

AMBION cDNA/cRNA Synthesis Protocol

DAY 1

First-Strand cDNA Synthesis

1.	A.	Total RNA/Nuclease-Free H ₂ O	11µl
	B.	T7 Oligo (dT) Primer	1µl
Total volume			12µl

- 70°C for 10min. Pulse spin, and keep on ice.

Making Master Mix, 1st Strand on Ice

2.	A.	10x 1 st Strand Buffer	2µl
	B.	Ribonuclease Inhibitor	1µl
	C.	dNTPs Mix	4µl
	D.	Reverse Transcriptase	1µl

3. Add **8µl** of mix above to sample in step 1, on ice.

4. Place sample on **42°C** of preheat PCR machine.

- **42°C** for **2hr**.

5. Pulse spin and place sample on ice for next step.

Second-Strand cDNA Synthesis

1. Making Master Mix in Order below, **ON ICE**.

A.	Nuclease-free H ₂ O	63µl
B.	10x 2 nd Strand Buffer	10µl
C.	dNTPs Mix	4µl
D.	DNA Polymerase	2 µl
E.	RNase H	1 µl
Total volume		80µl

2. Add **80µl** of above mixture to **20µl** cDNA Sample from 1st strand cDNA above.

3. Mix sample, and pulse spin.

- **16°C** for **2hr**.

4. Keep sample on ice, and proceed to cleaning up, or freeze at -20°C.

cDNA Purification

Preheat Nuclease-free H₂O at 50-60°C for ~10min.

Ethanol need to be added to cDNA Wash Buffer.

1. Place cDNA Filter Cartridge on 2ml wash tube.
2. Pipette **50µl of cDNA Binding Buffer** onto the Filter Cartridge.
3. Stand at **RTP** for **5min**, NO SPIN, to equilibrate the Filter.
4. Add **250µl of cDNA Binding Buffer** to **each cDNA sample**. Mix well.
5. Apply mixture above to equilibrate cDNA Filter.
6. Centrifuge for **~1min** at **9,800 RPM** (10,000xg).
7. Discard flow-through. No need to replace with new 2ml tubes.
8. Wash cDNA Filter Cartridge with **500µl of cDNA Wash Buffer (Ethanol need to be added)**.
9. Centrifuge **~1min** at **9,800 RPM**.
10. Discard flow-through, and spin the cartridge for an additional **1min**. Transfer Filter Cartridge **to new 2ml** tube.
11. Elude cDNA with **10µl of preheated Nuclease-free H₂O**. Let stand at **RTP** for **2min**.
12. Centrifuge **~1.5min** at **9,800 RPM**. Repeat step 11 above for a total of 20µl. But only about **16µl** of eluate was collected.
13. Put samples on ice or freeze at -20°C.

DAY 2

Biotin Labeled cRNA Synthesis

A.	cDNA Sample	16μl	(from previous step)
B.	10mM Biotin-11-CTP	7.5μl	
C.	10mM Biotin-16-UTP	7.5μl	
<hr/>			
	Total volume	~31μl	

1. Speed vacuum the samples to 18 μ l for ~3.5min. Check sample every 2 min or so.

Making IVT Master Mix at RTP in the Order below:

A.	T7 ATP Soln (75mM)	4 μ l	
B.	T7 CTP Soln (75mM)	3μl	***
C.	T7 GTP Soln (75mM)	4 μ l	
D.	T7 UTP Soln (75mM)	3μl	***
E.	T7 10x RXN Buffer	4 μ l	
F.	T7 Enzyme Mix	4 μ l	
<hr/>			
	Total volume	22μl	

2. Mix the mixture above well, and add 22 μ l to the **18 μ l cDNA/Biotin-CTP,-UTP** samples sitting at **RTP**.

➤ **37°C** for 6-14hr.

3. Add **2 μ l** of **DNase I** to each sample. Mix and pulse spin.

➤ **37°C** for **30min**.

4. Store at -20°C, or proceed to clean up.

5. **cRNA Cleaning Up**

Preheat either Elution Soln or Nuclease-free H₂O to 50-60°C for =>10min.

Add 100% Ethanol to aRNA Wash Buffer.

1. Add **60µl of normal cold Elution Soln** to cRNA sample to 100µl. Mix well.
2. **Place aRNA Filter Cartridge in aRNA Collection tube, and add 100µl aRNA Binding Buffer to center of Filter.**
3. Let stand at **RTP** for **5min**, NO SPIN.
4. Now add **350µl of aRNA Binding Buffer** to each cRNA sample. Mix well.
5. Add 250µl of 100% Ethanol to each cRNA sample. Mix well and go to the next step immediately.
6. Apply mixture to equilibrated aRNA Filter Cartridge, from steps 2-3.
7. Centrifuge for **~1min** at **9,800 RPM**.
8. Discard flow-through. No need to replace new Collection tube for aRNA.
9. Wash the aRNA Filter Cartridge with **650µl** of aRNA Wash Buffer.
10. Centrifuge at **9,800 RPM** for **1min**. Discard flow-through.
11. Spin the aRNA Filter Cartridge **for an additional 1min**. Transfer Filter Cartridge to new Collection tube.
12. Add to Filter **40µl of Elution Soln** or Nuclease-free H₂O, **preheated to 50-60°C for =>10min. Put Soln back into heater block.**
13. Let aRNA Filter Cartridge stand at **RTP** for **2-3min**.
14. Centrifuge at **9,800 RPM** for **1.5min**, and transfer to 1.5ml tube. This is **Eluate 1**.
15. Repeat with **another 80µl of Nuclease-free H₂O or Elution Soln**, steps 12-14.
This is **Eluate 2**. Keep the two elutes separate.
16. Dilute **4x**, 1µl of cRNA to 3µl of DEPC H₂O, for Agilent reading for eluate 1.