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)
- 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 1×10^6 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% CO_2 for 24 hours to allow them to adhere and reach approximately 70-80% confluency.

Transfection:

1. **Prepare DNA complexes (for each condition):**
    - **Condition 1 ($T7_{optm}$ only):**
        - Dilute 2.5 μg of $T7_{optmized}$ plasmid in 250 μL of OPTIMEM Serum-Free Medium.
    - **Condition 2 ($T7_{optm}$ + EVD68 molecular clone):**
        - Dilute 2.27 μg of $T7_{optm}$ plasmid and 227 ng of EVD68 molecular clone (10:1 ratio of $T7_{optm}$ to Molecular Clone) in 250 μL of OPTIMEM Serum-Free Medium.

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

2. **Add Transfection Reagent:**
    - For each DNA dilution prepared in step 1, add 7.5 μL of TransitLT1 transfection reagent.
3. **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.
4. **Add DNA-Lipid Complexes to Cells:**
    - After the 30-minute incubation, add the DNA-TransitLT1 complexes dropwise to the 293T cells.
5. **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: 10^6 TCID50/mL using this protocol with the EVD68 18952 molecular clone.