Material

MessageAmp aRNA Kit; Ambion, 1750

5M NH4OAC Buffer Kit; Ambion, 9010

Procedure

A. First strand cDNA Synthesis from total RNA or mRNA (time 1:30)

1. Thaw all necessary components and place them on ice
2. For 20 µl total RT reaction volume, we recommend using a thinwalled 0,5ml PCR tube and a thermal cycler for the first strand synthesis. Keep RNA always on ice and froze them immediately to –80°C until use.
3. Pipet the following components together and mix them well.

Components	Volume	Final Concentration
total RNA (mRNA)	x µl (max.11µl)	0,1 – 5 µg (0,10 to 1 µg)
T7 Oligo (dT) Primer	1 µl	
DEPC-H_2O	total volume of 12 µl	

Note: It is recommended to perform amplification experiments with constant amount of input RNA for all experimental samples in a project (eg: 2µg total RNA or 200ng mRNA).

4. Incubate at **70°C for 10 min** in a thermal cycler, then place the tube immediately on ice.
5. Pipet the following components in a master mix tube and check the volume.

Components	Volume 1x	Final Concentration
10 x First Strand Buffer	2 µl	1 x
Ribonuclease Inhibitor	1 µl	
dNTP-Mix	4 µl	

6. From the master mix, add 1x Volume (7µl) to the 12µl RNA/Primer reaction tube.
7. Mix gently and incubate in a thermal cycler at **42°C for 2min.**
8. Add **1µl Reverse Transcriptase** to each tube.
9. Mix gently and incubate in a thermal cycler at **42°C for 1 hours**
10. Starting immediately second strand synthesis, until, hold cDNA on ice.

B. Second strand synthesis (time 2:10)

1. Thaw all required components, mix them and place on ice.
2. On ice, pipet into master mix tube following components for second strand synthesis.and check the volume.

Components	Volume	Final Concentration
Nuclease-free water	63 µl	
10 x Second Strand Buffer	10 µl	1x
dNTP-Mix	4 µl	
DNA Polymerase	2 µl	
RNase H	1 µl	

3. From the master mix, add 1x Volume (80µl) to the 20µl first strand cDNA.
4. Mix gently and incubate at **16°C for 2 hours**.
5. Keep cDNA on ice until purification.

C. cDNA Purification (time 30min)

Kit contenting Filter Cartridges should centrifugate in all sections at 10.000g.

1. Preheat 5 ml Nuclease-free Water to 50°C for at least 10min
2. Equilibrating of cDNA Filter Cartridges: seated in 2 ml Wash Tubes and add 50 µl Binding Buffer onto the filters. Incubate at RT for 5 min.
3. Add 250 µl of cDNA Binding Buffer to each cDNA sample, mix gentle.
4. Pipet the cDNA/cDNA Binding Buffer onto the center of equilibrated filters. Centrifugate for 1 min at 10.000xg.
5. Discard the flow-through and replace filters in new 2 ml Wash Tubes.
6. Apply 500µl Wash Buffer (add 11,2 ml Ethanol before use) to each Filter, centrifugate 1min at 10.000g; discard flow-through and spin filter for additional 1 min to remove trace amounts of ethanol.
7. Transfer the filter to a cDNA elution tube (1.5 ml); apply 10 µl of preheated Nuclease-free water (50°C); leave it at RT for 2 min and centrifugate for 1,5 min at 10.000g; repeat the step with a second 10 µl water; Control the elution volume and bring each sample to volume of 16 µl (often 17-18µl, so take 16µl for IVT).
8. cDNA Samples should be stored at –20°C

D. In vitro Transcription to synthesize aRNA (time 6-14 hours)

9. Thaw all required components, mix them and place on ice.
10. On ice, pipet into master mix tube following components for the IVT (unmodified) and check the volume.

Components	Volume	Final Concentration
Each T7 NTP (75nM)	4 µl each	7.5 mM
T7 10 x Reaction Buffer	4 µl	1 x
T7 Enzyme Mix	4 µl	

11. From the master mix, add **1x Volume (24µl) to the 16µl cDNA**.
12. Mix and centrifugate the tube, incubate at **37°C for 6-14 hours.**add immediately **2 µl Dnase I** to each reaction mix, incubate at **37°C for 30 min** (continue immediately with next step (aRNA purification))

E. aRNA Purification (time 2:30)

13. add **60 µl elution solution/ or Nuclease-free water** to each sample to bring the final volume to 100 µl.
14. Keep aRNA on ice until purification.
15. Preheat 5 ml Nuclease-free Water to 50-60°C for at least 10min.
16. Equilibrating of aRNA Filter Cartridges: seated in aRNA collection tube and add 100 µl aRNA Binding Buffer onto the filters. Incubate at RT for 5 min
17. Add 350 µl of aRNA Binding Buffer to each aRNA sample, mix gentle.
18. Add 250 µl of 100% Ethanol to each aRNA sample, mix gentle and proceed immediately to the next step (attention:RNA precipitate after short time pipet all precipitates onto filter)
19. Pipet each sample mixture onto the center of equilibrated an aRNA filter. Centrifugate for 1 min at 10.000xg.
20. Do not Discard the flow-through (store in the collection tube until RNA is tested by Agilent-Chip)and replace filters in new aRNA collection tube.
21. Apply 650µl aRNA Wash Buffer (add 11,2 ml Ethanol before use) to each aRNA Filter, centrifugate 1 min at 10.000xg; discard flow-through and spin filter for additional 1 min to remove trace amounts of ethanol.
22. Transfer the filter to a fresh cDNA/aRNA collection tube; apply 50 µl of preheated Nuclease-free water (50°C); leave it at RT for 2 min and centrifugate for 1,5 min at 10.000xg; repeat the step with a second 50 µl water. End volume is about 90µl.
23. Measure concentration of aRNA dilution in Nanodrop and Agilent (optimal: 200ng). (In case of 2µg total RNA as amplification input, between 50-100µg aRNA would be expected. Make a dilution of 1:5 or 1:10 out of the aRNA eluat).

24. RNA storage in –80°C

F. Optional in case of low RNA concentrations:

25. For concentrating aRNA, precipitate samples with 1/10 Volume 5M NH4OAC (10µl) and 2,5 Volumes of 100% Ethanol (250 µl) to incubate at –20°C for minimal 1h.
26. Centrifugate at 4°C for 20min, remove Ethanol, wash the pellet with 500µl 70% Ethanol and repeat centrifugation step for further 5min.
27. Remove Ethanol exactly (first 200µl than 20µl Pipet) and dry the pellet at RT (check ethanol drops, RNA pellet should become clear, repeat this step if necessary).
28. Solve aRNA in 21 µl Nuclease-free water and incubate 5min at 50°C.
29. In case of amplifying 2µg total RNA, dilute aRNA 1µl ad 10µl with Nuclease-free water (1:10) and measure 1µl with Nanodrop (and if necessary with Agilent Bioanalyzer).
30. RNA storage in –80°C

Version	Tracking of changes	Name	Date