***Generation of P0 Virus***

*Walker Orr, 04/12/2024*

## Guidelines for Working with RNA

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| When working with RNA, it is very important to prevent nuclease contamination in your work areas, always keep RNA on ice, and store it at -80C.  Start by creating an RNAZone on your bench that will function similarly to a hood. Tape out an area of your bench where you will keep all your supplies that will be used to prepare the RNA. Think carefully about everything you will need—if you are spinning down RNA, for instance, your mySpin will need to end up in the RNAZone.  Use a different lab coat than the one you use for bacterial work, routine molecular biology, etc. Be liberal with glove changes; you can also spray your gloves down with RNAse-ZAP.  Take everything out of the RNA zone. Clean the area well with ethanol and then apply RNAse-ZAP. This surfactant kills RNAse enzymes that can degrade your RNA. Clean all tools (i.e., pipettes), scaffolding (i.e., tube racks, ice bucket, BioScion tube holders), and supplies (i.e., pipette tip boxes) with RNAse ZAP before bringing them into the RNAZone. Wipe off excess RNAse-ZAP with KimWipes.  When using microcentrifuge tubes, PCR strips, etc., take them from a freshly-opened bag of certified nuclease-free tubes. The DNA Lo Bind tubes, for instance, come in large bags of ~500 tubes and in smaller bags of <50. For working with RNA, use the smaller bags, as these will have less chance to experience contamination.  The quality of RNA preps can be readily assessed on a TapeStation RNA Screen Tape device. Make sure to use the correct buffer and ladder; there exist both High Sensitivity and standard RNA devices. |

## Linearization of DNA Template

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| 1. Digest 5 ug DNA using the correct restriction enzyme (this should cut downstream of the poly-A tail).   For EagI (4643): 5 ug DNA, 5 ul EagI, 10 ul rCutSmart, Nuclease-free water to 100 ul. |
| 1. Perform DNA Cleanup on the linearized DNA. (NEB Monarch PCR & DNA Cleanup; SOP #). Recall that it is a large fragment, so 2:1 binding buffer and 50C water for elution. |
| 1. Dilute 1 ul eluted cleaned-up DNA in 9 ul Nuclease-free water and measure using Qubit BR. |

## *In vitro* transcription

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| 1. Thaw components at room temperature. Vortex and spin reagents before use. Make a master mix as follows (1x) and keep at room temperature:    1. 2 ul 10x reaction buffer    2. 2 ul APT    3. 2 ul CTP    4. 2 ul GTP    5. 2 ul UTP    6. Common volume of nuclease-free water. |
| 1. Set up reactions in PCR strip tubes:   Master Mix: ≥ 10 ul  Template DNA: 1 ug  T7 RNA Polymerase: 2 ul  Additional nuclease-free water to 20 ul, as needed |
| 1. Mix thoroughly, spin down. Incubate 37C for 2 hours in a thermocycler. |

## DNAseI Treatment

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| 1. Add to each sample, on ice:   5 ul 5x DNAseI Reaction Buffer  2.5 ul DNAseI Enzyme  22.5 ul Nuclease-free water   1. Mix thoroughly, spin down. |
| 1. Incubate at 37C for 10 minutes. |

## Cleanup and QC

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| 1. Add 100 ul RNA Cleanup Binding Buffer to the 50 ul DNAseI-treated sample. |
| 1. Add 150 ul (1 volume) of 100% ethanol to this. |
| 1. Insert column into collection tube, load sample onto column, and close. Spin 1 minute at 16,000 x g, then discard flow-through. |
| 1. Wash with 500 ul RNA Cleanup Wash Buffer. Spin 1 minute, discard flow through. |
| 1. Repeat step 4. |
| 1. Spin dry. If there’s any wash buffer trapped between the cleanup column and collection tube, consider using a fresh collection tube and letting the side of the column dry. |
| 1. Transfer column to a clean 1.5-mL microcentrifuge tube. |
| 1. Add 100 ul of nuclease-free water to the center of the column and immediately spin to elute. |
| 1. Quantitate RNA on the nanodrop. |
| 1. Analyze RNA quality using TapeStation 4200. Make sure to use the correct buffer and ladder (there’s an HS and standard RNA TapeStation kit). Remember to preheat a thermocycler for the denaturation step. |

## TapeStation Analysis

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| 1. Thaw TapeStation RNA sample buffer at room temperature for 30 minutes; thaw ladder for 30 minutes on ice. |
| 1. Mix 5 ul sample buffer and 1 ul ladder or sample RNA for each sample. |
| 1. Vortex for 1 minute at 2000 rpm. Spin down. |
| 1. Heat denature for 3 minutes at 72C. Return to ice for 2 minutes. Spin down. |
| 1. Run the TapeStation RNA ScreenTape. |

## Transfection of RD cells with viral RNA

The day before, plate 2 million cells in a T25, or a proportionate amount in another vessel (scale all volumes that follow accordingly). The cells should be around 80% confluent upon transfection.

Before starting, prepare the following:

* OptiMEM, at room temperature
* Half-serum media and PBS at 37C
* TransIT mRNA and mRNA boost, at room temperature

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| 1. Wash cells with warm PBS and change media to half-serum. |
| 1. Vortex TransIT and mRNA Boost and spin down. |
| 1. For each T25 flask being transfected, add **630ul** OptiMEM to an microcentrifuge tube. |
| 1. Add **3.25 ug** of the respective RNA to each tube; pipette up and down. |
| 1. Add **6.5 ul** mRNA Boost (orange tube) to each tube; pipette up and down. |
| 1. Add **6.5 ul** TransIT (white tube) to each tube; pipette up and down. Rest for 3 minutes. |
| 1. Add this mixture dropwise to cells. Incubate at 37C for two days before harvest. |