

# Native MCV production

## Native MCV production/purification from plasmid DNA in 293TT cells

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### Primary reference:

Schowalter, R.M., D.V. Pastrana, and C.B. Buck, *Glycosaminoglycans and sialylated glycans sequentially facilitate merkel cell polyomavirus infectious entry*. *PLoS Pathog*, 2011. 7:e1002161

### Vector release and re-ligation of MCV genomic DNA

1. Digest 50 µg of pR17b [1, 2] with 12.5 µl BamHI-HF in 200 µl volume (2 hr at 37°C)
2. Add 1200 µl of Qiagen PB buffer to the digest and purify over two Qiaprep blue miniprep spin columns. Elute each column with 2x 100µl volumes of TE.
3. Prepare ligation reaction in a 50 ml conical: add the 400 µl of purified DNA, 8.6 ml of 1.05X T4 ligase buffer and 6 µl of high concentration T4 ligase (NEB). Incubate at 16°C overnight.
4. Ethanol precipitate the DNA:

- Add 4.5 ml of 7.5 M ammonium acetate (Sigma cat# A2706) and mix. Add 35 ml of 95% ethanol. Do not use absolute (100%) ethanol. Mix and incubate at 4°C overnight.

- Bring the sample back to room temperature. Centrifuge at ~5,000 x g (room temp or 16°C) for 60 minutes (e.g., Sorvall SH3000 swinging bucket rotor 4700 rpm).

- Wash pellet with 10 ml of 70% ethanol (incubate 10 min). Spin 10 min. Repeat. \*\*I found this part the most difficult to get right. I think it's important to treat the invisible pellet very delicately. Maintain the orientation of the tube in the bucket when you spin each time, and try not to jostle the tube a lot before you discard the supernatant. I prefer to pour the supernatant out, rather than aspirate. \*\*

- Remove the second 70% ethanol wash. Spin residual ethanol off the walls of the tube. Remove residual ethanol, then allow pellet to air dry for several minutes.

- Add 100 µl of TE to the dried pellet. Allow the DNA to redissolve for at least 10 minutes (overnight is good). Transfer dissolved DNA to a microfuge tube. Rinse the walls and bottom of the 50 ml tube using an additional 100 µl of water. Expect a yield of about 35µg of DNA.

### Transfection of 293TT cells, cell expansion and harvest:

1. In the late afternoon/evening, split 2.5 million 293TT cells into a T25 flask without hygromycin B.
2. The next morning, cells should be about 50% confluent. Using 27.5 µl Lipofectamine 2000 (1.1 µl/cm<sup>2</sup>), transfect 5 µg religated MCV isolate R17b DNA, 3.5 µg pMtB, and 4 µg pADL\* (0.5 µg DNA/ cm<sup>2</sup>) [3,4] \*\*I haven't yet tried using pMtBS for production. If I were to use pMtBS instead of pMtB, I would probably use less of that plasmid and more of the others. MCV small t is toxic and pMtBS makes more of the correct protein and is thus more toxic.\*\* After 6-8 hrs (overnight incubation might be better if the cells are >50% confluent) change the medium on the cells.
3. The following day or in two days when the transfected cells are nearly confluent, trypsinize the cells and transfer them to a T75 for continued expansion. When this flask is nearly confluent, transfer the cells into two T225 flasks. When these flasks are nearly confluent (usually 5-6 days after transfection), harvest using the standard protocol\*\*I generally add 1.4X volumes DPBS-Mg to the cell pellet and mix. Then, 25 mM ammonium sulfate from a 1M pH 9 stock. Then, 0.5% Triton X-100 from a 10% solution. Then, 0.1% Benzonase and 0.1% Plasmid Safe.\*\* Mix well and incubate at 37°C overnight. I prefer to harvest in the late afternoon and remove the sample from the water bath first thing the next morning.
4. Incubate ~15 min on ice, then add 0.17 volumes 5M NaCl. Mix and incubate on ice another ~15 min. In a 4°C centrifuge, spin for 10 min at 5,000 x g. If the supernatant doesn't look clear repeat spin. Move supernatant to a new siliconized tube. Resuspend the pellet with DPBS / 0.8 M NaCl, and spin again. Combine supernatant with previous, and spin the supernatant one more time as before. Load the resulting supernatant on the prepared Optiprep gradient.
5. After ultracentrifugation, collect fractions in siliconized tubes. Fractions can be analyzed for the presence of virus by Quant-iT Picogreen DNA Regent (Molecular Probes) and/or western blot for VP1. Pool the peak fractions and characterize your stock by quantitative PCR and western blot with comparison to pseudovirus standards for quantitation of VP1 (you may also be able to SYPRO Ruby stain if it's a good stock).
  - a. Picogreen: transfer 10 µl of each fraction into a black 96 well plate. Add 140 µl Picogreen reaction solution (prepared according to manufacturers instructions) + 0.1% proteinase K. Incubate at 70°C for 45 min, then at room temp in the dark for 1 hr to overnight prior to analyzing fluorescence. You should see a peak around fraction 4 or 5 that goes down before it goes back up. The first peak is where your virus is located, but the peak will likely be small and I often like to double check by western blot.

- b. QPCR: Digest 1-5 ul of the viral capsid in 100 ul solution containing 20 mM Tris, pH 8, 20 mM DTT, 20 mM EDTA, 0.5% SDS and 0.2% proteinase K. Incubate 20 min at 50°C. Add 600 µl of Qiagen PB buffer to the digest and purify over a Qiaprep purple spin column. Elute in 50 ul TE. One ul will be plenty for quantitation by PCR. Make different dilutions of known copy number pR17b plasmid for calculation of sample copies. I use this website for making dilutions: <http://www.thermoscientificbio.com/webtools/copynumber/>

I use VeriQuest SYBR Green qPCR Master Mix 2X (Affymetrix) and the primers I use are: GCTTGTTAAAGGAGGAGTGG and GATCTGGAGATGATCCCTTG.

#### Native virion propagation and amplification using 293-4T cells:

The native MCV genome exhibits very little transcriptional activity and fails to replicate efficiently in all cell lines we have tested so far. This makes it difficult to titer the infectivity of native MCV virions. To overcome this problem, we developed a 293TT cell line, called 293-4T, that stably expresses the MCV Large T and small t antigens. The ectopically expressed T antigens drive replication of the MCV genome and this leads to the production of new virions, at least in a fraction of the infected cells. Thus, native MCV virion stocks produced by transient transfection (see above) can be amplified and serially propagated in 293-4T cells.

Propagation and amplification of MCV virions has not yet been carefully optimized. Pilot experiments used ~50 µl of native MCV virions (produced as described above) to infect a ~25% confluent T-75 flask of 293-4T cells for several days. The culture was then passaged continuously for several weeks. New MCV virions were produced essentially continuously during this period. No gross cytolytic effects were observed. However, the infected cultures reproducibly show a small percentage of cells that are much larger than normal. Immunofluorescence experiments seem consistent with the idea that cells expressing VP1 remain adherent but are visibly swollen (sometimes extremely swollen).

In the few experiments where we have propagated MCV in 293-4T cells, we adopted a strategy of subjecting ~10% of the culture to rapid freeze-thaw (without any cryo-protectants) during each passage. We then reintroduced the freeze-thawed lysate into the passaged culture. Our theory is that MCV does not promote active lysis and may need to be actively released (e.g. by freeze-thaw lysis). We have not carefully tested whether the freeze-thaw approach facilitates MCV propagation or is unnecessary.

A potential problem with serially passaging MCV in 293-4T cells is that the cell line continuously provides the virus with T antigens. Thus, mutant viruses that lose large segments of the MCV early region may have a fitness advantage (smaller genome size can mean faster replication and more efficient packaging into new virions). Similar effects are observed when polyomaviruses are propagated at high multiplicity of infection [5].

#### References:

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