# Chapter 9

# Generation of Recombinant Modified Vaccinia Virus Ankara Encoding VP2, NS1, and VP7 Proteins of Bluetongue Virus

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## Abstract

Modified Vaccinia Virus Ankara (MVA) is employed widely as an experimental vaccine vector for its lack of replication in mammalian cells and high expression level of foreign/heterologous genes. Recombinant MVAs (rMVAs) are used as platforms for protein production as well as vectors to generate vaccines against a high number of infectious diseases and other pathologies. The portrait of the virus combines desirable elements such as high-level biological safety, the ability to activate appropriate innate immune mediators upon vaccination, and the capacity to deliver substantial amounts of heterologous antigens. Recombinant MVAs encoding proteins of bluetongue virus (BTV), an Orbivirus that infects domestic and wild ruminants transmitted by biting midges of the *Culicoides* species, are excellent vaccine candidates against this virus. In this chapter we describe the methods for the generation of rMVAs encoding VP2, NS1, and VP7 proteins of bluetongue virus as a model example for orbiviruses. The protocols included cover the cloning of VP2, NS1, and VP7 BTV-4 genes in a transfer plasmid, the construction of recombinant MVAs, the titration of virus working stocks and the protein expression analysis by immunofluorescence and radiolabeling of rMVA infected cells as well as virus purification.

**Key words** Recombinant modified vaccinia virus Ankara, Bluetongue virus, Viral-vectored vaccine, VP2, NS1 and VP7 proteins

## 1 Introduction

Vaccinia viruses engineered to express foreign genes are powerful vectors for production of recombinant proteins [1]. Modified vaccinia virus Ankara (MVA) was obtained from the Chorioallantois vaccinia virus Ankara (CVA) and was isolated following more than 500 passages in chick embryo fibroblasts. After this extensive propagation the viral genome suffered several major deletions and numerous small mutations resulting in replication defects in human and most other mammalian cells, as well as severe attenuation of pathogenicity [2–4]. This is why these viral vectors have shown

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excellent safety profiles (the vector can be used at biosafety level 1), significant immunogenicity against foreign expressed antigens and ability to induce protective immune responses [5]. Poxviruses can accommodate large fragments of foreign DNA and their replication occurs within the cytoplasm of infected cells, eliminating the risk of virus persistence and genomic integration in host DNA [3]. MVA has intrinsic adjuvant capacities ant it is being widely investigated as a safe smallpox vaccine and as an expression vector to produce vaccines against other infectious diseases and cancer [4]. Recombinant MVA (rMVA) expressing immunogenic viral proteins has been shown to induce both humoral and cell mediated immunity [1, 6].

Poxviruses have the ability to induce the expression of type-I and II interferons and to express soluble receptors capable of interacting with host antiviral mechanisms. This antagonist expression is minimized owe to the deletions in the rMVA genome, which contributes to the immunogenicity of this viral-vector used as a vaccine. Type-I interferons may act as a link between the innate and adaptive immune system, including humoral and cellular responses [7, 8]. MVA has been used to construct many vectored vaccines expressing different proteins from different kind of orbiviruses [3]. The transfer plasmid pSC11 [9] was designed to place the genes of interest (in our case from *bluetongue virus*) under the control of the vaccinia virus (VV) early/late promoter p7.5. Finally, rMVAs were generated after homologous recombination in permissive cells between the TK gene sequences of pSC11 and those of wild type MVA. In our laboratory, all of these recombinant vectors have been tested as potential vaccines in IFNAR$^{(-/-)}$ mice [10–15]. We engineered rMVAs expressing VP2, NS1, and VP7 proteins from BTV-4. IFNAR$^{(-/-)}$ mice were inoculated with DNA-VP2,-NS1,-VP7/rMVA-VP2,-NS1,-VP7 in an heterologous prime boost vaccination strategy, generating significant levels of antibodies specific of VP2, NS1, and VP7, including those with neutralizing activity against BTV-4. The vaccine combination expressing VP2, NS1, and VP7 proteins of BTV-4, elicited sterile protection against a lethal dose of homologous BTV-4 infection and induced cross-protection against lethal doses of heterologous BTV-8 and BTV-1, suggesting that the DNA/rMVA-VP2,-NS1,-VP7 marker vaccine is a promising multiserotype vaccine candidate against BTV [14].

This work details the methodology applied to generate the rMVAs encoding the proteins VP2, VP7, and NS1 of BTV-4. In addition, the chapter describes the protocols to analyze the BTV protein expression in DF-1 cells infected with these rMVAs by immunofluorescence assay and radiolabeling, immunoprecipitation and SDS-PAGE.

## 2 Materials

1. BTV serotype 4 (SPA2004/01).
2. Modified vaccinia virus Ankara (MVA) (generously provided by Professor Francisco Rodríguez, Centro Nacional de Biotecnología, CSIC, Madrid, Spain).
3. Kidney epithelial cells extracted from an African green monkey, *Chlorocebus* sp. (Vero cells) (ATCC, Cat. No. CCL-81).
4. Chicken embryo fibroblast (DF-1 cells) (ATCC, Cat. No. CRL-12203).
5. Serum and antibiotic free Dulbecco's modified Eagle's medium.
6. DMEM with 2 mM glutamine, 10 % fetal calf serum (FCS), and 1 % Penicillin/Streptomycin (complete DMEM).
7. TRI Reagent Solution (Ambion).
8. 10× RT buffer, 25 mM $MgCl_2$, 0.1 M DTT (Life Technologies).
9. SuperScript® III Reverse Transcriptase (200 U/μL) (Life Technologies).
10. RNaseOUT™ (40 U/μL) (Life Technologies).
11. 10× PCR Buffer II, 10 mM dNTPs, specific primer (VS and RS) (Table 1) (Life Technologies).
12. AmpliTaq DNA Polymerase (1.25 U/50 μL) (Life Technologies).
13. 1 % agarose gel (Tris–acetate–EDTA buffer (TAE) and 1 % agarose).
14. Midori green DNA stain (Nippon Genetics Europe GmbH).

**Table 1**

**Primer used for amplification of BTV genes**

| Gene | | Sequence |
|---|---|---|
| VP2 | VS | 5′-CGCCCGGGATGAACTAGGCATCCCAG-3′ |
| | RS | 5′-CGCCCGGGCATACGTTGAGAAGTTTTGTTA-3′ |
| NS1 | VS | 5′-CGCCCGGGATGGAGCGCTTTTTGAGAAAATAC-3′ |
| | RS | 5′-CGCCCGGGCTAATACTCCATCCACATCTG-3′ |
| VP5 | VS | 5′-CGCCCGGGATGGGTAAAGTCATACGATC-3′ |
| | RS | 5′-CGCCCGGGTCAAGCATTTCGTAAGAAGAG-3′ |
| VP7 | VS | 5′-CGCCCGGGATGGACACTATCGTCGCAAG-3′ |
| | RS | 5′-CGCCCGGGCTACACATAGCGCGCGCGTGC-3′ |

*Sma*I restriction site *underlined*

15. Qiaex II Gel Extraction Kit (Qiagen).
16. pSC11 plasmid (kindly provided by Professor Francisco Rodríguez, Centro Nacional de Biotecnología, CSIC, Madrid, Spain).
17. *Sma*I restriction endonuclease, shrimp alkaline phosphatase (SAP), and T4 ligase enzyme.
18. Luria-Bertani (LB) agar plates and media.
19. Ampicillin sodium salt.
20. QIAprep® Spin Miniprep Kit (Qiagen).
21. Lipofectamine® Reagent (Invitrogen).
22. Noble agar (Difco Noble Agar (DB) and distilled water).
23. X-Gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside).
24. Complete DMEM-0.6 % Noble agar with X-Gal (0.4 μg/μL) (complete DMEM–agar–X-Gal).
25. Formaldehyde 10 %.
26. Crystal violet in 80 % methanol.
27. Acetone–methanol solution (40 %/60 %).
28. Phosphate buffered saline 1× (PBS 1×).
29. PBS 1× 20 % FCS (blocking solution).
30. Mouse polyclonal antibody against BTV-4.
31. Alexa Fluor® 594 goat anti-mouse IgG (H + L) (Invitrogen).
32. ProLong Gold antifade reagent (Life Technologies).
33. RIPA buffer: 50 mM Tris–HCl pH 7.4, 300 mM NaCl, 0.5 % sodium deoxycholate, 1 % Triton X-100, protease inhibitors.
34. Methionine-free DMEM cell culture medium.
35. [$^{35}$S] Methionine (800 Ci/mmol). (Amersham).
36. Dynabeads® Protein G system (Life Technologies).
37. SDS-PAGE buffer: 0.125 M Tris–HCl, 4 % SDS, 20 % v/v glycerol, 0.2 M DTT, 0.02 % bromophenol blue, pH 6.8.
38. 36 % sucrose cushion and sucrose gradient.
39. SW 28 centrifuge tube (50 mL).

## 3 Methods

These methods describe the generation of the recombinants MVAs encoding BTV-4 VP2, VP7 and NS1 proteins, the screening of positive recombinants, the upgrowth and quantification of virus stock, the analysis of BTV protein expression by immunofluorescence assay and radiolabeling, immunoprecipitation and SDS-PAGE in

infected DF-1 cells as well as the purification of the rMVAs generated in DF-1 cells.

### 3.1 Cloning of VP2, NS1, and VP7 BTV-4 Genes for Generation of Recombinant MVAs

Segments 2, 5, and 7 corresponding to VP2, NS1, and VP7 proteins will be amplified from total RNA of BTV-4 infected cells. To generate the MVA transfer plasmids pSC11-VP2, pSC11-NS1, and pSC11-VP7, the restriction site *Sma*I, must be introduced into the 5′ and 3′ ends of the PCR products, unique restriction site contained into pSC11.

1. Infect confluent Vero monolayers in M24-well plates ($1.67 \times 10^4$ cells/well) with BTV serotype 4 (BTV-4) with a multiplicity of infection (MOI) of 1.
2. After virus adsorption for 1.5 h at 37 °C, 5 % $CO_2$, remove the medium, add 1 mL of complete DMEM and incubate for 24 h at 37 °C.
3. At 24 h post infection (h.p.i), when a clear cytopathic effect is observed, remove the supernatant and extract total RNA from infected cells with TRI Reagent Solution, according to the method recommended by the manufacturer (*see* **Note 1**).
4. The recovered RNA can be stored at −80 °C in small aliquots for later processing. RNA stored at this temperature is stable for prolonged periods of time (over 1 year).
5. Denature 5 μg of RNA in presence of 1 μL of 2 μM Reverse Sense (RS) BTV gene-specific primer (Table 1), 1 μL of 10 mM dNTP mix in a final volume of 10 μL by heating to 65 °C for 5 min and then rapidly cool on ice.
6. Add 2 μL 10× RT buffer, 4 μL 25 mM $MgCl_2$, 2 μL of 0.1 M DTT, 1 μL RNaseOUT™ (40 U/μL), and 1 μL SuperScript® III Reverse Transcriptase (200 U/μL).
7. Incubate the reaction for 1 h at 50 °C. Then, inactivate the reverse transcriptase by heating at 85 °C for 5 min. Chill on ice.
8. The cDNA produced is stable at 4 °C for short term storage, −20 °C for prolonged storage or used for Polymerase Chain Reaction (PCR) immediately.
9. Amplify the VP2, NS1, and VP7 cDNAs by PCR. Use 10 μL of 10× PCR Buffer II, 2 μL of 10 mM dNTPs, 2 μL of each specific primer (VS and RS) including *Sma*I site (Table 1), 4 μL of 25 mM $MgCl_2$ solution, 0.6 μL of AmpliTaq DNA Polymerase (1.25 U/50 μL), and 5 μL of cDNA template in a final volume of 100 μL.
10. Amplification cycle parameters are: 94 °C for 2 min (1×); 94 °C for 45 s, 55 °C for 1 min, and 72 °C for 2 min (30×); 94 °C for 15 min (1×).

11. Analyze the PCR products on a 1 % agarose gel stained with Midori green DNA stain (or other intercalating dye) and purify the PCR products with Qiaex II Gel Extraction Kit.
12. Digest the plasmid pSC11 and the purified PCR products VP2, NS1, and VP7 (containing the restriction site *Sma*I into the 5′ and 3′ ends) with the restriction enzyme *Sma*I as per manufacturer instructions.
13. Proceed to dephosphorylation of digested pSC11 with shrimp alkaline phosphatase (SAP) according to the method recommended by the manufacturer in order to prevent the plasmid self-ligation.
14. Purify the digested PCR products and the digested and dephosphorylated plasmid with Qiaex II Gel Extraction Kit.
15. Ligate the purified digested VP2, NS1, and VP7 with the purified digested plasmid pSC11 with T4 ligase enzyme according to the manufacturer's instructions. Perform the ligation at 16 °C overnight with a molar ratio of vector to insert of 1:3.
16. Transform the ligation products into chemically competent DH10B bacterial cells and plate out the transformants on LB agar with selection in the presence of ampicillin (100 μg/mL).
17. The next day select single colonies and grow in LB with ampicillin. Isolate the plasmid following the QIAprep Miniprep Handbook and analyze the presence and right orientation of the cloned VP2, NS1, and VP7 genes into the transfer plasmid pSC11 by sequencing (*see* **Note 2**).

### 3.2 Construction of Recombinant MVAs

The next step of the procedure is the generation of recombinant MVAs (*see* Fig. 1). The MVA transfer plasmids pSC11-VP2, pSC11-NS1, and pSC11-VP7 should contain the VP2, NS1, and VP7 BTV genes, flanked by thymidine kinase (TK) sequences of MVA, under the control of the vaccinia virus (VV) early/late promoter p7.5. Infection of cells with MVA and subsequent transfection with pSC-11 plasmids will generate recombinant viruses

#### 3.2.1 Infection/Transfection of DF-1 Cells with MVA wt and pSC11 Plasmid Respectively

1. Plate DF-1 cells in p35 or six-well plates 1 day prior to infection in a 2 mL volume of complete DMEM.
2. DF-1 cells that are 60–80 % confluence are needed for infection and transfection.
3. Add 100 μL of MVA wild type (wt) in serum and antibiotic-free DMEM at 0.1 or 1 of MOI.
4. Incubate the cells at 37 °C and air–5 % $CO_2$ atmosphere for 1.5 h. After virus adsorption, DF-1 infected cells are transfected with pSC11-VP2, pSC11-NS1 or pSC11-VP7.
5. Mix 2 μg of plasmid in 50 μL of serum and antibiotic-free DMEM. Add to this mixture 9 μL of Lipofectamine® reagent

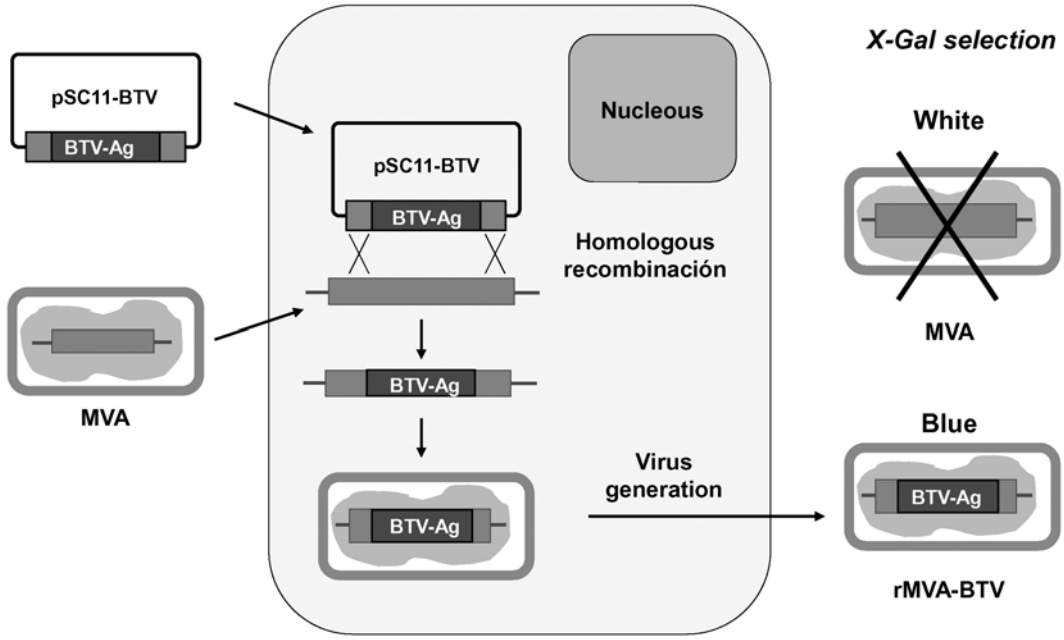


**Fig. 1** General procedure for the generation of recombinant MVA. Genes VP2, NS1, and VP7 of BTV-4 were cloned into the vaccinia transfer plasmid pSC11 downstream of the p7.5 vaccinia promoter. DF-1 cells were infected with MVA virus (MOI 0.01 p.f.u./cell). After adsorption, cells were transfected with pSC11-VP2, pSC11-NS1, or pSC11-VP7 plasmids. Recombinant MVA viruses were generated by homologous recombination at the Thymidine kinase locus, allowing the analysis by using the LacZ marker

in 250 μL of serum and antibiotic-free DMEM and incubate at room temperature for 30 min.

6. Add 0.7 mL of serum and antibiotic-free DMEM to the lipid–plasmid complex. Add the total volume (≈1 mL) to the cells.
7. Incubate at 37 °C and air–5 % $CO_2$ atmosphere for 5 h, shaking the plate every 30 min.
8. Remove the lipid–plasmid complex and add 1 mL of complete DMEM.
9. Incubate at 37 °C and air–5 % $CO_2$ atmosphere for 72 h.
10. When the cytopathic effect (CPE) is apparent, harvest cells and supernatants by disruption of the monolayer (*see* **Note 3**). Carry out three cycles of thawing–freezing and sonicate twice for 10 s to disrupt the cells and release viruses.
11. Centrifuge at 2500 × *g* for 1 min. The supernatant will be used for the plaque analysis to look for recombinant MVAs as described below.

### *3.2.2 Plaque Purification of Recombinant MVA Viruses*

In this step, we try to find clear, well-separated plaques for isolating and screening cloned viruses (*see* Fig. 2).

1. Plate DF-1 cells in six-well plates and incubate until they reach 80 % confluence.

2. Use the supernatants from the infected-transfected cells to do tenfold dilutions on the DF-1 cells from undiluted to $10^{-7}$ (*see* **Note 4**).
3. Allow the viruses to adsorb at 37 °C for 1 h.
4. Aspirate the supernatants and add 1.5 mL of complete DMEM.
5. Incubate at 37 °C and air–5 % $CO_2$ atmosphere for 72 h.

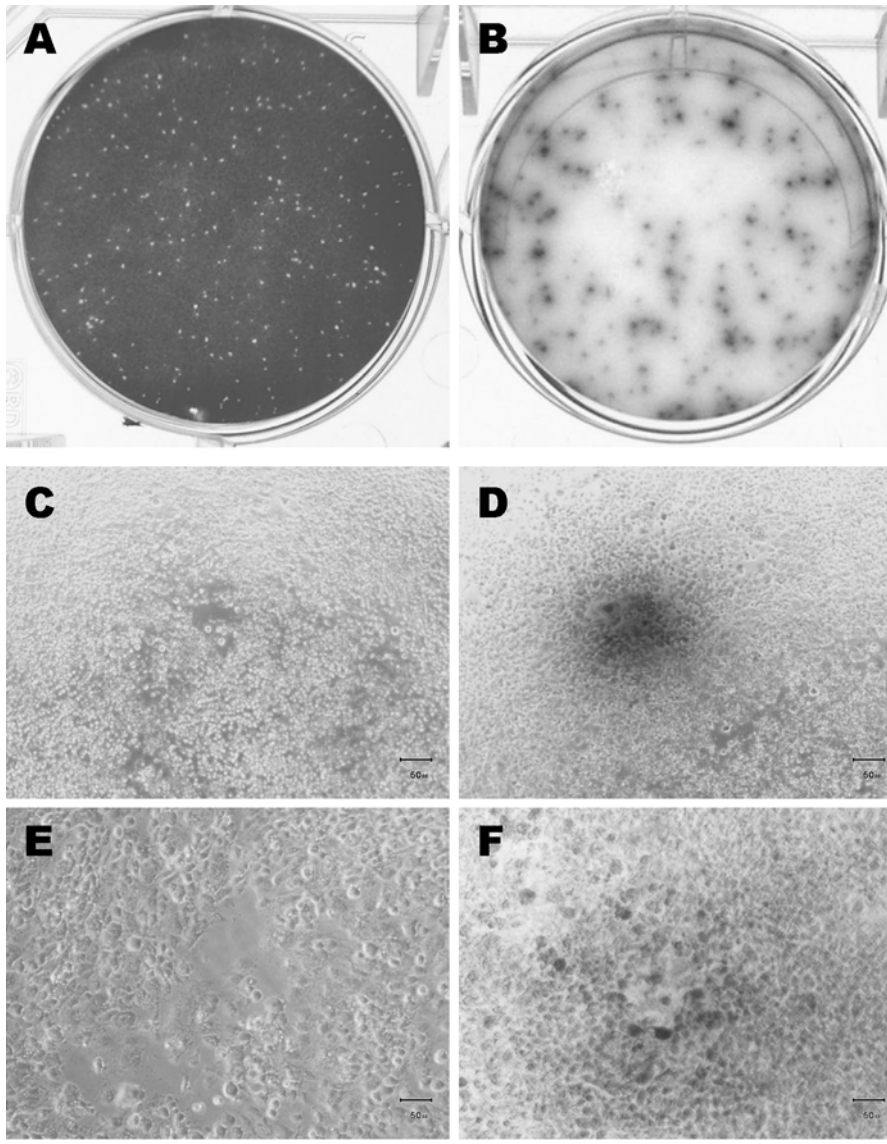


**Fig. 2** Plaque formation of rMVA-VP2 and wild-type MVA in avian DF-1 cells. DF-1 cells were infected with 100 pfu/well of rMVA-VP2 (**b**, **d**, and **f**) or MVA-wt (**c** and **e**). 72 h post infection, titration was performed (**a**) or DMEM-0.6 % and Noble agar with X-Gal was added over the monolayer (**b**–**f**). *White plaques*: MVA-wt; *Dark plaques*: rMVA-VP2

6. Remove the medium and add complete DMEM–agar–X-Gal (*see* **Note 5**) over the monolayer.
7. Allow the overlay to solidify.
8. Incubate at 37 °C for 8 h.
9. Pick only well-separated blue plaques, about six per clone (*see* **Note 6**). To pick the plaques, insert the tip of the micropipette into the agar overlay just over the plaque, and draw the agar plug into the pipet. Transfer it into a small tube with 0.5 mL of complete DMEM and pipette up and down a few times to ensure that the plug does not remain in the pipette tip.
10. Carry out three cycles of thawing–freezing and sonication.
11. Repeat this cloning procedure (**steps 9** and **10**) about six times with each clone.
12. Amplify the cloned plaques using DF-1 cells.

### 3.3 Preparation and Titration of Virus Working Stocks

1. Passage DF-1 cells in 175 $cm^2$ flasks such they are confluent in 1 or 2 days.
2. Remove the old medium, add fresh medium and inoculate 0.1 MOI (*see* **Note 7**) of seed virus stock and Incubate DF-1 cells at 37 °C for 2–3 days until all cells show clear CPE; often, most cells will be floating.
3. Remove part of the medium (*see* **Note 8**), detach the cell monolayer and disrupt the cells with three cycles of thawing–freezing at –80 °C, transferring the medium and the disrupted cells to a new tube.
4. Sonicate in water bath the tube twice for 10 s, aliquot the rMVAs working stocks in volumes suitable for your purposes. We usually prepare aliquots of 1 mL each. Store at –80 °C.
5. For plaque assays of stocks prepare DF-1 cells in six-well plates 1 or 2 days prior to virus titration.
6. Thaw virus in 37 °C water bath, and make tenfold dilutions of the stock in complete DMEM. Each dilution must be mixed carefully and pipet tips changed between tubes (*see* **Note 4**). Transfer 100 μL of each dilution in each well.
7. Adsorb virus at 37 °C for 1.5 h, gently tilting back and forth every 15–20 min.
8. Aspirate the inoculum from higher to lower dilution wells, add 1.5 mL of complete DMEM from higher to lower dilution wells, and incubate for 3 days at 37 °C and air–5 % $CO_2$ atmosphere.
9. Fix the plates with 1 mL of 10 % formaldehyde for 30 min. Remove the medium and add 1 % crystal violet to stain the monolayers and count plaque numbers to calculate the virus titers (*see* **Note 9**).

### 3.4 Protein Expression Analysis

There are various methods to analyze the expression of the BTV proteins in DF-1 cells infected with the generated rMVAs. The two most common methods used in our laboratory are the immunofluorescence assay (*see* Fig. 3) and the immunoprecipitation of proteins in radiolabeled infected cells (*see* Fig. 4).

#### 3.4.1 Immunofluorescence Assay

1. Plate DF-1 cells in 24-well plates with coverslips and incubate until they reach 80 % confluence.
2. Infect these cells with the rMVAs at an MOI of 1.
3. After 24 h of infection, fix the infected cells with acetone–methanol and store the plate at −20 °C for 20 min.
4. Remove acetone–methanol and wash once with 1 mL of PBS 1× (*see* **Note 10**).
5. Incubate the fixed cells with 1 mL blocking solution for 1 h.

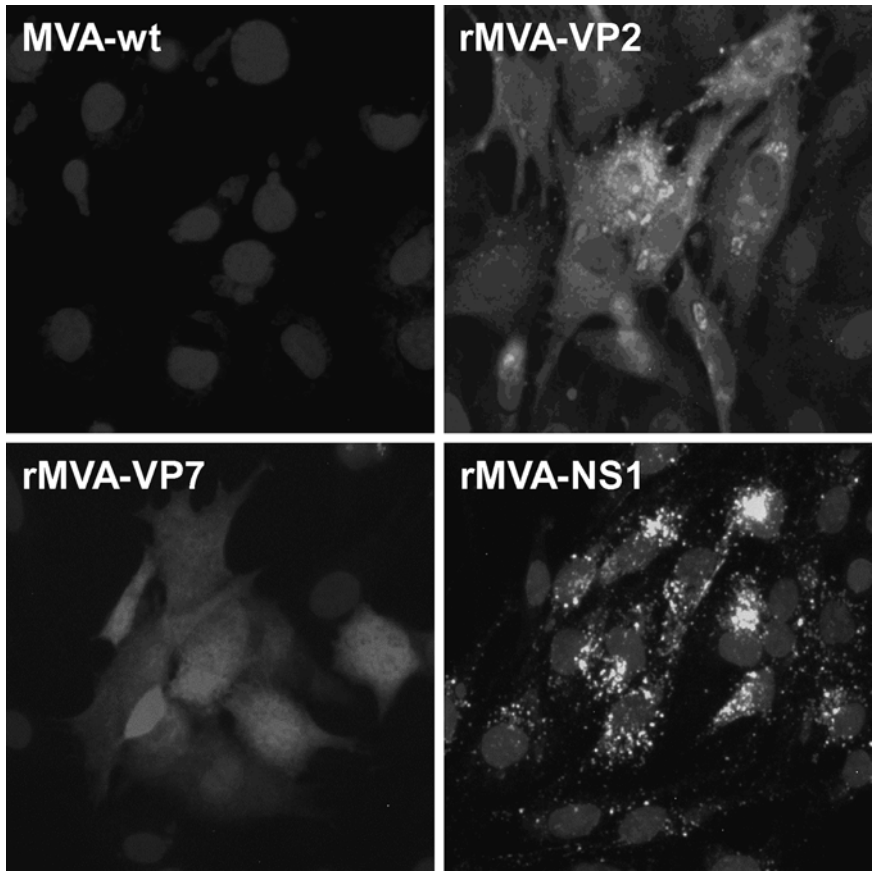


**Fig. 3** Analysis of BTV-4 VP2, NS1, and VP7 expression by immunofluorescent staining. DF-1 cells were infected with recombinant MVA containing BTV-4 VP2, NS1, or VP7 genes. At 24 h.p.i, cells were fixed and analyzed by immunofluorescence by using a mouse polyclonal antisera specific of BTV-4

6. Remove the blocking solution and add the primary antibody. We use a mouse polyclonal antibody against BTV-4 diluted 1:500 or 1:1000 in blocking solution (it depends on each stock of sera) (*see* **Note 11**). We usually use 250 μL/well. Incubate at 4 °C o/n or room temperature for 3 h.
7. Remove the polyclonal antibody and wash with PBS 1× three times for 10 min preferably with shaking.
8. Add the conjugated secondary antibody (Alexa Fluor® 594 goat anti-mouse IgG (H + L)) specific of mouse primary antibody diluted 1:1000 in blocking solution over the cells. Incubate for 30 min at room temperature in dark conditions.
9. Remove the secondary antibody and wash with PBS 1× three times for 10 min with shaking preferably.
10. Mount the coverslips on slides using ProLong Gold antifade reagent and visualize using an immunofluorescence microscope.

### *3.4.2 Analysis of BTV Proteins Expression by Radiolabeling, Immunoprecipitation, and SDS-PAGE*

Radiolabeling followed by immunoprecipitation is useful to analyze the expression of BTV proteins. VP2 protein contains conformational epitopes and polyclonal antibodies specific of BTV are not able to recognize the denatured protein by immunoblot. VP2, NS1, and VP7 can be immunoprecipitated with BTV-specific polyclonal antibodies from either BTV or MVA-VP2, MVA-NS1, and MVA-VP7 infected cells (*see* Fig. 4).

1. Infect DF-1 cells in 35-mm dishes with MVA-VP2, MVA-NS1, or MVA-VP7 at an MOI of 1.
2. After 90 min of virus adsorption, remove the culture medium and rise the cell monolayers with PBS 1× and once with methionine-deficient medium.
3. Add 1.5 mL of fresh methionine-deficient medium and incubate for 60 min (to starve of methionine).
4. At the end of starvation period, replace the medium and add medium containing [$^{35}$S] methionine (100 μCi/mL).
5. Incubate the cells for 16 h at 37 °C.
6. At the end of the incubation, remove the labeling medium and rinse the cells twice with PBS 1×.
7. Add 300 μL of RIPA buffer to each dish.
8. Leave the dishes on ice for 10 min.
9. Harvest the cell lysate to microfuge tubes. Vortex for 5 s and incubate on ice for another 10 min.
10. Centrifuge the tubes for 10 min at 8,050 × *g* to remove the cell debris and nuclei.
11. Transfer the supernatant to new microfuge tube and stand on ice or store at −20 °C.

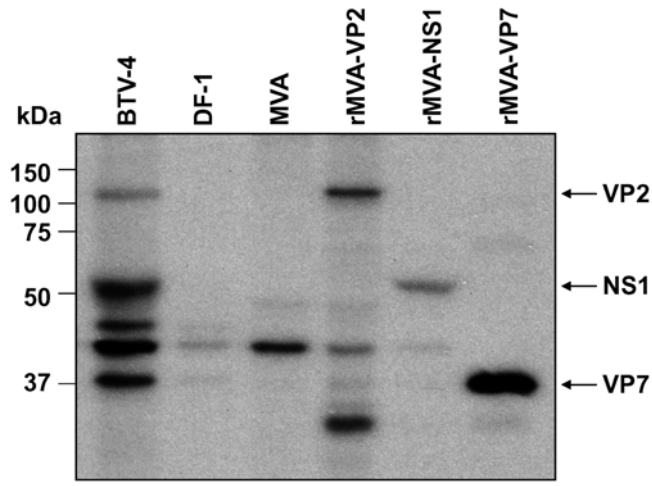


**Fig. 4** Analysis of BTV protein expression by radiolabeling, immunoprecipitation, and SDS-PAGE. [$^{35}$S] methionine-labeled BTV proteins were isolated by immunoprecipitation using polyclonal antibody specific of BTV-4. The expression of BTV proteins in DF-1 cells infected with rMVA-VP2, rMVA-NS1, or rMVA-VP7 was then analyzed by SDS-PAGE

12. Immunoprecipitate BTV proteins with 10 μL of mouse polyclonal antibody specific of BTV-4 by using the Dynabeads® Protein G system and according to the protocol recommended by the manufacturer.
13. After the immunoprecipitation process, boil the beads in SDS-PAGE buffer for direct characterization of proteins on SDS-PAGE.

### *3.5 Purification of rMVAs by Using a Sucrose Gradient*

There are various forms to purify and to separate viruses. Sucrose gradient is frequently used for separating virus, and the use of a sucrose cushion allows the possibility to concentrate the virus.

1. Layer 19 mL of the sonicated lysate onto 19 mL of a 36 % sucrose cushion (in PBS) in a sterile SW 28 (or SW 27) centrifuge tube (50 mL). Centrifuge for 90 min at 30,000 × *g* (SW 28 rotor) at 4 °C. Aspirate and discard the supernatant.
2. Resuspend the viral pellet in 0.5 mL of PBS 1× for a T150 flask (*see* **Note 12**).
3. Sonicate once for 1 min, and prepare a sterile 24–40 % continuous sucrose gradient in a sterile SW 27 centrifuge tube the day before it is needed by carefully layering 6.8 mL each of 40, 36, 32, 28, and 24 % sucrose. Let it sit overnight in the refrigerator.
4. Overlay the sucrose gradient with 1 mL of sonicated viral pellet and centrifuge for 50 min at 26,000 × *g* (11,500 × *g* an SW 27 rotor), 4 °C.

5. Observe the virus as a milky band near the middle of the tube. Aspirate the sucrose above the band and discard. Carefully collect the virus band (~10 mL) with a sterile pipet, place in a sterile tube, and save.
6. Collect aggregated virus from the pellet at the bottom of the sucrose gradient after aspirating the remaining sucrose from the tube. Resuspend the viral pellet by pipetting up and down in 1 mL of 1 mM Tris·Cl, pH 9.0.
7. Sonicate the resuspended pellet once for 1 min, reband the virus from the pellet as in **steps 5** and **6** and pool band with band from **step 6**. Add 2 volumes of 1 mM Tris·Cl, pH 9.0, and mix. Transfer to sterile SW 27 centrifuge tubes (*see* **Note 13**).
8. Centrifuge for 60 min at 32,900 × *g*, 4 °C, then aspirate and discard supernatant, resuspending the virus pellets in 1 mL of 1 mM Tris·Cl, pH 9.0. Sonicate as in last steps and divide into 200–250-μL aliquots. Store at −80 °C.

## 4 Notes

1. We found that 1 mL of TRI reagent is suitable for lysis of $5–10 \times 10^6$ cells.
2. Sequencing was performed by using a plasmid specific primer located 214 nucleotides downstream of the *Sma*I restriction site: *pSC11-A(VS): GTGGTGATTGTGACTAGCGTAG*.
3. The CPE caused after MVA infection consist of vacuolae formation spreading the cell cytoplasm. By using a Pipetman is easy to disrupt the monolayer, transferring the supernatants to a new tube to facilitate virus processing. It is important to use negative controls (infecting cells with MVA wt and MVA wt + Lipofectamine) and include a positive control (recombinant plasmid pSC11 + Lipofectamine).
4. We usually add 20 μL of the virus stock diluted in 180 μL of complete medium, and successively transfer 20 μL of the prior dilution to 180 μL of complete medium until dilution $10^{-7}$).
5. In order to allow cell spreading, we use a proportion 1:1 between agar and medium. Do not try to do too many assays at the same time because the agar–DMEM mixture could solidify.
6. It is advisable to confirm the presence of the plaques by light microscopy.
7. In order to obtain a high viral titer is convenient to use low MOI to avoid a prompt damage in the cells.
8. We usually maintain 4 mL of medium/flask.

9. It is convenient to count the plaques of the intermediate dilutions, because it is easier to count (the size of MVA plaques are small).
10. In this step is possible to stop the procedure and the coverslips can be stored in PBS 1× at 4 °C for at least 1 month.
11. It is convenient to do tenfold dilutions of the sera in order to find the best dilution.
12. At this stage, the virus may be sufficiently pure for some purposes—e.g., isolation of DNA.
13. The total volume should be ~60 mL, which is enough to fill two SW 27 centrifuge tubes. If less volume is obtained, fill the tubes with 1 mM Tris–HCl, pH 9.0.

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