RT Protocols to Test

B) and **C)** are based on the **TSO Revision 1 Protocol** with the following modifications:

B) Hot Start (No Ice)

Same as the original protocol, but keep everything at **55°C**. First, anneal primer and keep at **55°C**. Then, make dNTP/enzyme mix, warm at **55°C**, then add to primer tube.

C) Touchdown RT reaction

Replace **Primer Annealing** with the following:

Primer Annealing:

5X Hyb Buffer Recipe

Reagent
1.5 M NaCl
50 mM Tris pH 7.5
10 mM EDTA

1. Make a primer/template mix for each sample as detailed below:

Reagent	Volume (uL)
total RNA	10 ug (14 uL)
5X Hyb buffer	3.75 uL
1 ug primer pool	1 uL
Total	18.75 uL

2. In thermo-cycler, heat to **90°C** then cool to **55°C** over 30 min.
3. Prepare the dNTP Enzyme mix as described, warming to **55°C** before adding to template/primer mix. Proceed with rest of reaction as described.

D) Rocketscript Protocol

1. Add the following reagents:

Reagent	Volume (uL)
5X Rocketscript buffer	3.75 uL
1st strand primer pool	1 uL
total RNA	10 ug (14 uL)
Total	18.75 uL

2. Place samples in thermo-cycler and run the following cycle: **70°C 1 min, 60°C hold**
3. Make the enzyme dNTP mix for each sample as detailed below:
4.

Reagent (final concentration per protocol in parentheses)	Volume (uL)
5X Rocketscript buffer	4
25 mM dNTP mix (25 mM of each dNTP; final conc = 1 mM mcSCRB-seq protocol, 0.5 mM Dwyer protocol, 1 mM Smart3Seq protocol, 0.5 mM Thermo protocol -> try 1 mM for now)	1.6
TSO 50 uM stock (2 uM mcSCRB-seq, 1 uM Smart3Seq protocol -> try 1 uM for now)	1.6
200 U/uL RocketScript Reverse Transcriptase (2 U/uL mcSCRB-Seq, 10 U/uL Thermo protocol -> try 10 U/uL for now)	1.5
ddH2O	11.3
Total	**20**

1. Follow same cycling protocol/conditions for Maxima RT, except allow reaction to proceed at **60°C** for 90 min.

E) RapiDxFire Protocol

1. Add the following reagents:

Reagent	Volume (uL)
10X Thermostable RT Buffer	1.67 uL
1st strand primer pool	1 uL
total RNA	10 ug (14 uL)
Total	16.67 uL

2. Place samples in thermo-cycler and run the following cycle: **70°C 1 min, 60°C hold**
3. Make the enzyme dNTP mix for each sample as detailed below:
4.

Reagent (final concentration per protocol in parentheses)	Volume (uL)
10X Rocketscript buffer	2
25 mM dNTP mix (25 mM of each dNTP; final conc = 1 mM mcSCRB-seq protocol, 0.5 mM Dwyer protocol, 1 mM Smart3Seq protocol, 0.5 mM Thermo protocol -> try 1 mM for now)	1.6
TSO 50 uM stock (2 uM mcSCRB-seq, 1 uM Smart3Seq protocol -> try 1 uM for now)	1.6
200 U/uL RocketScript Reverse Transcriptase (2 U/uL mcSCRB-Seq, 10 U/uL Thermo protocol -> try 10 U/uL for now)	1.5
ddH2O	13.3
Total	**20**

F) Capping Protocol

Protocol

1. Combine RNA and Nuclease-free H2O in a 1.5 ml microfuge tube to a final volume of 15.0 µl.
2. Heat at 65°C for 5 minutes.
3. Place tube on ice for 5 minutes.
4. Add the following components in the order specified:

Reagent	Volume (uL)
Denatured RNA (from above)	15
10X Capping Buffer	2
GTP (10 mM)	1
SAM (2 mM, dilute 32 mM stock to 2 mM)	1
Vaccinia Capping Enzyme	1
Total	20

5. Incubate at 37°C for 30 minutes.
6. Try to use without purification in the RT extension.