\*Begin with 100ng-1000ng of RNA – 5uL max for each sample

 - If sample exceeds 5uL, may vacuum-dry the sample down to 5uL

Heat samples at 70C for 5 minutes

***Prepare polyadenylation master mix***

|  |  |  |
| --- | --- | --- |
|  |  |  1X |
| NF Water |  | 1.5 |
| 10X Poly(A) Tailing Buffer | 1 |
| Rnase Inhibitor |  | 1 |
| Poly(A) Tailing ATP | 0.5 |
| PAP |  | 1 |
| \*Pipette MM up & down to mix, centrifuge, place on ice |
| Add 5uL of MM to each RNA sample. |
| Pipette up and down 2-3 times, centrifuge |
|

|  |
| --- |
| Place samples in the thermo cycler: |
| **Temp** | **Time** | **Cycles** |
| 37C (lid off) | 15 min | 1 |
| 4C | forever |  |
|  |  |  |

 |

***Reverse Transcription (1st strand cDNA Synthesis) – Assemble at room temperature***

|  |  |  |
| --- | --- | --- |
|  |  | 1X |
| NF Water |  | 3 |
| T7 Oligo(dT) Primer | 1 |
| 10X First Strand Buffer | 1 |
| dNTP Mix |  | 4 |
| ArrayScript |  | 1 |
| \*Pipette MM up & down to mix, centrifuge, place on ice |
| Add 10uL of MM to each RNA sample. |
| Pipette up and down 2-3 times, centrifuge |
|  |  |  |
| Place samples in the thermo cycler: |
| **Temp** | **Time** | **Cycles** |
| 42C (lid off) | 2 hrs. | 1 |
| 4C | forever |  |

***2nd strand cDNA Synthesis – Assemble on ice***

|  |  |  |
| --- | --- | --- |
|  |  |  1X |
| NF Water |  | 63 |
| 10X Second Strand Buffer | 10 |
| dNTP Mix |  | 4 |
| DNA Polymerase |  | 2 |
| RNase H |  | 1 |
| \*Pipette MM up & down to mix, centrifuge, place on ice |
| Add 80uL of MM to each sample. |
| Pipette up and down 2-3 times, centrifuge |
| Place samples in the thermo cycler: |
|  |  |  |
| **Temp** | **Time** | **Cycles** |
| 16C (lid off) | 2 hrs. | 1 |
| 4C | Forever |  |
| \*Place on ice immediately after, or -20 freezer…for no more than 1hr  |

***cDNA Purification***

|  |
| --- |
| \*All centrifugation at 10,000g |
| Preheat H2O to 55C – 18uL/sample |
| \*Make sure precipitate is not visible in cDNA Binding Buffer, or else warm to 37C |  |
| Add 250uL of cDNA Binding Buffer to each sample. |
| Pipette up and down 2-3 times, centrifuge |
| Proceed quickly to next step: |
| Pipet samples onto the center of the cDNA Filter Cartridge |
| Centrifuge for 1 min and discard flow-through. |
|

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| Add 500uL of Wash Buffer to each cartridge. |
| Centrifuge for 1 min and discard flow-through. |
| Centrifuge for 1 min again and transfer filter to cDNA elution tube. |

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| Apply 18uL of preheated NF water to center of filter |
| Leave at room temp for 2 minutes and centrifuge for 1 min (~16uL) |
| \*Double-stranded cDNA can be stored at -20C overnight if necessary |

|  |
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| ***In Vitro Transcription to Synthesize Amino Allyl-Modified aRNA – Prepare at room temp.*** |
| Pipette 16uL of each sample into PCR tubes. |
|  |
|

|  |  |  |
| --- | --- | --- |
|  |  |  1X |
| aaUTP (50 mM) |  | 3 |
| ATP, CTP, GTP Mix |  | 12 |
| UTP Solution (50mM) | 3 |
| T7 10X Reaction Buffer | 4 |
| T7 Enzyme Mix |  | 4 |
| \*Pipette up and down to mix, centrifuge, and place on ice. |
| Transfer 26uL of IVT MM to each sample. |
| Pipette up & down 2-3 times, flick 3-4 times, centrifuge |
| Place samples in the thermo cycler: |
| **Temp** | **Time** | **Cycles** |
| 37C (lid: 100-105C) | 4-14hrs | 1 |
| 4C | Forever |  |

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| Add 58uL NF water to each aRNA sample (final vol=100uL) |
| Transfer to 1.5mL NF tube |  |
| \*May store at -20C freezer |  |
|  |  |  |
| ***aRNA Purification*** |  |  |
| \*All centrifugation at 10,000g |
|  |  |  |
|  |  |

 |  |  |
| Add 350uL of aRNA Binding Buffer to each aRNA sample. |
| \*Proceed immediately to next step |
|  |
| Add 250uL of 100% ethanol to each aRNA sample and pipette up and down 3 times.  |
| \*Do not vortex or centrifuge |
| \*Proceed immediately to next step |

|  |
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| Pipet each sample onto the center of the aRNA filter |
| Centrifuge for 1 minute and discard flow-through |
|  |
| Apply 650uL Wash buffer to each aRNA Filter cartridge |
| Centrifuge for 1 minute, discard flow-through, and centrifuge again for 1 minute. |
| Transfer filter to a fresh aRNA Collection Tube. |
| Add 200uL of NF water to center of filter. |
| Incubate the samples at 55C for 10 min |
| Centrifuge for 1.5 min |

\*Bioanalyze or nandrop samples to verify the synthesis of aRNA.

May store in -20C at this point

***Dye-Coupling Reaction***

|  |
| --- |
| Add 11uL of DMSO to each Cy3 or Cy5 dye and vortex |
| \*Keep in dark for up to an hourPlace 5-20ug of amino allyl-modified aRNA into individual wells of a 96-well plate and vacuum-  |

 dry until no liquid remains. Be careful not to over-dry!

|  |
| --- |
| Add 9uL Coupling Buffer and vortex gently |
| Add 11uL of prepared dye and vortex gently (Cover with aluminum foil) |
| Incubate 30 min at room temp in the dark |
| Add 4.5uL 4M Hydroxylamine and vortex gently |
| Incubate 15 min at room temp in the dark |
| Add 5.5uL NF water to each sample (Final vol = 30uL) |

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| ***Dye Labeled aRNA Purification*** |
| \*All centrifugation at 10,000g |
| Preheat H2O to 50-60C – 20uL/sample |
|  |
| Add 105uL of aRNA Binding Buffer to each aRNA sample in new eppendorf tubes |
| \*Proceed immediately to next step! |
| Add 75uL of 100% ethanol to each sample, and pipette up and down 3 times |
| \*Do not centrifuge or vortex! |
| Proceed immediately to next step

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| --- |
| Pipet each sample onto center of filter in aRNA Filter Cartridge |
| Centrifuge for 1 min and discard flow-through

|  |
| --- |
| Apply 500uL Wash Buffer to each labeled aRNA Filter Cartridge. |
| Centrifuge for 1 min, discard flow-through and centrifuge again for 1 min. |
| Transfer labeled aRNA filter carridge to a labeled elution tube. |
| Add 10uL of NF water (preheated) to center of filter and leave at room temp for 2 min. |
| Centrifuge for 1.5 min and add 10uL of preheated water again (repeat previous steps) |
| \*Labeled aRNA now in NF water (~20uL)Nanodrop and record concentrations and dye-intensities of each sample\*May store overnight in -20C freezer |

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