



Title: *siRNA Transfection/Viral Infection- ELISA protocol for 96-well plates* No: RTLP-siRNA-4

Location: *Old CCRC Tripp Lab* Approval Date: 10 September 2004 Supersedes Date:
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|----------------|-----------------------------|----------------------|------------------|
| •Lab coat | •Glutamine | •96-well flat bottom | •Pipettes |
| •Gloves | •MEM | plate | •Pipetteman |
| •MDCK cells | •37°C, 5% CO ₂ | •dH ₂ O | •Pipette Aid |
| •Optimem I | incubator | •10% formalin | •Pipetteman tips |
| •Transit TKO | •TPCK-treated trypsin | (buffered) | • |
| •siRNA to test | •1° Antibody (biotinylated) | • | • |
| •Virus Stock | • Alkaline-Phosphatase | • | |
| | Streptavidin kit | • | |
| | | • | |

Reagents:

TKO- TransIT-TKO Transfection Reagent – *Mirus (cat# MIR2150)*

Opti-MEM I: from *Invitrogen (cat#51985-034)*

TPCK-treated Trypsin

1 ml/ml TPCK-treated Trypsin (Worthington #3740)

Dissolved in PBS o/n @ 4°C, filter sterilized

1 ml aliquots, stored frozen @ -20°C

MEM-Q: MEM (Mediatech-MT-15-010-CV) + 1% L-glutamine.

Procedure:

Cell preparation

1. Plate MDCK cells in 96-well plates, 10,000 cells /well in ~0.2ml complete medium (D-5).
2. The next morning cells should be ~65% confluent; prepare and transfect cells.

Transfection

1. Bring Optimem I (Invitrogen) and Transit TKO (Mirus) to room temperature.

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2. For 80 wells: combine **720ul Optimem I + 20ul TKO** in 2ml Sarstedt tubes. Mix by flicking or gently vortexing. Incubate 5-20 minutes @ RT.
3. Thaw siRNA stocks and prepare 50uM stocks if necessary.
4. Add **3.0ul of siRNA** to Optimem/TKO. Mix by gentle pipetting or flicking tube. Incubate 5-20 minutes @ RT.
This equates to: 9uL OptiMEM/well; 0.25uL TKO/well; 0.0375uL siRNA/well
5. Dump off complete medium from 96-well plates, wash with PBS (0.2mL per well); dump off PBS, and replace with **44uL/well complete media (D-5)**.
6. Add **10ul siRNA/reagent** complexes to each well of experimental design. Gently tap plate to mix evenly.
7. Incubate in 37°C 5%CO₂ incubator for 24 hours.

Infection

1. Thaw A/PR/8/34 (egg prep)-Virus stock is $\sim 3 \times 10^8$ PFU / ml. **NOTE:** Or just used equivalent amount of virus stock desired for testing.
2. Dump transfection medium, wash 1X with MEM-Q.
3. Titrate A/PR/8 (or desired stock virus) down plate with a final volume of 100uL (ex; 10uL virus + 90uL MEM-Q); be sure to leave row H without virus (=negative ctrl).
4. Incubate in 37°C 5%CO₂ incubator for 2 hours.
5. Dump media, wash 1X with MEM-Q.
6. Add 200uL/well of MEM-Q+ 0.25ug/mL TPCK treated Trypsin.
7. Incubate in 37°C 5%CO₂ incubator for 24-48hrs.
8. Dump media; Fix with 10% Formalin, buffered at room temperature for 45 minutes. Proceed with ELISA.

ELISA Protocol

1. Wash wells 2X with PBS/0.2% Tween-20.
2. Block wells with 1 X StartingBlock for 1 minute at RT.
3. Wash wells 2X with PBS/0.2% Tween-20.
4. Dilute 1^o Antibody 1:2000 in 1x PBS, need 50 µl/well.
1^o Antibody = mouse anti-influenza A biotinylated monoclonal antibody from Chemicon-
use either: MAB8257B-- conc.= 1.0mg/mL or MAB8258B-. = 1.0mg/mL

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5. Add 1^o Antibodies to wells (0.05uL/well)
6. Incubate at RT for 1 hour.
7. Wash wells 3X with PBS/0.2% Tween-20.
8. Dilute 2^o Antibody (Alkaline-Phosphatase Streptavidin- *Vector Laboratories cat#: SA 5100*) 1:1000 in 1X PBS, need 100 µl/well.
9. Add 2^o Antibody to wells (0.1mL/well).
10. Incubate at RT for 45min.
11. Wash wells 3X with PBS/0.2% Tween-20.
12. Develop Reaction using pNpp Phosphatase Substrate (*KPL cat#50-80-00*). Need 100 µl/well.
13. Add substrate to wells.
14. Incubate at room temperature for 5-45 minutes; take readings every 10minutes.
15. Measure Absorbance at 405 nm.