**Part 1: SmartSeq**

* **INPUT:** total RNA, ranging between picograms to 10ng. 2.6uL will be used per sample.
* **ANNEAL PRIMERS:** ON ICE, add each sample (2.6uL total RNA) to a thin-walled 0.2mL PCR tube and add:

|  |  |  |  |
| --- | --- | --- | --- |
| **Item** | **Volume (uL)** | **\_\_\_\_\_\_xMM** | **Lot#** |
| oligo-dT primer (10uM) | 1 |  |  |
| dNTP mix (10mM) | 1 |  |  |

* + Mix, spin down, **incubate @72C for 3 minutes**, spin down, put on ice
* REVERSE TRANSCRIPTION: add the following ON ICE:

|  |  |  |  |
| --- | --- | --- | --- |
| **Item** | **Volume(uL)** | **\_\_\_\_\_\_xMM** | **Lot#** |
| SuperScriptII reverse transcriptase (200U/uL) | 0.50 |  |  |
| RNAse inhibitor (40U/uL) | 0.25 |  |  |
| SuperscriptII first-strand buffer (5x) | 2 |  |  |
| DTT (100mM) | 0.50 |  |  |
| Betaine(5M)\* | 2 |  |  |
| Nuclease-free H2O | 0.06 |  |  |
| TSO(100uM)\*\* | 0.10 |  |  |
| **Total volume:** | 5.41 |  | N/A |

\*stored at 4C

\*\*template-switching oligos, stored in -80C

* Mix, spin down, and incubate in thermocycler on following settings:

|  |  |  |
| --- | --- | --- |
| **Step** | **Temp(C)** | **Time(hh:mm:ss)** |
| 1 | 42 | 01:30:00 |
| 2 | 50 | 00:02:00 |
| 3 | 42 | 00:02:00 |
| 4 | Return to step 2, 5x |  |
| 5 | 70 | 00:15:00 |
| 6 | 4 | hold |

* PCR PRE-AMPLIFICATION: Prepare Master Mix during Reverse Transcription rxn.

|  |  |  |  |
| --- | --- | --- | --- |
| **Item** | **Volume(uL)** | **\_\_\_\_\_\_xMM** | **Lot#** |
| First-strand rxn (previous step) | 10 |  | N/A |
| Kapa HiFi HotStart ReadyMix (2x) | 12.5 |  |  |
| IS PCR primers (10uM) | 0.25 |  |  |
| H2O | 2.25 |  |  |
| **Total volume:** | 25 |  | N/A |

* Add 15uL PreAmp Master Mix to each sample, seal, mix, spin down, and incubate in thermocycler on following settings:

|  |  |  |
| --- | --- | --- |
| **Step** | **Temp(C)** | **Time(hh:mm:ss)** |
| 1 | 98 | 00:03:00 |
| 2 | 98 | 00:00:20 |
| 3 | 67 | 00:00:15 |
| 4 | 72 | 00:06:00 |
| 5 | Return to step 2 | 17x (18 cycles total) |
| 6 | 72 | 00:05:00 |
| 7 | 4 | hold |

* Do a 1:1 ratio bead cleanup (add 25uL beads to each sample)
	+ 5 minute bead incubation
	+ 80% EtOH wash 1
	+ 80% EtOH wash 2
	+ Elute with 17.5uL H2O, keep 15uL
* Quant the cleaned cDNA on Tapestation, using D5000 reagents

**Part 2: Custom QXT**

**A: Fragmentation and Adaptor-tagging**

* **FIRST, IF POSSIBLE:** Normalize cDNA samples to 2x the input mass (for 1ng cDNA input will be 2ng/10.13uL, for the 5ng input will be 10ng/10.13uL). Run quants (picogreen) to assess concentration. If normalization was successful, add one volume of water to the samples to bring it back to 1x concentration and continue with 10.16uL.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **1ng cDNA input:*** **INPUT**: 1ng cDNA in 10.16uL water
* **QXT DILUTION:** **1:20** dilution of QXT enzyme:Storage Soln., at least 2uL of dilution/sample
* **MASTER MIX:**

|  |  |  |
| --- | --- | --- |
| **Reagent** | **1 rxn (uL)** | **\_\_\_\_rxn** |
| cDNA | 10.16 | N/A |
| 5x TD Buffer | 8.84 |  |
| QXTEnzyme | 0.1 |  |
| StorageSoln | 1.9 |  |
| **Total:** | 21 |  |

 | **5ng cDNA input:*** **INPUT**: 5ng cDNA in 10.16uL water
* **QXT DILUTION:** **1:6** dilution of QXT enzyme:Storage Soln., at least 2uL of dilution/sample
* **MASTER MIX:**

|  |  |  |
| --- | --- | --- |
| **Reagent** | **1 rxn (uL)** | **\_\_\_\_rxn** |
| cDNA | 10.16 | N/A |
| 5x TD Buffer | 8.84 |  |
| QXTEnzyme | 0.33 |  |
| StorageSoln | 1.67 |  |
| **Total:** | 21 |  |

 |

* Place samples in thermocycler on “DNA Fragmentation” program:

|  |  |  |
| --- | --- | --- |
| **Step** | **Temp(C)** | **Time(hh:mm:ss)** |
| 1 | 55 | 00:10:00 |
| 2 | 4 | 00:01:00 |
| 3 | 4 | Hold |

* Add 5uL 0.2%SDS and 24uL water/sample

|  |  |  |
| --- | --- | --- |
| **Reagent** | **1 rxn (uL)** | **\_\_\_\_rxn (uL)** |
| 0.2%SDS | 5 |  |
| H2O | 24 |  |
| **Totals:** | 29 |  |

* Seal, vortex, incubate at RT for 1 minute.

**B: Ampure bead purification 1**

|  |  |
| --- | --- |
| **Single cleanup*** Vortex beads and incubate @ RT for 30minutes
* Add 49uL bead mix/sample (1.0x)
* Seal samples, vortex, briefly spin down
* Incubate samples @RT 5 minutes
* Put samples on magnet rack, allow to clear, discard supernatant
* Wash 1 with 200uL 80% EtOH
* Wash 2 with 200uL 80% EtOH
* Remove last of the EtOH, allow samples to air dry for at least 10 minutes.
* Elute with 24uL H2O, incubate for 2 mins @RT
 | **Double SPRI cleanup** **(experimental)*** Vortex beads and equilibrate @ RT for 30minutes
* **0.5x cleanup (>600bp fragment removal):** Add 25uL beads to each 50uL sample, vortex, briefly spin
* Incubate samples 5 mins @RT
* Add samples to magnet rack, allow solution to clear, **save supernatant to new clean tube** (optional: save beads)
* **0.2x cleanup** (removal of <150bp fragments, rounding out total cleanup to 0.7x): Add 10uL bead mix/sample, seal, mix, incubate for 5 mins @RT
* Put samples on magnet rack, allow to clear, discard supernatant
* Wash 1 with 200uL 80% EtOH
* Wash 2 with 200uL 80% EtOH
* Remove the last Ethanol and allow samples to air dry for at least 10 minutes
* Elute with 24uL H2O, incubate for 2 mins @RT
 |

**C: Amplification**

* Add 24uL Kapa Hotstart Master Mix/sample
* Add 1uL i7 primer and 1uL i5 indexing primer, or 2uL from a multiplex plate. **NOTE THE USE OF CUSTOM PRIMERS[[1]](#footnote-1)**
* Seal, vortex, briefly spin down samples, put in thermocycler on “Pre-Capture PCR” protocol

|  |  |  |
| --- | --- | --- |
| **Step** | **Temp(C)** | **Time(hh:mm:ss)** |
| 1 | 68 | 00:02:00 |
| 2 | 98 | 00:02:00 |
| 3 | 98 | 00:00:30 |
| 4 | 57 | 00:00:30 |
| 5 | 72 | 00:01:00 |
| 6 | Return to step 3 |

|  |  |
| --- | --- |
| **13x for 1ng cDNA inputs** | **11x for 5ng cDNA inputs**  |

 |
| 7 | 72 | 00:05:00 |
| 8 | 4 | hold |

**D: Ampure bead purification 2**

* Make sure beads are equilibrated to room temp
* Add 32.5-35uL Ampure beads/sample (0.65x-0.7x beads:sample ratio), seal, mix, brief spin
* Incubate sample/bead mix @RT for 5 minutes
* Move to magnet stand, allow solution to clear, discard supernatant
* Wash 1 with 200uL of 80% EtOH
* Wash 2 with 200uL of 80% EtOH
* Elute in 20uL water. Add water to samples, take off magnet, seal, mix, spin, incubate @RT for 2 minutes
* Transfer samples back to magnet stand, allow to clear, save supernatant in separate, clean tubes.
* REPEAT Ampure bead purification 2 for a total of 2x bead cleanups
* Evaluate on Tapestation
1. **Avoid S511 QXT i5 primer** [↑](#footnote-ref-1)