**EVD68 Production using 293T cells**

**Purpose:** To provide a standardized method for production of recombinant EVD68 with high rescue efficiency. Protocol is based on a preprint by the Freeman lab.

Preprint: **https://www.biorxiv.org/content/10.1101/2024.12.20.629498v1.full.pdf**

**Materials:**

* EVD68 molecular clone with a T7 promoter. ~227 ng per reaction in 6 well plate.

Note: Trial experiment described here was performed with rUSA/IL/2014-18952 but should work with all molecular clones of EVD68 even Fermon according to the preprint.

* T7opt in pCAGGS (Addgene Plasmid #65974): ~2273 ng per reaction in 6 well plate.
* 293T cells. (CRL-3216)
* RD cells (ATCC or Andino)
* 6 well plate.
* DMEM growth media (#1965092) supplemented with 10% Fetal Calf Serum (FCS) and 1% Pen/Strep.
* Opti-MEM™ I Reduced Serum Medium (#31985062)
* Transit-LT1 transfection reagent. (MIR 2300)

Note: Trial experiment described here was performed with 6 well plate but could theoretically be scaled up. Transit-LT1 protocol:

**https://tools.mirusbio.com/assets/protocols/ml001\_transit\_lt1\_transfection\_reagent.pdf**

**Procedure:**

**Cell Culture:**

1. **Plate 293T cells:** Seed 1×106 293T cells in a 6-well plate containing 2.5 mL growth medium supplemented with 10% Fetal Calf Serum (FCS) and 1% Pen/Strep.
2. **Incubate:** Culture the cells at 37°C with 5% CO₂ for 24 hours to allow them to adhere and reach approximately 70-80% confluency.

**Transfection:**

1. **Prepare DNA complexes (for each condition):**
   * **Condition 1 (T7optm only):**
     + Dilute 2.5 µg of T7optmized plasmid in 250 µL of OPTIMEM Serum-Free Medium.
   * **Condition 2 (T7optm + EVD68 molecular clone):**
     + Dilute 2.27 µg of T7optm plasmid and 227 ng of EVD68 molecular clone (10:1 ratio of T7optm to Molecular Clone) in 250 µL of OPTIMEM Serum-Free Medium.

**Note:** Adjust DNA amounts and OPTIMEM volume proportionally for different culture vessel sizes

1. **Add Transfection Reagent:**
   * For each DNA dilution prepared in step 1, add 7.5 µL of TransitLT1 transfection reagent.
2. **Incubate DNA-TransitLT1 Complexes:**
   * Mix the DNA-TransitLT1 solutions gently by pipetting up and down.
   * Incubate the mixtures at room temperature for 30 minutes to allow the formation of transfection complexes.
3. **Add DNA-Lipid Complexes to Cells:**
   * After the 30-minute incubation, add the DNA-TransitLT1 complexes dropwise to the 293T cells.
4. **Incubation for Virus Rescue:**
   * Incubate the transfected cells at **33°C** until cytopathic effect (CPE) is observed, typically within 3-4 days.
   * Monitor the cells daily for signs of CPE, such as cell rounding, detachment, and lysis.

**Post-Transfection:**

Important Note: RD cells (preferably Andino’s) at **33°C** are the best for virus passaging after virus production using 293T cells.

1. **Harvest Supernatant (if CPE is observed):** Once significant CPE is visible, perform two freeze-thaw cycles of the 6 well plate. Collect the media and perform centrifugation at 2000x g for 5 minutes. Collect the supernatant for further analysis.
2. **Virus titration:** Perform titration (TCID50) using RD cells at **33°C**.
3. **Virus growth:** Perform passaging of passage 0 stock using RD cells at **33°C**. MOI 1 for 24 hrs or MOI 0.01 for 72 h produces the best titers according to the preprint. P0 stock is added to cells at low inoculum (~400ul) and cells are rocked at room temperature for 1 hour to adsorb virus. After 1 hr, cell growth media is replenished (~2.1mL).

Note: In preprint, they remove inoculum and replenish media. Virus adsorption can be carried out at room temperature or potentially at **37°C** (according to Adam’s protocol).

**Preliminary results:** I was able to obtain for passage 0 stocks: 106 TCID50/mL using this protocol with the EVD68 18952 molecular clone.