Round A/B Random Amplification of RNA Protocol

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Turn on UV after completing every step where amplifiable product is handled. Set up all master mixes AFTER UV IRRADIATING the work area and **BEFORE** samples are brought into the hood. Remove reagent tubes and store in freezer box as soon as the master mixes are made. No reagent tubes should remain in the hood during irradiation!

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BEFORE STARTING

Before beginning place the following into the BSC:

1) New Ziploc bag for tip waste (or use the baggie your tubes aliquots are in)
2) Get new tube aliquots (1.5ml, 0.5ml, and 0.2ml), new pipette tips (P10, P200, P1000)
3) Get 2.0 ml tube "adaptors" for the centrifuge. (Makes it easier to spin 0.5 ml tubes.)
4) Sufficient aliquots of all necessary reagents in the freezer (keep enzymes at -20 until needed)
5) Adequate racks (for each tube size)
6) UV irradiate for at least 15 minutes!

I. Round A

In Tissue Culture Room, Hood #2, reverse transcription with randomized primer.

1. Make sure the centrifuge inner lid is facing up. Then, turn on UV in Rd A hood for at least 15 min (30 min if someone else has done a Round A that day.) Turn on the RT Thermocycler in the TC room to warm up (65°C Soak):

<File> 65 <Enter>
<Start>

2. Make master mix (2X enzyme mix) and set aside (do not add this to your tubes until step #7):

3.2 μl □H_2O
4 μl 5X RT Buffer
0.8 μl 12.5 mM dNTP mix (final conc 500 uM each nucleotide)
2 μl RT (Promega)

Master Mix	6	7	8	9	10	11	12	13
H_2O	19.2	22.4	25.6	28.8	32	35.2	38.4	41.6
5X RT Buffer	24	28	32	36	40	44	48	52
12.5mM dNTP	4.8	5.6	6.4	7.2	8.0	8.8	9.6	10.4
Promega RT	12	14	16	18	20	22	24	26

3. Label 500 μL tubes (lid with sample #, side with date). **Always include H_2O only (negative) and positive control (e.g. 50 ng of Mock HeLa).** It is best to use 2 water controls – the first and last tubes (the first tube screens for contaminated reagents; the last tube screens for carry over from tube to tube.)

4. Aliquot H_2O and 1 μl primer A (40 pmol/μl). When the RNA (50ng-200ng, 100ng, is optimal) is added, the final volume will be 10 μl.

5. Put stocks away (Change Gloves) and go get RNAs. Add RNA (do not mix RNA sample by flicking the tube, may cause contamination) to primer A/ H_2O mix by gently pipetting up and down.

6. Heat the RNA/ primer mix 65°C, for 4 min. Cool at RT, in a rack in the hood, for 4 min. (This allows the RNA to denature and the primer to randomly anneal.) **Do NOT heat your master mix, or you will kill your enzyme.** While your samples are cooling, reprogram the RT Thermocycler (42°C Soak):

<Stop> <Stop>
<File> 42 <Enter>
<Start>

7. Perform a quick spin (6 seconds) of the samples. Add 10 μL of 2X enzyme master mix to each tube and pipette up and down to mix (Master Mix from Step 2).

8. Heat at 42°C for 30 min. While sample is incubating, **turn on UV** and return samples to -80°C.

9. Program RT Thermocycler in the TC room for 65°C as in step 1 and heat to 65°C 4 min.

10. While your samples are heating, make an aliquot of RT with enough enzyme for your samples (1μl each sample plus 1μl extra) (to avoid contaminating the master RT tube). Cool samples at RT 4 min and perform a quick spin.

11. Add 1 μL RT enzyme, program RT Thermocycler for 42°C as in step 6 and heat at 42°C for 30 min. **Turn on UV during 30 min incubation. This can go longer or be used as a STOPPING POINT if necessary.**

2nd strand synthesis with Sequenase "RdA"

12. Program DNA Thermocycler to program #81
<Stop> <Stop>
<File> 81 <Enter>
<Start>

13. Make Sequenase Mix:
2 □l 5X Sequenase Buffer
7.7 □l H_2O
0.3 □l Sequenase

Program #81:
94°C, 2 min
10°C, 5 min
} Step #14
10°C to 37°C over 8 min
37°C, 8 min
94°C, 2min
} Step #15
10°C, 5 min
10°C to 37°C over 8 min
37°C, 8 min
94°C, 8min
} Step #16

Sequenase Mix	6	7	8	9	10	11	12	13
H_2O	46.2	53.9	61.6	69.3	77	84.7	92.4	100.1
5X seq. Buffer	12	14	16	18	20	22	24	26
Sequenase	1.8	2.1	2.4	2.7	3.0	3.3	3.6	3.9

14. Heat sample (Rd A product) to 94°C 2 min. After the temperature reaches 10°C, spin down the samples and add 10 μL Sequenase mix for a total RXN volume of 30 μL. If you have >6 samples, add Sequenase mix to half of the samples at a time (so that the samples don't warm up too much while they are out of the DNA Thermocycler.). You may need to pause the cycler so it does not continue from 10 – 37 degrees C before you have added all your mix.

15. **Turn on UV after adding Sequenase mix.** (Program 81 will now ramp from 10°C to 37°C over 8 min, hold at 37°C for 8 min, heat to 94°C for 2 min and cool to 10°C.)

16. After the temperature reaches 10°C, spin down the samples and add 1.2 μl of diluted Sequenase (4:1 dilution).

17. Program DNA Thermocycler to program #82
    <Stop> <Stop>
    <File> 82 <Enter>
    <Start>
(Program 82 will now ramp from 10°C to 37°C over 8 min, hold at 37°C for 8 min, heat to 94°C over 8 min.)

	1	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
Dilute buffer	0.9	5.4	6.3	7.2	8.1	9.0	9.9	10.8	11.7
Sequenase	0.3	1.8	2.1	2.4	2.7	3.0	3.3	3.6	3.9

18. Label this tube with the sample ID on the top and the date (mo/day/yr) on the side. Store unused RdA product in the small ⁻20°C Rd A freezer in the back freezer room.

II. Round B PCR amplification

1. In the Rd A hood, make Rd B master mix (See data sheet for calculations) Add another water control at this step also:

76 µl H_20
10 µl 10X PCR Buffer
4 µl 50 mM MgCl2
2 µl 12.5 mM dNTP
1 µl Accuprime Taq (Hot Start)

Master Mix	6	7	8	9	10	11	12	13
H_20	456	532	608	684	760	836	912	988
PCR Buffer	60	70	80	90	100	110	120	130
MgCl2	24	28	32	36	40	44	48	52
12.5mM dNTP	12	14	16	18	20	22	24	26
Accuprime Taq	6	7	8	9	10	11	12	13

2. Aliquot 93 µl of Rd B master mix to Rd B PCR tubes.

3. Add 1µl of tagged (barcoded) Primer B to each sample.

4. Place tagged (barcoded) Primer Bs back in freezer and **Change Gloves**.

5. Perform a quick spin of the RdA product. Add 6 µl of RdA template ($^1/_5$ of Rd A) to each PCR tube.

6. Turn on the DNA Thermocycler (#1 – 3) in the lab (GeneAmp PCR System 9700) to warm up. Program GeneAmp to hs40x:

<run>
scroll to hs40x
<start> <start>

Program hs40x:
94°C, 2 min
94°C, 30 sec
40°C, 30sec
50°C, 30sec
72°C, 1min
} 40 cycles
10°C, Soak

The run will take about 3 hr. If you leave it to run in the PCR overnight, make sure to have it hold at 10°C forever.

6. Seal Ziploc bag with tip waste and dispose of in “dirty bay” trash—NOT in TC room!!! UV irradiate RdA hood at least 20 min.

7. Transfer 10 µl of Rd B sample to a new set of tubes and use this to run a 1% agarose gel. Use Low Mass Ladder (2 µl) as reference.