Chapter 7

BacMam Platform for Vaccine Antigen Delivery

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Abstract

Recombinant baculo viruses based on Autographa californica multiple nuclear polyhedrosis virus carrying vertebrate cell active expression cassettes, so-called BacMam viruses, are increasingly used as gene delivery vectors for vaccination of animals against pathogens. Different approaches for generation of BacMams exist and a variety of transfer vectors to improve target protein expression in vivo have been constructed. Here we describe a use of transfer vector which contains an insect cell-restricted expression cassette for the green fluorescent protein and thus enables easy monitoring of BacMam virus rescue, fast plaque purification of recombinants and their convenient titer determination and which has been proven to be efficacious for gene delivery in vaccination/challenge experiments.

Key words BacMam technology, Baculo virus transfer plasmids, Green fluorescent protein expression, In vitro and in vivo transduction

1 Introduction

The baculovirus *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV), belongs to the genus *Nucleopolyhedrovirus* of the family *Baculoviridae* [1]. Its large double-stranded DNA genome has size of about 134 kb and purified virus DNA is infectious. In the enveloped virions the size of the circular genome determines the length of the rod-shaped nucleocapsid which surrounds the DNA, enabling integration of large DNA sequences into the viral genome [1]. With respect to biosafety and biosecurity, Baculovirus vectors are regarded as safe because of their highly specific host range for productive replication, and lack of detectable AcMNPV-promoter driven gene expression in mammalian cells [reviewed in ref. [2]]. AcMNPV has been used successfully for the high level synthesis and purification of proteins in infected

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insect cells since the early 1980s with expression of the heterologous open reading frames (ORFs) driven by the baculoviral polyhedrin or p10 promoters [3]. In the mid-ninties, Hofmann et al. [4] and Boyce and Buchner [5] showed that recombinant AcMNPV with mammalian promoters regulating the expression of the protein of interest were suitable for delivery into and expression of genes in hepatic cells. Following these ground braking developments, a number of mammalian cell types, cells of avian [6] and even piscine [7] origin have been reported to be transduceable by the so called BacMam method which is also known as BacMam technology [for review *see* ref. 8].

Different commercially available systems for generation of recombinant baculoviruses make BacMam viruses easy to produce. These gene delivery vectors have a broad in vitro host range, are suitable for both transient and stable gene transfer, and, if mass application is envisaged, are cost-effective in comparison to chemical gene transfer procedures [2]. These benefits are mirrored by the increasing number of publications dealing with application of the BacMam technology. Surprisingly, publications reporting application of this technology in immunization challenge/experiments against pathogens are still limited so far [9–11] although direct vaccination with BacMam viruses can result in the induction of significant humoral and cell-mediated immunity against animal and zoonotic pathogens, including influenza virus, porcine reproductive and respiratory syndrome virus, porcine circovirus type 2, West Nile virus, RABV, and hepatitis C virus [*see* ref. 12 for references].

Developments for next-generation BacMam vectors for vaccination purposes are aimed at enhancement of the transduction efficacies in vivo by increasing the antigen expression or by display of specific ligands on pseudotyped virus particles [9, 12, 13].

Here, we describe construction of BacMam viruses relying on the FastBacDual system (Invitrogen, Karlsruhe, Germany) using the new baculovirus transfer vector pMamBac-CAGGS (Fig. 1) which is based on pBacMamMCMVdual-ie [13] and contains the strong mammalian cell-active CAGGS enhancer/promoter element [14].

To facilitate isolation, plaque purification and titer determination of BacMam viruses, pMamBac-CAGGS also transfers an insect cell-restricted, polyhedrin promoter driven green fluorescent protein (GFP) expression cassette into the Baculovirus genome (Fig. 1). Thus, in insect cells, infected with respective recombinants, GFP is expressed and infection can be easily monitored using a fluorescence microscope. In vertebrate cells, however, the polyhedrin promoter is inactive.

2 Materials

Prepare all solutions using ultrapure water with a conductivity of <0.06 μS/cm and analytical grade reagents. Autoclave at 121 °C for 20 min or filtrate through a 0.2 μm filter unit. Use sterile disposable equipment for cell culture. Heat-sterilize all glass materials. Standard equipment for molecular biology laboratories is implied.

2.1 Cloning Procedures

1. Restriction enzyme *EcoR*I including 10× reaction buffers.
2. Plasmid pBacMam-CAGGS (available from the authors).
3. Calf intestine alkaline phosphatase (CIP) including 10× reaction buffer.
4. Phage T4 DNA ligase.
5. QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany).
6. 60 mM ethylene glycol tetraacetic acid (EGTA), pH 7.0.
7. TE buffer: 10 mM Tris–HCl , 1 mM EDTA, pH 7.5.
8. TE-saturated phenol.
9. Chloroform–isoamyl alcohol 24:1 (v/v).
10. 3 M sodium acetate, adjusted to pH 4.8 with acetic acid.
11. Basic enzyme buffer (10× TA): 330 mM Tris–HCl, 660 mM potassium acetate, 100 mM magnesium acetate, adjusted to pH 7.9 with acetic acid, 1 mg/mL bovine serum albumin, 5 mM dithiothreitol.
12. Appropriate chemically competent *E. coli* strain.
13. LB-medium: dissolve 10 g tryptone, 5 g yeast extract, and 8 g NaCl per liter. For LB agar petri dishes add 15 g Bacto-Agar. Autoclave and cool to 56 °C in a water bath. Pour 10–15 mL into 10 cm petri dishes and invert plates after solidification. Store at 4 °C. For selection add ampicillin to a final concentration of 100 μg/mL.
14. Qiagen Plasmid Mini kit and Plasmid Midi kit (Qiagen, Hilden, Germany).

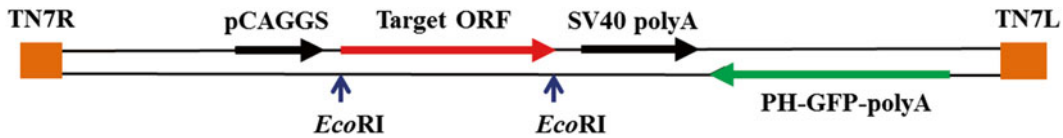

Fig. 1 Schematic of the pFastBac-Dual-based BacMam transfer plasmid pBacMamCAGGS with inserted target ORF. The hybrid human cytomegalovirus/chicken β-globin enhancer/promoter element (CAGGS) and the SV40 consensus sequence for polyadenylation (SV40 polyA) which control expression of the target ORF are indicated, as are the target ORF flanking *EcoRI* restriction enzyme cleavage sites. Also depicted is the insect cell-active, polyhedrin promoter regulated expression cassette for GFP (PH-GFP-poly A). TN7R and TN7L denote sequences required for transposition from the transfer plasmid into the bacmid. Note: Not drawn to scale

2.2 Agarose Gel Electrophoresis

1. 50-fold concentrated Tris-acetate buffer: 2 M Tris–HCl, 0.25 M sodium acetate, 0.05 M EDTA adjusted to pH 7.8 with acetic acid.
2. Agarose for gel electrophoresis.
3. Ethidium bromide solution of 10 mg/mL in water (*see* **Note 1**).
4. Electrophoresis buffer for agarose gel electrophoresis is 1× Tris-acetate. Add ethidium bromide in water to a final concentration of 100 ng/mL.
5. DNA loading buffer: 40 % sucrose, 1 mM EDTA pH 7.5, 0.05 % bromophenol blue, 0.1 % SDS.
6. Suitable DNA size marker.
7. Agarose gel electrophoresis equipment.
8. UV-transilluminator (254 and 302 nm) (*see* **Note 2**).

2.3 Transposition of the Transfer Plasmid into the Baculovirus Bacmid (Bac-to-Bac System, Invitrogen)

1. Chemically competent *E. coli* DH10Bac (Invitrogen).
2. SOC-medium (per 100 mL): 2 g tryptone/L, 0.5 g yeast extract/L, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgSO_4$, 10 mM $MgCl_2$, 20 mM glucose.
3. LB medium (*see* Subheading 2.1, **item 13**) with 10 μg/mL tetracycline, 50 μg/mL kanamycin, 7 μg/mL gentamycin.
4. LB-gar plates (*see* Subheading 2.1, **item 13**) with 10 μg/mL tetracycline, 50 μg/mL kanamycin, 7 μg/mL. gentamycin, 20 μg/mL. For blue/white selection spread 40 μL X-Gal (20 mg/mL) and 40 μL 100 mM IPTG evenly on the plate.
5. Solution 1: 10 mM EDTA, pH 8.0, 20 mM Tris–HCl, pH 8.0, 50 mM glucose. Before use add 2 mg/mL lysozyme.
6. Solution 2: 0.2 M NaOH, 1 % SDS in H_2O.
7. Solution 3: 3 M sodium acetate, pH 4.8.
8. TE-buffer with 50 μg/mL RNase A.

2.4 Insect Cell Culture

1. *Trichoplusia ni* High Five cells, grown in serum free "Insect Express Sf9-S2 with l-glutamine Medium" (PAA) in appropriate disposable tissue culture vessels.
2. *Spodoptera frugiperda* SF9 cells, grown in Grace's insect culture medium with 10 % FCS in appropriate disposable tissue culture vessels.
3. Serum free double concentrated Grace's insect culture medium.
4. Low melting point agarose (2 %) in H_2O.

2.5 Rescue of BacMam Viruses from Bacmid DNA

1. High Five cells.
2. FuGENE HD transfection reagent (Roche).
3. SF9 cells.

2.6 Transduction of Vertebrate Cells with BacMam Viruses

1. Vertebrate cells in adequate culture vessels in appropriate cell culture medium.
2. PBS^+. Contents per 1 L: 137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.4 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.2. Sterilize by filtration. PBS^- does not contain $CaCl_2$ and $MgCl_2$ (*see* **Note 3**).
3. 1 M Na-butyrate pH 7.0.
4. Low speed centrifuge with a plate rotor.

3 Methods

3.1 Agarose Gel Electrophoresis

We use self-constructed agarose gel apparatuses with buffer recirculation. Nevertheless, the specifications given below can be easily adapted to other formats. Thus, assemble the device as appropriate.

1. Prepare a 0.6 % gel by boiling 3 g agarose in 490 mL water in a microwave oven until fluidized and cool to 56 °C in a water bath. Replenish evaporated water (*see* **Note 4**).
2. Add 10 mL 50× Tris-acetate buffer and 5 μL of 10 mg/mL ethidium bromide solution and leave in a 56 °C water bath until use.
3. Pour the gels according to the specifics of your system (*see* **Note 5**). For examination of control of for example restriction enzyme cleavage reactions make small (6 cm × 4 cm ($l \times w$)), ~5 mm thick gels. For purification of DNA fragments larger gels (25 cm × 15 cm ($l \times w$)) and 5 mm thick) may be more appropriate. Use combs as necessary.
4. After solidifying cover the gels with electrophoresis buffer containing 0.1 μg/mL ethidium bromide and draw out the comb. Pipette the samples into the wells. Include one well with the DNA size marker.
5. The small gels run at 8 V/cm distance between the electrodes for 25 min; the large gels at 4 V/cm distance between the electrodes for 3–6 h (*see* **Note 6**).
6. Place the gel on a UV transilluminator (254 nm for documentation only, 302 nm for excision of fragments) after electrophoresis to visualize the DNA fragments. Take a picture for documentation (*see* **Note 2**).

3.2 Insertion of the Target ORF into the BacMam Transfer Vector

Among a large number of BacMam transfer plasmids, to our knowledge only those developed in our laboratory carry an expression cassette for GFP whose expression is driven by the polyhedrin promoter and thus restricted to insect cells [13]. We so far did not observe any negative effects of the presence of this cassette on

BacMam virus propagation in insect cells and infectious virus yield. Advantages of GFP expression in insect cells are monitoring of BacMam rescue, easy plaque purification and fast titer determination. For insertion of target ORFs into the transfer vector pBacMamCAGGS (*see* Fig. 1) use of the *Eco*RI cleavage site is recommended.

1. Isolate the target ORF DNA fragment, flanked by *Eco*RI cohesive ends or blunt ends, after agarose gel electrophoresis using the QIAquick Gel Extraction Kit according to the manufacturer's instructions. Elute the DNA with 50 µL 5 mM Tris–HCl/1 mM EDTA pH 7.0.
2. For the vector preparation, cleave 5 µg of plasmid pBacMamCAGGS (*see* Fig. 1) using 5 µL of 10× *Eco*RI reaction buffer, 5 U *Eco*RI enzyme, and water to a final volume of 50 µL. Incubate for 2 h at 37 °C.
3. For dephosphorylation of 5′ ends with calf intestine phosphatase (CIP) add 25 µL 10× CIP buffer, 174 µL ultra-pure water, and 1 µL CIP. Incubate for 30 min at 37 °C. Add another 1 µL CIP and incubate for 30 min at 56 °C. Add 50 µL of 60 mM EGTA and incubate for 30 min at 65 °C to inactivate the phosphatase. Add 30 mL 10 % SDS, 1 µL proteinase K (10 mg/mL) and incubate at 56 °C for 30 min.
4. Add 300 µL of TE-saturated phenol to the DNA sample. Shake vigorously 20–30 s. Centrifuge the sample for 2 min at room temperature to separate the phases (*see* **Note 7**).
5. Transfer the aqueous phase into a new 1.5 mL Eppendorf tube and add 300 µL of a 1:1 mixture TE-phenol and chloroform–isoamyl alcohol. Mix thoroughly and centrifuge the sample for 2 min at room temperature to separate the phases.
6. Transfer the aqueous phase into a 1.5 mL Eppendorf tube and add 1 mL chloroform–isoamyl alcohol. Mix thoroughly and centrifuge the sample for 2 min at room temperature to separate the phases.
7. Transfer the aqueous phase into a new 1.5 mL Eppendorf tube and determine the volume. Add TE to 360 and 40 µL 3 M sodium acetate, pH 7.0, and 1 mL 100 % ethanol. Mix thoroughly and incubate at −80 °C for about 30 min.
8. Pellet the precipitated DNA by centrifugation for 15 min at room temperature and remove the ethanol and wash the pellet with 1 mL 70 % ethanol and centrifuge for 10 min. Remove the ethanol and dry the pellet for 5–10 min by incubating the open tube at 56 °C.
9. Resuspend the dried pellet in 50 µL of TE by incubation at 56 °C for 15 min.

10. Into a new 1.5 mL Eppendorf tube, pipette 5 μL of the purified vector, up to 24 μL of the purified target ORF fragment, 5 μL BSA, 5 μL 10× TA, 5 μL 100 mM DTT, 5 μL 10 mM ATP, and 0.1 U T4 ligase (*see* **Note 8**). Adjust to 50 μL with H_2O. As a control, prepare the same ligation mixture but use water instead of the purified target ORF fragment. Incubate for 5 min at 37 °C, 1 h at 25 °C, and overnight at 4 °C (*see* **Note 9**).
11. To transform chemically competent bacteria, incubate freshly thawed aliquots on ice for 5 min and add 1–10 μL of the ligation mixtures to the recommended amount of bacteria.

    Incubate for 20 min on ice, 2 min at 42 °C, and again on ice for 5 min.
12. Add 200 μL LB medium + 2 μL 1 M KCl and 2 μL 2 M $MgSO_4$ per tube, incubate for 1 h at 37 °C and plate on LB-agar petri dishes containing ampicillin. Incubate overnight at 30 °C and then at 37 °C if colonies are too small (*see* **Note 10**).
13. Pick 6–24 (ore even more) colonies (*see* **Note 11**) with sterile pipette tips, sterile toothpicks, or an inoculation loop into 3 mL LB-medium with ampicillin and incubate overnight at 37 °C on a gyratory shaker.
14. Prepare plasmid DNA using the Qiagen Plasmid Mini kit according to the manual.
15. Determine the DNA concentration by UV spectrophotometry at 260 nm.
16. Cleave 5–20 μL plasmid DNA with appropriate enzymes for 1–2 h in the recommended buffer to determine the correct orientation of the target ORF.
17. Separate cleavage products by 0.6 % agarose gel electrophoresis for 3 h at 4 V/cm and identify correct clones.
18. Transform chemically competent *E. coli* with 1 μL plasmid DNA from a correct clone and incubate in 50 mL LB with ampicillin overnight at 37 °C on a gyratory shaker.
19. Prepare plasmid DNA with the Qiagen Plasmid Midi Kit according to the manual. Determine the DNA concentration by UV spectrophotometry at 260 nm (1 OD_{260} corresponds to a DNA concentration of 50 μg/mL) and verify identity and purity of the preparation by cleavage of 0.5 μg plasmid DNA with an appropriate restriction enzyme for 2 h followed by 0.6 % agarose gel electrophoresis.
20. Add 1.5 μg of the appropriate, purified BacMam transfer plasmid to 100 μL chemically competent *E. coli* DH10Bac in an Eppendorf tube, mix thoroughly and leave for 20 min on ice. Transfer tube to 42 °C for 2 min and chill on ice for 5 min.

21. Add 900 µL SOC medium, incubate for 4 h at 37 °C on a gyratory shaker at 300 rpm.
22. Prepare a 10^{-3} dilution in SOC medium and incubate overnight at 37 °C 300 rpm.
23. Dilute overnight culture 10^{-3}, 10^{-4} and 10^{-5} in SOC medium (500 µL each) and incubate at 37 °C, 300 rpm for 2 h.
24. Prepare IPTG und X-Gal containing agar petri dishes.
25. Plate 200 µL of the 10^{-3}, 10^{-4} and 10^{-5} dilutions on the agar plates. Incubate plates at 37 °C for 24 h and leave at room temperature for another day.
26. Pick four to six "white" colonies into 3 mL LB-medium with kanamycin, gentamycin, tetracycline and incubate overnight at 37 °C on a gyratory shaker.
27. Centrifuge 1 mL overnight culture at $4500 \times g$ for 1 min.
28. Resuspend bacteria in pellet in 100 µL "solution 1", add 100 µL "solution 2" and mix. Add 150 µL "solution 3", mix thoroughly and incubate for 20–60 min on ice.
29. Centrifuge for 5 min at $20{,}000 \times g$ and transfer the supernatant into a new tube.
30. Add 1 mL –20 °C 100 % ethanol, mix thoroughly and incubate for 15 min at –80 °C.
31. Centrifuge for 10 min at $20{,}000 \times g$ and discard supernatant.
32. Wash DNA pellet with 1 mL 70 % ethanol. Centrifuge tube at $20{,}000 \times g$ for 5 min. Dry the pellet 10 min by incubating the open tube at 56 °C for about 10 min.
33. Resolve pellet in 40 µL TE-buffer with RNase A at 56 °C for 5 min followed by shaking at 37 °C on a gyratory shaker at 1400 rpm for 30 min.
34. Determine OD_{260} and OD_{280} and use the clone with the 260/280 ratio closest to 2.0 for rescue of virus.

3.3 Rescue of BacMam Viruses from Bacmid DNA

1. Seed 1×10^6 High five cells in 1 mL "Insect Express medium" into a well of a 6 well plate and let cells attach for 1 h at 27 °C.
2. Pipette 5 µL bacmid DNA to 95 µL sterile water and add 6 µL of Fugene® HD transfection reagent. Incubate for 40 min at room temperature.
3. Wash cultures once with cell culture medium and cover cells with 1 mL cell culture medium.
4. Add 900 µL cell culture medium to the transfection mixture, mix gently and add it drop wise to the cells. Incubate plate for 5 h at 27 °C, replace supernatant with 2 mL fresh cell culture medium and incubate further at 27 °C.

5. Monitor transfected High five culture daily for appearance of autofluorescing cells and/or foci. Under optimal conditions, single autofluorescing cells (*see* Fig. 2) become visible at about 24 h after transfection (*see* **Note 12**).

3.4 Plaque Purification of BacMam Viruses

The BAC system is based on the well-studied Escherichia coli F factor. Replication of the F factor in *E. coli* is strictly controlled. However, the F plasmid is maintained in one to four copies per cell [15]. In addition, recent reports have shown that BAC vector sequences can be spontaneously excised from bacmid-derived vectors upon passage in insect cells which appears to occur primarily when foreign protein expression is interfering with virus replication. In summary, plaque purification is indispensable to as far as possible ascertain obtainment of a homologous virus isolate.

1. When a substantial amount of the transfected cells autofluoresce (usually 3 days after transfection), transfer 150 μL transfected-cell culture supernatant into an Eppendorf tube and prepare 10^{-1} and 10^{-2} dilutions. Seed each 7.5×10^5 SF9 cells in 2 mL Grace's insect culture medium with 10 % FCS into the wells of a 6-well plate and let cells attach for 20–30 min. Inoculate 100 μL of each dilution directly into one separate well and incubate for 1–2 h at 27 °C.
2. For the agarose overlay liquefy 2 % low melting agarose in a microwave oven and cool in water bath to 45 °C. Equilibrate double concentrated Grace's insect culture medium with 20 % FCS to room temperature (*see* **Note 13**) and mix directly before use 1:1 with LMP agarose.
3. Remove supernatant from the infected SF9 cells, mix equal amounts of agarose and 2× medium and overlay the cells quick but gentle with 2.5 mL per well.
4. After solidification of the overlay (approximately 10 min at room temperature) incubate plate at 27 °C and check for appearance of autofluorescing plaques.
5. Usually 3 days later, circle discrete located autofluorescing plaques on the bottom of the plate by passing a marker pen (e.g., Edding 404 black) along the objective from below (*see* **Note 14**). Aspirate cells from individual plaques using for example a Gilson P1000 (volume to 50 μL) and flush into a reaction tube with 1 mL SF9 medium. Shake for 30 min or freeze/thaw to release virions from picked infected cells.
6. Seed 1.25×10^6 SF9 cells in 4 mL medium into a 25 cm^2 tissue culture bottle and add the 1 mL plaque isolate. Incubate for 5–7 days at 27 °C until complete CPE.
7. For titer determination dilute BacMam preparations from 10^{-1} to 10^{-8} and transfer 100 μL of each dilution into the wells of a 96-well plate. Prepare four to six parallel columns and add about 1.4×10^4 SF9 cells in 25 μL Grace's insect culture

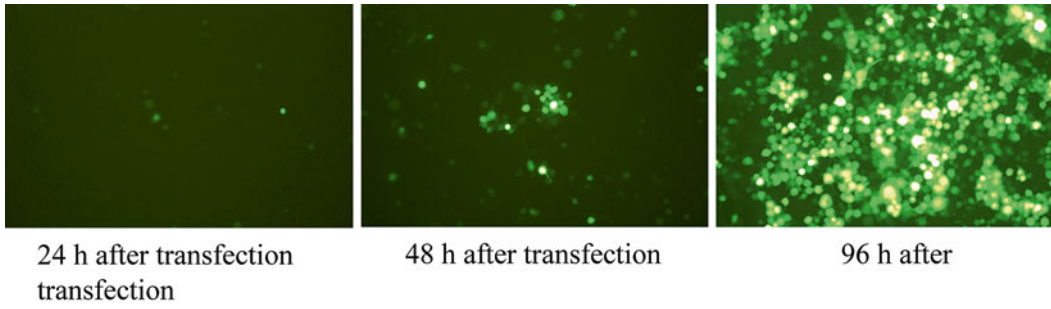

Fig. 2 Dissemination of autofluorescing cells in a High Five cell culture transfected with bacmid DNA indicates generation of BacMam viruses containing the sequences to be transferred. Cells were photographed using a Nikon fluorescence microscope and the FITC filter set

medium to the virus dilutions. Incubate for 3–5 days at 27 °C, determine wells with autofluorescent cells and calculate the $TCID_{50}$ using the Spearman and Kärber [16] algorithm.

3.5 Verification of Target Protein Expression in Vertebrate Cells

Verification of target protein expression by the individual BacMam plaque isolates should be performed prior to large scale propagation and immunization of animals. This is can be done by transduction of widely used cell lines like HEK 293T, Vero, BHK, Hela, and others reported to be efficiently transduced (reviewed by ref. [8]) followed by antigen detection using Western blotting, indirect immunofluorescence, ELISA, etc. Optimal transduction conditions need to be determined for each cell line but the following protocol should as a first approximation be applicable for most mammalian cell lines (*see* also Fig. 3)

1. Seed cells in desired plate format in a density that the cultures are confluent 24 h later (*see* **Note 15**).
2. Remove culture medium and wash cells twice with PBS^+ (*see* **Note 16**). Add 25 $TCID_{50}$ BacMam viruses per cell in PBS. Final volumes should be 2 mL per well in 6 well plates and accordingly less for other plate formats.
3. Centrifuge plates at 600 × *g* for 1 h at 27 °C. If a centrifuge with a plate rotor is not available incubate cultures for 4–6 h on a gyratory shaker with low speed agitation at 25–27 °C.
4. Replace inoculum with normal cell culture medium supplemented with 5 mM Na-butyrate [13] and incubate cultures for 24–48 h under normal conditions (*see* **Note 17**).
5. Remove medium, wash monolayers with PBS twice and process cells according to the envisaged detection method.

3.6 Large Scale Production and Concentration of BacMam Virions for In Vivo Experimentation

1. Infect 10^7 detached SF9 cells in 35 mL culture medium at a multiplicity of infection of 0.1 and seed into cell culture flasks with 150–162.5 cm growth area. Incubate at 27 °C until CPE is complete (usually 6–8 days).

2. Transfer contents of the flasks into 50 mL conical tubes and centrifuge at 4000 × *g* for 20 min. If applicable, pool supernatants from multiple tubes, remove an aliquot for titration and overlay a 7.5 mL 25 % sucrose cushion in PBS^-, filled into Beckman SW32 rotor ultracentrifuge tubes, with 25 mL of the infected culture supernatant. Centrifuge for 90 min at 25,000 rpm and 4 °C.
3. Aspirate carefully first the culture medium and then the sucrose cushion. Add 1 mL PBS+ to the pellets, seal tubes with Parafilm and leave overnight on ice.
4. Resuspend pellets and homogenize carefully using a Dounce homogenizer. Ten strokes should be sufficient. Store in aliquots at −70 °C.
5. Thaw an aliquot, sonicate for 5 s in an ultrasonic water bath at 40 W (*see* **Note 18**). Titrate together with sample taken prior ultracentrifugation and calculate recovery rate which normally is around 50 %.

3.7 In Vivo Transduction

Suitability of BacMam viruses as delivery vector for vaccines has been demonstrated, e.g., for mice [12], chicken [17] or pigs [10]. The doses for vaccination varied between 10^7 infectious units for pigs, given three times intra muscular (i.m.) in 15 days intervals and 10^9 infectious units for mice and chicken, immunized i.m. twice in a 3 week interval.

Since protocols for vaccination/challenge experiments depend markedly on specifics of the disease, of the target animal and of the laboratory, a general instruction appears not reasonable (*see* **Note 19**).

In the example for a vaccination/challenge experiment depicted below, rabbits were immunized i.m. with 5×10^8 PFU BacMam/VP60 (*see* also Fig. 4) at days 0, 7, and 12, and challenged with a lethal dose RHDV on day 42. All vaccinated animals developed VP60-specific serum antibodies and survived the challenge infection—demonstrating the potential of BacMam viruses as safe vaccine delivery vector.

4 Notes

1. Ethidium bromide powder is highly toxic when inhaled. Ethidium bromide is suspected to be a mutagen. Thus, solutions should be prepared under a fume hood and regarded as hazardous. Wear gloves while handling.
2. Short-wave UV light is hazardous for your eyes. Wear safety goggles or a face shield when examining or cutting out DNA fragments on a transilluminator to prevent damage to the eyes.

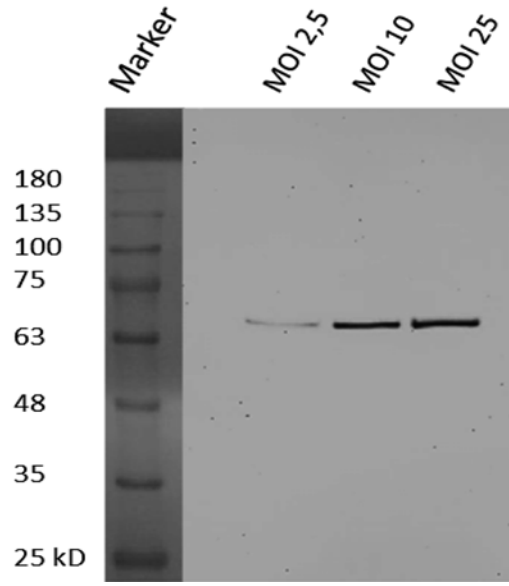

Fig. 3 Determination of BacMam transduction efficacy. Rabbit kidney cells (RK13) were transduced with BacMam/RHDV_VP60 expressing the rabbit hemorrhagic disease virus protein 60 (VP60) at the indicated MOI. Cells were harvested 24 h after transduction, lysed and proteins were transferred to nitrocellulose membranes after size separation by SDS-10 % PAGE. VP60 expression was visualized by monitoring chemiluminescence using a polyclonal rabbit serum against VP60, peroxidase labeled a-rabbit IgG serum and the Supersignal West Pico chemoluminescent kit (Pierce, Rockford, Il.) as recommended by the supplier

3. Be sure that the PBS^{+} contains calcium and magnesium. These are frequently absent in commercially available PBS solutions. Do not autoclave. Insoluble Ca^{2+} complexes may form.
4. Do not use Erlenmeyer flasks to boil agarose gels because bumping may lead to superheating may lead to sputtering of the agarose solution due to a chimney effect. Using glass beakers is safer.
5. Air bubbles on the gel surface are mainly an aesthetical problem. They can be removed with the flame of a pocket lighter or a Bunsen burner or punching with a toothpick, pipette tip, etc.
6. Electrophoresis time depends on the DNA fragment sizes (VP60) of the sample. Easy distinguishable DNA fragments require shorter, complex fragment compositions longer running times.
7. Unless otherwise noted, centrifugation of 1.5 mL Eppendorf tubes means centrifugation at about 16,000–20,000 × *g*.
8. Use 1 U T4 ligase for blunt end ligations.
9. We prefer incubation of plasmid ligations overnight at 4 °C instead of the frequently used overnight incubation at 16 °C.

10. Incubation of plates overnight at 37 °C may result in large ampicillin resistant colonies which exhaust the antibiotic and enable growth of non-transformed bacteria.
11. The number of clones to be analyzed depends on the number of colonies grown on control plates and on target plates. If for example the latter contain 30 colonies versus 5 colonies on the controls, it is theoretically sufficient to test 6 clones for presence of an insert in correct orientation.
12. In cases in which the low level expression of the target protein in insect cells [13] interferes with replication of the BacMam virus, appearance of autofluorescing cells may be delayed. However, if no autofluorescing cells appear after 3 days, at least bacmid preparation and transfection should be repeated. Although we do it not routinely, it may be worthwhile to include the empty vector as a positive process control.

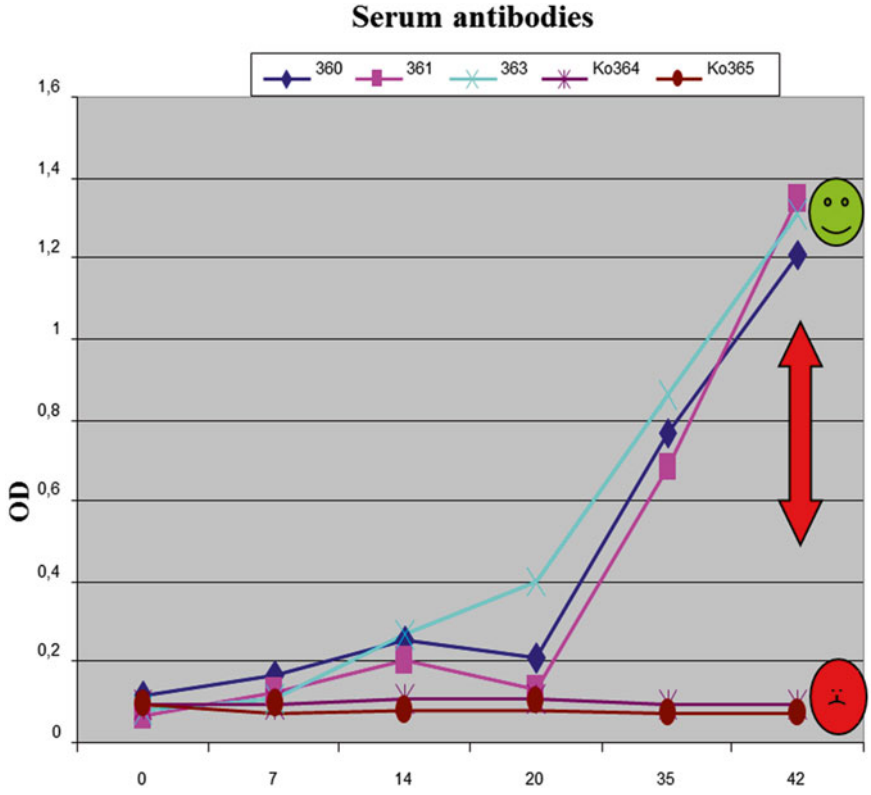

Fig. 4 Protection of rabbits against RHDV by BacMam virus vaccination. Rabbits were vaccinated i.m. with 5×10^8 PFU BacMam/VP60 at days 0, 7, and 12 (#360, #361, and #363) or left unvaccinated (#364 and #365) and challenged with a lethal dose RHDV on day 42 (*red double arrow*). Animals were bled at the indicated days and VP60-specific antibodies were quantified using an in-house indirect ELISA [18]. After challenge, all vaccinated rabbits survived (*green emoticon*), whereas the non-vaccinated animals died within 2–3 days

13. The given water bath temperature is valid if the room temperature is about 20 °C and must be reduced at significantly higher room temperatures to avoid heat damage of the SF9 culture.
14. To see the tip of the marker pen it is necessary to turn on visible light slightly.
15. We use 6-well plates if Western Blotting or antigen-ELISA is envisaged and 24-well plates for immunofluorescence.
16. It is essential to use PBS with calcium and magnesium. Otherwise most cell types will detach during the following incubation.
17. For some cells butyrate is toxic after prolonged incubation. Butyrate containing medium should then be replaced by normal cell culture medium after first signs of cytopathicity.
18. Concentrated baculovirus preparations tend to form aggregates which can be dissipated by sonication. Longer treatment times, however, reduce biologic activity.
19. If an adjuvant is foreseen to be used, its effect on transduction efficacy needs to be clarified. We observed total inactivation of BacMams by products recommended for enveloped virus-containing live vaccines.

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