Outline for mRNA preparation **AMGS**

I. DNA template prep
   A. Gradient PCR amplification from mutated plasmid, small scale (24ul per reaction)
   B. Run 1% agarose gel to check best Tm
   C. Large scale PCR using best Tm from gradient (50ul per reaction)
   D. Pull together all reactions and do PCR cleanup (Clonetech kit)
   E. Run 1% agarose gel
   F. Gel extraction/ DNA purification from gel (Zymo kit)
   G. Optional: Check 260/280 and 260/230 ratios. If not acceptable, do PCR cleanup (Clonetech kit).

II. In vitro transcription

III. DNAse treatment

IV. mRNA purification using LiCl

V. mRNA capping and 2'O-Methylation

VI. mRNA purification using phenol/chloroform extraction and ethanol precipitation

I. DNA template prep

A. Gradient PCR amplification from mutated plasmid, small scale (24ul per reaction)
Forward primer = T7 or T7 + 5'UTR of interest
Reverse primer = Nluc + 60T

B. Run 1% agarose gel to check best Tm

C. Large scale PCR using best Tm from gradient (50ul per reaction)

D. Pull together all reactions and do PCR cleanup (Clonetech kit)

E. Run 1% agarose gel

F. Gel extraction/ DNA purification from gel (Zymo kit)

G. Optional: Check 260/280 and 260/230 ratios. If not acceptable, do PCR cleanup (Clonetech kit).

II. In vitro transcription

1. The following protocol will allow you to obtain 10 to 50 times the amount of RNA that what the input DNA was. For example, if the DNA input is 1ug, the RNA output will be ~50ug.
2. Mix the following in a tube (use calculations in spreadsheet):

Component	Volume (ul) needed for 1X reaction	Final concentration
Template DNA	x	Minimum: 500ng Recommended: 1-2ug
10x T7 RNA Buffer (NEB)	5	1x
ATP [100mM]	2.5	5mM
CTP [100mM]	2.5	5mM
UTP [100mM]	2.5	5mM
GTP [100Mm]	2.5	5mM
BSA [10mg/ml]	0.5	1x
DTT [0.1M] *fresh*	4.5	9mM (+buffer DTT = 10mM total)
$MgCl_2$ [2M] *RNase-free*	0.625	5x total rNTP = 25mM
T7 RNA polymerase (NEB)	4	4U/ul (stock at 50U/ul)
Murine RNAse inhibitor (NEB)	1.25	1U/ul (stock at 40U/ul)
Water	X	-
Total volume	50	-

3. Incubate at 37°C anywhere from 2h to overnight. *do 4 hours

III. DNAse treatment

1. Add 5.5ul RQ1 DNAse buffer and 1.25ul RQ1 DNase directly intro in vitro transcription reaction.
2. Incubate for 30min at 37°C.

IV. LiCl purification

1. Add EQUAL volume of 7.5M LiCl/50mM EDTA as volume of ivt reaction and mix well.
2. Incubate at -20C for 30min-1hr. 1hr is best.
3. Centrifuge at top speed (15K rpm) for 20 mins.
4. Observe bottom of tubes. You should see a small white pellet. This is your RNA.
5. Remove supernatant carefully using pipette.
6. Wash pellet 2 times with 70% EtOH. To do this, add 1ml of 70% EtOH to the pellet, vortex for 10 seconds to break pellet and spin at 4C, 15K rpm for 5 minutes. Repeat.
7. After second wash, remove ALL ETHANOL using pipette. Let pellet dry for 5 to 20 minutes.
8. Resuspend pellet with 50 µl warm water. -> 40-50C

V. Capping and 2'O-Methylation

1. Prepare 15ul of 10ug RNA stock (use spreadsheet calculations):

2. Mix the following:

Component	Volume (ul) needed for 1X reaction	Final concentration
RNA	15	Min: 10ug Max: ∞
10x capping buffer	2	1x
GTP [100Mm]	1	10mM
SAM *fresh*	1	4mM
Vaccinia D1/D2 (Capping enzyme) (NEB #: M2080S)	0.5	-
Vaccinia VP39 (2'O Methyltransferase) (NEB #: M0366S)	0.5	-
Total volume	20	-

3. Incubate at 37°C for 2h.

VI. Zymo cleanup kit OR phenol chloroform

a. Zymo kit is recommended. Preferred kit: Zymo RNA clean and concentrator kit-5 (without DNAse)
b. Phenol-Chloroform is fine for downstream in vitro assays, but will affect results in cells because there may always be trace amounts of phenol.

A. Zymo RNA clean and concentrator kit-5 (without DNAse)

1. Follow kit's protocol: https://www.zymoresearch.com/collections/rna-clean-concentrator-kits-rcc/products/rna-clean-concentrator-5
2. Elute in 15 ul water.

B. Phenol/chloroform extraction and ethanol precipitation

1. Clean up by phenol chloroform extraction.
   a. WARNING: DO THESE STEPS UNDER THE HOOD. PHENOL/CHLOROFORM IS VERY TOXIC.
   b. Up the capping reaction volume to 400ul using water.
   c. Add phenol chloroform reagent (Name: Acid Phenol: CHCl3, 5:1 solution, pH: 4.5) (Location: common 4C fridge) to 400ul capping reaction. Mix thoroughly by pipetting up and down. Close tubes very well to avoid phenol chloroform leaks.
   d. Vortex each tube thoroughly (for 1 minute each).
   e. Centrifuge at 4C, 15K rpm, 20mins.
   f. Remove tubes from centrifuge. Let sit in rack in the hood for 1 minute.
   g. Transfer top layer to a new tube. Use 200ul tip to transfer. Avoid taking phenol layer (bottom layer).

1. Add 1ul glycogen, 1/10th volume of 3M NaOAc and 1ml of 100% EtOH to top layer that was transferred to new tube.
2. Put at -80°C overnight.
3. The next morning, spin 20min. Observe bottom of tubes. You should see a small white/blue pellet. This is your RNA.
4. Remove supernatant carefully using pipette.
5. Wash pellet 2 times with 70% EtOH. To do this, add 1ml of 70% EtOH to the pellet, vortex for 10 seconds to break pellet and spin at 4C, 15K rpm for 5 minutes. Repeat.
6. After second wash, remove ALL ETHANOL using pipette. Let pellet dry for 5 to 20 minutes.
7. Re-suspend pellet in 20ul water (adjust to obtain 1-2ug/ul of RNA).