

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.	RNase 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 60°C for ~10min or more.

Ethanol needs to be added to cDNA Wash Buffer.

Check cDNA Binding Buffer for precipitates, if so warm up at 37°C.

1. Place cDNA Filter Cartridge on 2ml wash tube, if not coming with pre-assembly.
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 equilibrated cDNA Filter.
6. Centrifuge for **~1min** at **9,800 RPM** (10,000xg).
7. Discard the 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 the flow-through, and spin the cartridge for an additional **1min**. Transfer Filter Cartridge **to new 1ml cDNA Elution 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 another 10µl of **preheated Nuclease-free H₂O**. But only about **~16µl** of eluate was collected.
13. Put samples on ice for next step or freeze at -20°C for later cRNA labeling.

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	
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	Total volume	~31μl	

1. Speed vacuum the samples to **18 μ l** for **~3-6min.**

Making In Vitro Transcription (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	
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	Total volume	22 μ l	

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

➤ **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.

Notes: I make my IVT Master Mix on ice. First I thaw the reagents, and then keep everything on ice except the T7 10x RXN Buffer at RTP. Warm the 10x Buffer at 37°C if precipitates form. Add the 10x Buffer to the entire mix on ice and aliquot to the 0.5ml RXN samples on ice.

cRNA Cleaning Up

Preheat either Elution Solution or Nuclease-free H₂O to 60°C for 10min or so.

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. Warm aRNA Binding Buffer if precipitates form. **aRNA Binding Buffer contains β-Mercapto Ethanol, work in the fume hood and collect waste in Buffer RLT bottle. They have similar components. Ambion has said all the wastes can go down the sink, but call Ambion if there is a concern in their reagents.**
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 transfer to the equilibrated aRNA Filter Cartridge from steps 2-3 above.
6. Centrifuge for **~1min** at **9,800 RPM**.
7. Discard the flow-through. No need to replace new Collection tube for aRNA.
8. Wash the aRNA Filter Cartridge with **650µl** of aRNA Wash Buffer.
9. Centrifuge at **9,800 RPM** for **1min**. Discard the flow-through.
10. Spin the aRNA Filter Cartridge **for an additional 1min**. Transfer Filter Cartridge to new aRNA Collection tube.
11. Add to the Filter with **40µl of Elution Soln** or Nuclease-free H₂O, **preheated to 60°C for ~10min or more. Put Soln back into heating block.**
12. Let aRNA Filter Cartridge stand at **RTP** for **2-3min**.
13. Centrifuge at **9,800 RPM** for **1.5min**, and transfer to 1.5ml tube. This is **Eluate 1**.
14. Repeat steps 11-13 with **another 80µl of Elution Soln** or Nuclease-free H₂O. This is **Eluate 2**. Keep the two elutes separate freezing at -80°C.

15. Dilute **4x**, 1 μ l of cRNA to 3 μ l of DEPC H₂O, for Agilent reading for eluate 1.