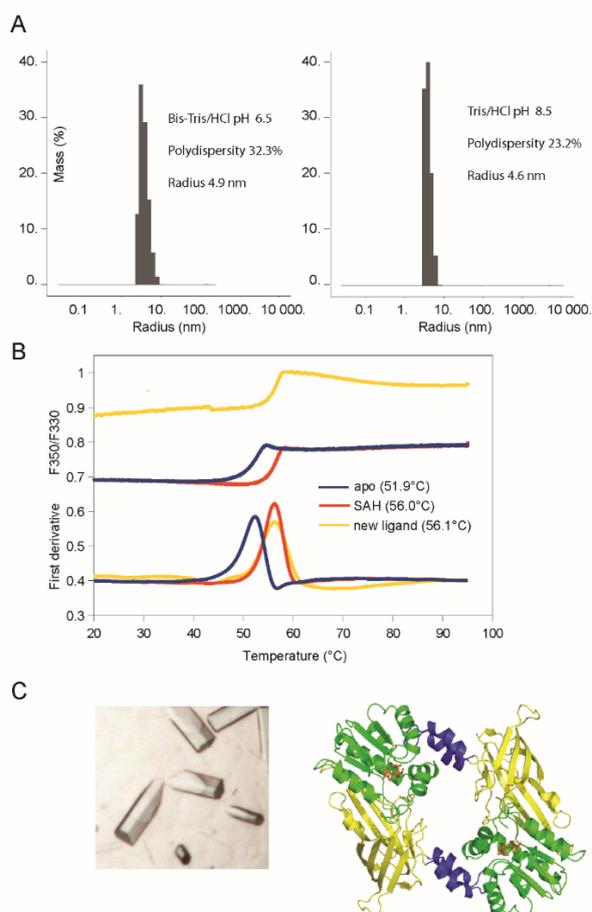


Figure 5. Quality control. (A) Dynamic light scattering analysis. Particle size distribution of drPRMT2 recorded at pH 6.5 and 8.5 in absence of substrate. At pH 6.5 the distribution is monomodal but the polydispersity is high. At pH 8.5 the polydispersity is slightly above the 20% threshold but still acceptable. Crystals of drPRMT2 were obtained in presence of substrate analogues at this pH. **(B) nano Differential Scanning Fluorimetry.** Effect of ligand binding on protein stabilization monitored by Nano DSF. Melting curves are derived from 350/350 nm fluorescence emission ratios. Melting temperatures (T_m) are calculated from the peaks of the first derivatives. T_m values for each ligand are indicated in brackets. Apo: protein without ligand. SAH and New ligand: protein incubated in the presence of S-Adenosyl-homocysteine or with substrate analogue, respectively. In most cases, ligand binding shifts T_m towards higher temperatures, showing that the purified protein is functional. **(C) Crystals and X-ray structure** of drPRMT2 [43].

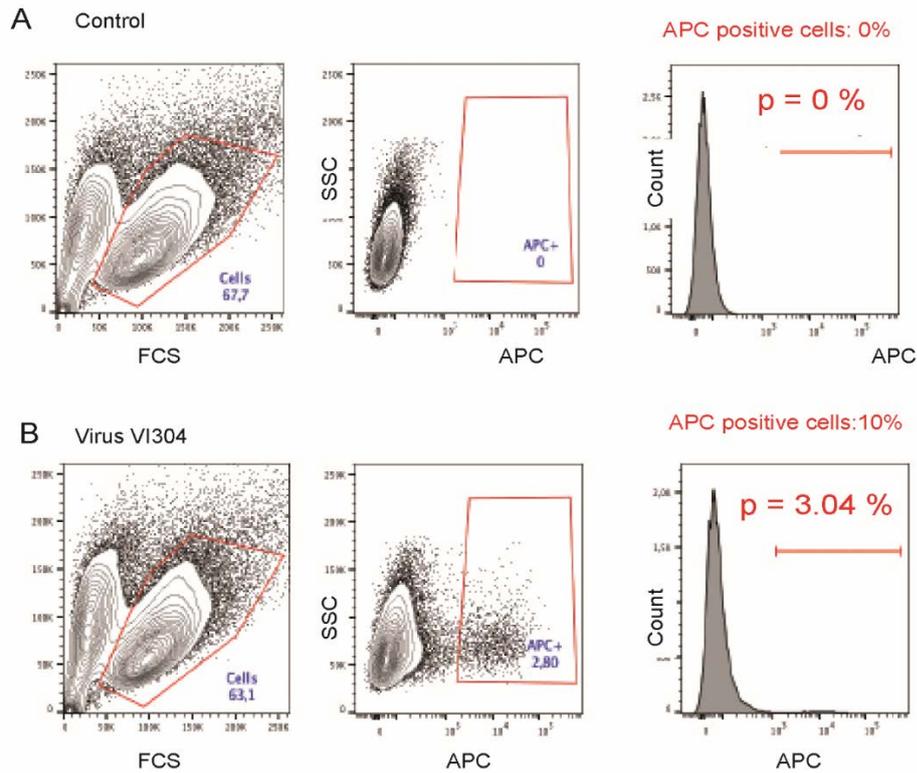


Figure 6. Baculovirus titration using a flow cytometric assay. Shown is the gating strategy and representative flow cytometry plots from non-infected (**A, control**) and from cells infected with a 1:4 10^3 (=0.00025) dilution of the VI304 viral stock (**B, virus VI304**) after staining with APC conjugated anti-gp64 antibody. The percentage of gp64 positive cells is obtained from the graph where the number of events is plotted against the APC fluorescence intensity.

Meta-analysis servers	
EXPASY SIB Bioinformatics Resource Portal [44]	http://www.expasy.org/
MPI Toolkit for protein sequence analysis [45]	http://toolkit.tuebingen.mpg.de/
BIP server of the LSCF Bioinformatics Unit from the Weizmann institute	https://bip.weizmann.ac.il/toolbox/overview.html
EMBL Bioinformatic Institute	https://www.ebi.ac.uk/services

Table 1: Resource portals providing access to software tools and databases for selection of construct boundaries.

Plasmid	Affinity tags	
pAC8-6His	Nter 6xHis tag	
pAC8-GST	GST tag	
pAC8-Flag	Flag tag	
pAC8-HA	HA tag	
pAC8-GFP	GFP	Control plasmid
pAC8-0GW	-	Gateway cassette
pAC8-6His-3C-GW	Nter 6xHis tag, optional Cter c-myc	Gateway cassette
pAC8-10His-3C-GW	Nter 10xHis tag, optional Cter c-myc	Gateway cassette
pAC8-Flag-3C-GW	Nter Flag tag, optional Cter c-myc	Gateway cassette
pAC8-GST-3C-GW	Nter GST tag, optional Cter c-myc	Gateway cassette
pAC8_DsRed-6His-3C-GW	Nter 6xHis tag	Co-expression of DsRed
pAC8_DsRed-GST-3C-GW	GST tag	Co-expression of DsRed
pAC8_DsRed-Flag-3C-GW	Flag tag	Co-expression of DsRed
pAC8_DsRed-HA-3C-GW	HA tag	Co-expression of DsRed

Table 2: Transfer vectors for expression screening.

Primer name	Primer Sequence
Gene_FW	GAAAAATCCGTCCTCTCCCAATCCGTG
Gene_RV	ACAGAGTCGACGCTGTCAAACGAAATCAAA
PH_FW (bac1)	ACCATCTCGCAAATAAATAA
PH_RV	TGGTATGGCTGATTATGATC

Table 3: Oligonucleotides.

	Sf9*	Sf9 Oxford 3	ExpiSf9™	Sf21	High-five™
Use	Virus generation Initial evaluation	Amplification, Production	Production	Production	Production
Growth	Semi-Adherent	Suspension	Suspension	Suspension	Suspension
Medium	Grace, 10%FBS	SF900-II or Insect-XPRESS	ExpiSf™ CD	SF900-II	Express-five
Max cell density	1.5 10 ⁵ cells/cm ² 80% confluency	5 10 ⁶ cells/ml	7 10 ⁶ cells/ml	10 x 10 ⁶ cells/ml	3 x 10 ⁶ cells/ml
Seed density for maintenance	2.0 10 ⁴ cells/cm ² 1:3 - 1:4 dilution	0.8 10 ⁶ cells/ml	0.5 10 ⁶ cells/ml	0.5 10 ⁶ cells/ml	0.3 10 ⁶ cells/ml
Seed density for infection	2.0 10 ⁴ cells/cm ² 1:3 - 1:4 dilution	1.0 10 ⁶ cells/ml	5.0 x 10 ⁶ cells/ml	1.0 x 10 ⁶ cells/ml	1.0 x 10 ⁶ cells/ml
Doubling time*	24-30 h	24-30 h	18-24 h	18-24 h	18-24 h
Agitation	Na	110 rpm	110 rpm	110 rpm	110 rpm
Freezing cells					
Cryopreservation Medium	70% Grace, 20% FBS, 10 % DMSO	45 % SF900-II, 45% CD SF900-II, 10 % DMSO	45% ExpiSf, 45% CD ExpiSf 10 % DMSO	60% Sf900-II, 30 %FBS, 10 % DMSO	45% Express Five, 45% CD Express Five, 10 % DMSO
Density (cell/vial)	>1. 10 ⁷	>1. 10 ⁷	>1. 10 ⁷	>1. 10 ⁷	>3. 10 ⁶

Table 4: Culture parameters for Sf9, Sf21 and High-Five™ cells. Sf9 or Sf21 but not High Five cells are suitable to produce or amplify virus. High Five cell lines exhibit higher (tenfold) susceptibility to baculovirus infection than Sf9 cells, whereas Sf9 cells show a higher (100-fold) capacity for production of infectious virus particles [46]. CD Conditioned.

Sample	Dilution	Virus Stock volume	Diluting medium volume	Total volume
Dilution 1	1 : 10	120 μ L undiluted virus	1.08 mL	1.2 mL
Dilution 2	1 : 10 ²	120 μ L of dilution 1	1.08 mL	1.2 mL
Dilution 3	1 : 10 ³	150 μ L of dilution 2	1.35 mL	1.5 mL
Dilution 4	1 : 4 10 ³	400 μ L of dilution 3	1.2 mL	1.6 mL
Dilution 5	1 : 4 ² 10 ³	400 μ L of dilution 4	1.2 mL	1.6 mL
Dilution 6	1 : 4 ³ 10 ³	400 μ L of dilution 5	1.2 mL	1.6 mL

Table 5: Serial dilutions of the virus for titration.

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