

Video Article

Propagating and Detecting an Infectious Molecular Clone of Maedi-visna Virus that Expresses Green Fluorescent Protein

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Abstract

Maedi-visna virus (MVV) is a lentivirus of sheep, causing slowly progressive interstitial pneumonia and encephalitis¹. The primary target cells of MVV *in vivo* are considered to be of the monocyte lineage². Certain strains of MVV can replicate in other cell types, however^{3,4}. The green fluorescent protein is a commonly used marker for studying lentiviruses in living cells. We have inserted the *egfp* gene into the gene for dUTPase of MVV. The dUTPase gene is well conserved in most lentivirus strains of sheep and goats and has been shown to be important in replication of CAEV⁵. However, dUTPase has been shown to be dispensable for replication of the molecular clone of MVV used in this study both *in vitro* and *in vivo*⁶. MVV replication is strictly confined to cells of sheep or goat origin. We use a primary cell line from the choroid plexus of sheep (SCP cells) for transfection and propagation of the virus⁷. The fluorescent MVV is fully infectious and EGFP expression is stable over at least 6 passages⁸. There is good correlation between measurements of TCID₅₀ and EGFP. This virus should therefore be useful for rapid detection of infected cells in studies of cell tropism and pathogenicity *in vitro* and *in vivo*⁸.

Video Link

The video component of this article can be found at <https://www.jove.com/video/3483/>

Protocol

1. Transfection

The molecular clone is contained in two plasmids, p8XSp5-*egfp* and p67r, of 12 kb and 4.5 kb respectively.^{9,10}

1. Cut equimolar quantities of the two plasmids, i.e. 4.4 µg of p8XSp5-*egfp* and 1.6 µg of p67r with XbaI and ligate before transfection.
 2. For ligation, use 1 Weiss unit of ligase in a 50 µl reaction and incubate at 16°C overnight.
 3. When ligation is completed, incubate the ligation mix at 65°C for 15 min for inactivation of the ligase. This step may be omitted.
 4. Two days before transfection, seed 10⁶ SCP cells in a T25 tissue culture flask, and grow cells for two days in 5 ml DMEM + 10% lamb serum and 2mM glutamine without antibiotics to a monolayer of 90% confluency. If the cells have not reached 90% confluency after two days, leave the cells to grow one additional day.
- Note: The lamb serum can be substituted by fetal bovine serum (FBS). We routinely use lamb serum, since some strains of MVV are inhibited by FBS; this MVV strain is not inhibited by FBS, however.
5. When the cells are ready for transfection, change medium to DMEM with 1% serum (lamb serum or FBS) without antibiotics. We use Lipofectamin 2000 (Invitrogen) for transfection.
 6. Dilute the DNA in 500µl Opti-MEM medium.
 7. Dilute 20µl Lipofectamin 2000 in 500µl Opti-MEM medium and leave at room temperature (RT) for 5 min.
 8. After the 5 min incubation, combine the diluted DNA with diluted Lipofectamine 2000 (total volume = 1000 µl). Mix gently and incubate for 20-30 min at RT.
 9. Add the transfection mix (1 ml) to the T25 flask containing SCP cells and medium (5 ml). Mix by rocking gently.
 10. Incubate at 37°C and 5% CO₂ overnight (18 – 24 h).
 11. Change medium the following day to DMEM with 1% serum, 2mM glutamine, 100IU penicillin and 100IU streptomycin.
 12. Monitor the production of GFP in the SCP cells by inverted fluorescent microscopy. Low transfection efficiency can be expected with these primary cells, usually 5 – 15%.
 13. Incubate further at 37°C, 5% CO₂ for several days to allow spreading of the virus in the flask. It usually takes 7-14 days for full infection.
 14. Harvest the virus by aliquoting the supernatant into Eppendorf tubes. Spin in a microfuge at 3000 rpm for 5 min to remove cell debris. Transfer the supernatant containing the virus to new tubes. Store at -80°C.

2. Titration of virus

1. Seed 3×10^4 SCP cells in 100 μ l DMEM medium supplemented with 10% serum, 2mM glutamine, 100IU/ml penicillin and 100IU/ml streptomycin, per well in a 96 well plate. Incubate at 37°C, 5% CO₂.
2. If the cells are confluent the following day, change medium to DMEM with 1% serum, 2mM glutamine, 100IU/ml penicillin and 100IU/ml streptomycin, 100 μ l in each well.
3. Dilute the virus in the following way:
 1. Add 180 μ l DMEM with 1% serum, 2mM glutamine, 100IU/ml penicillin and 100IU/ml streptomycin per well to 4 x 10 wells in a 96 well round bottom or V-bottom plate.
 2. For serial tenfold dilutions in quadruplicate, add 20 μ l of virus to each of 4wells in the first column of the plate, and using a four-channel pipette, pipette up and down to ensure that the samples are thoroughly mixed. Change pipette tips and transfer 20 μ l to the next 4 wells and so on. Leave the last wells without virus for a negative control.
4. Add 100 μ l of the virus dilutions to the SCP cells and medium.
5. Incubate at 37°C and 5% CO₂. Observe for GFP fluorescence and cytopathic effects for two weeks.
Cytopathic effects consist of cells becoming rounded and less transparent than normal cells with processes stretching out, ending with multinuclear cells appearing and cell death.
6. Calculate virus titer (TCID₅₀) using the Reed Muench method¹¹.

3. FACS analysis of infected cells

Note: For rapid monitoring of replication of the virus, infected cells can be examined by flow cytometry.

1. Trypsinize infected cells (ca 10^5), wash 1x with PBS and resuspend in 500 μ l PBS.
2. Add 500 μ l 4% paraformaldehyde solution (2% final concentration), incubate at RT for 30 min.
3. Pellet cells in a microfuge at 3000 rpm for 5 min.
4. Wash with PBS 2x5 min and analyze on a FACScan analyzer. Count 10,000 events.

4. Representative results:

This virus should be fully infectious and replicate to a titer of $10^6 - 10^7$ TCID₅₀/ml. Infected cells are fluorescent and can be detected by flow cytometry and fluorescence microscopy. Typically, GFP can be detected in more than 60% of the cells in the infected cell cultures using flow cytometry (Fig. 1). Likewise, when visualized by fluorescence microscopy, a majority of the cells are fluorescent (Fig. 2A and B).

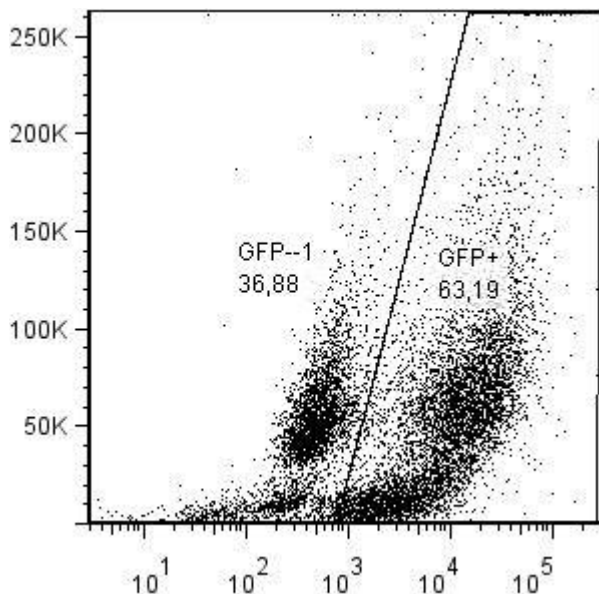


Figure 1. FACScan analysis of SCP cells infected with KV1772-egfp virus after 7 days of infection.

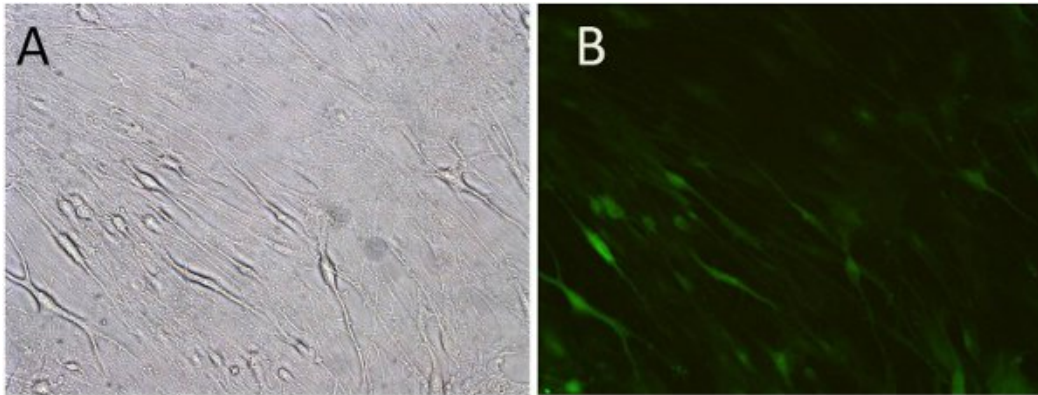


Figure 2. Phase contrast microscopy of SCP cells infected with KV1772-egfp after 7 days of incubation (A). Same cells visualized by fluorescence microscopy (B).

Discussion

The GFP expressing molecular clone of MVV presented here should be useful for analyzing the host-cell interactions and pathogenicity of MVV both in vitro and in vivo. The virus obtained after 7-14 days of replication has undoubtedly acquired some mutations due to the inaccuracy of reverse transcriptase. However, there are limitations to the use of this clone as a single cycle vector. One is that use of the clone is restricted to cells of sheep and goat origin. The transfection efficiency of these cells using our construct is only 5-15%, and using a pEGFP-N3 vector the transfection efficiency is 15-20% compared to over 90% using 293-T cells. 293-T cells have been tested as packaging cells for MVV vectors, but the resulting virus particles proved non-infectious both in sheep cells and in 293-T cells¹² probably due to unidentified restriction factors. The infectious clone is contained in two plasmids, which is another shortcoming. This is due to the fact that the full-length clone is unstable in *E. coli*. However, it should be possible to clone the full-length clone in a low copy plasmid.

Plasmids and cells can be obtained from the authors.

Disclosures

No conflicts of interest declared.

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