

Microarray protocol

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RNAqueous

- 1) Pellet cells and remove media
- 2) Add 200 μ L of Lysis/binding buffer from RNAqueous kit. (STOP)
- 3) Heat an aliquot of elution solution \sim 70 μ L/sample to 100° C
- 4) Add equal volume of 64% EtOH to lysate and mix
- 5) Apply the lysate/EtOH mixture to the RNAqueous filter cartridge
- 6) Centrifuge for 1 min on 12000 g
- 7) Discard flow through
- 8) Wash with 700 μ L of wash solution #1
- 9) Centrifuge for 1 min on 12000g
- 10) Wash twice with 500 μ L of wash solution #2/3
- 11) Spin dry
- 12) Elute RNA using 2x 38.5 μ L into a fresh collection tube at 12000 g for 1 min

DNase Treatment

- 1) To the RNA solution add the following reagents to scale to 100 μ L
 - 10 μ L DNase
 - 10 μ L 10x DNase buffer
 - 3 μ L RNase Inhibitor
- 2) Incubate for 30 min at RT
- 3) Stop the reaction with 10 μ L of EDTA

Phenol:chloroform:isoamyl extraction

- 1) Centrifuge the phase lock tubes for 1 min at 5000 g
- 2) To the RNAqueous isolates add 100 μ L of shaken p:c:I solution
- 3) Apply to phase lock tubes and centrifuge for 5 min at 15000g at RT
- 4) Place top layer into a fresh tube then add .5 μ L of glycogen, 40 μ L of 3M NaOAc, and 300 μ L of cold 100% EtOH
- 5) Place samples in -80° C for a minimum of 2 hours. (STOP)
- 6) Centrifuge sample for 20 minutes at 15000g
- 7) Wash pellet in 75% EtOH and centrifuge for 10 min at 15000g
- 8) Remove all ethanol with a pipette and air dry briefly.
- 9) Resuspend in 10 μ L nuclease free water.

MessageAmp aRNA Synthesis

First Strand

- 1) Mix RNA and 1 μ L T7 Oligo (dT) Primer and bring the volume to 12 μ L with nuclease free water.
- 2) Incubate 10 minutes at 70° C (melt 70) and place at 42° C
- 3) In a second tube prepare the RT master mix and incubate at 42° C for 2 minutes:
 - 2 μ L 10x First Strand Buffer
 - 1 μ L Ribonuclease Inhibitor
 - 4 μ L dNTP Mix
- 4) Transfer 7 μ L of RT master mix to each RNA sample (keep @ 42° C)
- 5) Add 1 μ L of reverse transcriptase
- 6) Incubate for 2 hours at 42° C on PCR block (42 C)

Second Strand

- 1) Add the following to the cDNA sample (20 μ L) from above:
 - 63 μ L Nuclease-free water
 - 10 μ L 10x Second strand buffer
 - 4 μ L dNTP mix
 - 2 μ L DNA polymerase
 - 1 μ L RNase H
- 2) Incubate mixture for 2 hours at 16° C (ME 2nd)

cDNA Purification

- 1) Preheat nuclease-free water (from kit) to 50° C for at least 10 min
- 2) Preheat binding buffer to 37° C to dissolve crystals
- 3) Place filter cartridge in a 2 mL tube and add 100 μ L of cDNA binding buffer and let sit at RT for 5 min
- 4) Add 250 μ L of cDNA binding buffer to each cDNA sample
- 5) Apply the cDNA mixture to the filters and centrifuge at 10,000g for ~1min
- 6) Discard the flow through
- 7) Wash the filter with 650 μ L of cDNA wash buffer
- 8) Spin Dry
- 9) Elute cDNA twice with 50 μ L of 50° C nuclease-free water
- 10) Concentrate the purified cDNA to \leq 8 μ L
- 11) Adjust the volume to 8 μ L (STOP)

In Vitro Transcription of aRNA- Unmodified aRNA

- 1) To the 8 μ L double stranded cDNA tube add the following reagents and mix
 - 2 μ L T7 ATP soln (75 mM)
 - 2 μ L T7 CTP soln (75 mM)
 - 2 μ L T7 GTP soln (75 mM)
 - 2 μ L T7 UTP soln (75 mM)
 - 2 μ L T7 10x reaction buffer

- 2 μL T7 enzyme mix
- 1 μL Nuclease-free water

- 2) Incubate the reaction for 6-14 hours at 37° C
- 3) Add 2 μL DNase I to each reaction, mix, and incubate 30 min at 37° C

aRNA purification

- 1) Preheat nuclease-free water or aRNA elution solution to 50° C for 10 min
- 2) Place a filter cartridge in a 2 mL tube and add 100 μL of aRNA binding buffer
- 3) Add 78 μL of aRNA elution solution to each aRNA sample
- 4) Add 350 μL of aRNA binding buffer to each sample
- 5) Add 250 μL of 100% EtOH
- 6) Apply mixture to filter and centrifuge for 1 min at 10,000 g
- 7) Wash filter with 650 μL of aRNA wash buffer and centrifuge for 1 min at 10,000 g
- 8) Spin dry
- 9) Elute aRNA with 2x 50 μL of heated nuclease-free water or aRNA elution solution

Second Round of Amplification

Synthesis of First-Strand cDNA

- 1) Reduce volume of aRNA to 10 μL
- 2) Add 2 μL of second round primers (final volume of 12 μL)
- 3) Incubate mixture at 70° C for 10 min
- 4) At RT add the following mixture to the aRNA/Primer mixture:
 - 2 μL 10x first strand buffer
 - 1 μL Ribonuclease inhibitor
 - 4 μL dNTP mix
 - 1 μL Reverse transcriptase
- 5) Incubate the first strand synthesis mixture at 42° C for 2 hours (program?)
- 6) Add 1 μL RNase H and incubate at 37° C for 30 minutes
- 7) Add 5 μL T7 Oligo(dT) primer and incubate at 70° C for 10 minutes
- 8) Add the second strand cDNA synthesis reagents below to the first strand synthesis mixture for a total volume of 100 μL
 - 26 μL First strand mixture
 - 58 μL Nuclease-free water
 - 10 μL 10x second strand buffer
 - 4 μL 5 mM dNTP mix
 - 2 μL DNA polymerase
- 7) Incubate the second strand synthesis mixture at 16° C for 2 hours (ME 2nd)

cDNA Purification

- 1) Preheat nuclease-free water to 50° C for at least 10 min
- 2) Place filter cartridge in a 2 mL tube and add 100 μL of cDNA binding buffer and let sit at RT for 5 min

- 3) Add 250 μL of cDNA binding buffer to each cDNA sample
- 4) Apply the cDNA mixture to the filters and centrifuge at 10,000g for ~1min
- 5) Discard the flow through
- 6) Wash the filter with 650 μL of cDNA wash buffer
- 7) Spin dry
- 8) Elute cDNA twice with 50 μL of 50° C nuclease-free water
- 9) Concentrate the purified cDNA to $\leq 8 \mu\text{L}$
- 10) Adjust the volume to 8 μL

In Vitro Transcription of aRNA – Biotin aRNA

- 1) For each sample add 3.75 μL of 10 mM biotin-11-CTP and 3.75 μL of biotin-16-UTP in a nuclease-free tube and dry to completion.
- 2) To the dried biotin tube add the following reagents and mix
 - 8 μL double stranded cDNA
 - 2 μL T7 ATP soln (75 mM)
 - 1.5 μL T7 CTP soln (75 mM)
 - 2 μL T7 GTP soln (75 mM)
 - 1.5 μL T7 UTP soln (75 mM)
 - 2 μL T7 10x reaction buffer
 - 2 μL T7 enzyme mix
 - 1 μL Nuclease-free water

- 3) Incubate the reaction for 6-14 hours at 37° C
- 4) Add 2 μL DNase I to each reaction, mix, and incubate 30 min at 37° C

aRNA purification

- 1) Preheat nuclease-free water or aRNA elution solution to 50° C for 10 min
- 2) Place a filter cartridge in a 2 mL tube and add 100 μL of aRNA binding buffer
- 3) Add 78 μL of aRNA elution solution to each aRNA sample
- 4) Add 350 μL of aRNA binding buffer to each sample
- 5) Add 250 μL of 100% EtOH
- 6) Apply mixture to filter and centrifuge for 1 min at 10,000 g
- 7) Wash filter with 650 μL of aRNA wash buffer and centrifuge for 1 min at 10,000 g
- 8) Spin Dry
- 9) Elute aRNA with 2x 50 μL of heated nuclease-free water or aRNA elution solution

aRNA Fragmentation

- 1) Spec aRNA to determine concentration
- 2) Set concentration to at or above .6 $\mu\text{g}/\mu\text{L}$ (final concentration of 20 μg of aRNA)
- 3) Add 5x fragmentation buffer and water to a maximum of 40 μL
- 4) Incubate at 94° C for 35 minutes and place on ice to stop