*Preparing RNA for RNA-Seq*

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Adapted from E. Bess

Adapted from Jordan Bisanz.

**Total RNA Extraction**

|  |  |
| --- | --- |
| Reagents: | Location |
| TRI Reagent (Sigma Aldrich Cat # T9424) | Flammables |
| PureLink RNA Mini Kit (Life Technologies Cat # 12183025) | Kit Cabinet |
| PureLink DNase Set (Life Technologies Cat # 12185010) | 4**°**C |
| Bead-beating tubes with glass bead and lysing matrix E (MP Biomedicals, 116914050) | Kit Cabinet |
| Chloroform (Sigma Cat# 288306-1L) | Flammables |
| 100% ethanol | Flammables |
| TURBO DNase (Ambion; ThermoFisher Cat# AM2238) | -20**°**C |
| Agencourt RNA Clean XP Beads (Cat# A63987) | 4**°**C |
| AMPure XP beads (cat # A63881) | 4**°**C |
| RNAse Zap (cat # AM9780M) | Kit Cabinet |
| RiboZero (24 reactions) MRZB12424 | 4**°**C & **-80°C** |
| Kapa Biosystems Quantification (KK4824) | -20**°**C |
| Qubit reagents | 4**°**C and RT |
| Bioanalyzer RNA Nano chips and kit (or TapeStation RNA tape) | 4**°**C and RT |
| Bioanalyzer hsDNA chips and kit (or TapeStation hsDNA tape) | 4**°**C and RT |

**Part I. Extraction of Total RNA**

*For steps 1 – 6, work in the fume hood to contain the vapors. Trizol and chloroform are toxic irritants, so be careful when working with them. Dispose of any plastics that have been in contact with Trizol/chloroform in the hazardous waste containers in the fume hood.*

1. To a bacterial pellet, add 1 mL of TRI Reagent and vortex to resuspend. Incubate samples at room temperature for 10 minutes.
2. Transfer cell suspension to 2 mL Lysing Matrix E tubes (MP Biomedicals, 116914050), which contain glass beads. Lyse and homogenize in bead-beater for 5 minutes at room temperature.
3. Add 200 uL of chloroform to each sample. Vortex samples for 15 seconds each and incubate at room temperature for 10 minutes.
4. Centrifuge mixture at 16,000 x *g* for 15 minutes at 4 °C.
5. Transfer 500 uL of the upper aqueous phase to a new RNase-free microcentrifuge tube.
6. Add 500 uL of 100% ethanol to the new tube that contains the aqueous phase, and vortex to mix well.
7. Transfer 500 uL of the new mixture to a Purelink spin column and spin at ≥ 12,000 x *g* for 30 seconds. Discard flow through.
8. Repeat step 7 until all of the water/ethanol mixture has been applied to the column.
9. Add 350 uL of wash buffer I to the spin column and centrifuge at ≥ 12,000 x *g* for 30 seconds. Discard flow through.
10. Add 80 uL of PureLink DNase mix (see below; in 4**°**C). Incubate at room temperature for 15 minutes.

|  |  |  |
| --- | --- | --- |
|  | 1 reaction | To make \_\_\_\_\_\_ rxn |
| 10x reaction buffer | 8 ul |  |
| DNase | 10 ul |  |
| RNase-free water | 62 ul |  |

1. Add 350 uL wash buffer I, and centrifuge for 30 seconds at ≥ 12,000 x *g*.
2. Move the column to a new collection tube.
3. Add 500 uL wash buffer II (with EtOH added upon opening the kit), and centrifuge for 30 seconds at ≥ 12,000 x *g*.
4. Discard flow through. Repeat step 13, discarding flow through.
5. Centrifuge the column for 1 minute at ≥ 12,000 x *g* to dry the column.
6. Move the column into a recovery tube and add 50 uL RNase-free water.
7. Incubate at room temperature for 1 minute.
8. Centrifuge for 1 minute at ≥ 12,000 x *g* and discard the column, keeping the flow-through, which contains purified total RNA.

*You can stop here and store the samples in -80****°****C.*

**Part II. DNAse treatment and RNA purification**

*(taken from PureLink RNA Mini Kit instructions manual)*

1. Add 6 uL of TURBO DNAse in buffer to each sample. See master mix formula below.

|  |  |  |
| --- | --- | --- |
|  | 1 reaction | To make \_\_\_\_\_\_ rxn |
| 10x TURBO-DNAse buffer | 5 ul |  |
| TURBO-DNAse | 1 ul |  |

1. Incubate at 37°C for 30 min.
2. To one volume of liquid sample (56 uL), add one volume of Lysis Buffer (56 uL) and one volume of 100% ethanol (56 uL).
3. Mix by vortexing.
4. Transfer sample to a spin cartridge with collection tube.
5. Centrifuge at 12,000 x *g* for 30 seconds at room temperature. Discard the flow-through.
6. Add 350 uL wash buffer I, and centrifuge for 30 seconds at ≥ 12,000 x *g*.
7. Move the column to a new collection tube.
8. Add 500 uL wash buffer II (with EtOH added upon opening the kit), and centrifuge for 30 seconds at ≥ 12,000 x *g*.
9. Discard flow through. Repeat step 10, discarding flow-through.
10. Centrifuge the column for 1 minute at ≥ 12,000 x *g* to dry the column.
11. Move the column into a recovery tube and add 30 uL RNase-free water.
12. Incubate at room temperature for 1 minute.
13. Centrifuge for 1 minute at ≥ 12,000 x *g* and discard the column, keeping the flow-through, which contains purified total RNA.

*You can stop here and store the samples in -80****°****C.*

**Part III. Quality Control**

1. Quantify sample using Nanodrop (record 260/280 and 260/230) or Qubit (more sensitive).
2. Run 100-600 ng of your RNA on a 1.5% agarose gel to check for intact/non-degraded RNA. Alternatively, run an RNAnano chip on the bioanalyzer instrument in the Diabetes Center (10th floor).
3. Optional: PCR to check for genomic DNA. Primers: 515F and 806R. Control: 1ng gDNA. Check on 1.5% agarose gel that the bands, if present, and weaker than the positive control.

**rRNA depletion with Ribo-Zero (Illumina)**

Reagents:

Illumina Ribo-Zero Bacterial rRNA depletion (Cat #: MRZB12424)

Follow the manufacturer’s protocol.

Quality Control:

Run an RNAnano Agilent (Cat# 5067-1511) chip following rRNA depletion.

**RNA Fragmentation, cDNA Synthesis, and Library Preparation**

Reagents:

NEBNext Ultra RNA Library Prep Kit for Illumina (Cat #: E7770S – kit of 24 rxns at -20**°**C)

* Magnetic rack
* 80% ethanol
* agencourt ampure beads
* 0.1X TE, pH 8.0
* 10 mM Tris-HCl, pH 7.5 -8.0
* 10 mM NaCl (optional)

NEBNext Multiplex Oligos for Illumina, Dual Index Primers Set 1 (Cat #: E7600 – kit of 96 rxns at -20**°**C)

Follow the protocol given in Chapter 3 of the instruction manual.

Quality Control:

Run bioanalyzer hsDNA chip (Agilent Cat# 5067-4626) as called for in the NEB protocol.

**Quantify Each Library Prep & Pool**

Reagents:

Kapa Biosystems Quantification (KK4824)

Follow protocol for the Quantification Kit. Pool libraries so that there are approximately equal amount of each library in a single eppendorf.

Sample Timeline for Workflow

For 6- 12 samples

|  |  |  |
| --- | --- | --- |
| Day 1 |  |  |
|  | Extraction of Total RNA | 2.5 – 3h |
|  | DNAse Treatment and RNA Purification | 1.5 - 2h |
|  | Quality Control (need to check that Bioanalyzer available) | 2h |
| Day 2 |  |  |
|  | ribosomal RNA depletion with RiboZero | 3h |
|  | Quality Control (need to check that Bioanalyzer available) | 2h |
| Day 3 |  |  |
|  | NEBNext Ultra II RNA Library Prep, Part 1 (4.1 – 4.4) | 3.5h – 4h |
| Day 4 |  |  |
|  | NEBNext Ultra II RNA Library Prep, Part 2 (4.5 – 4.7) |  |
|  | NEBNext Ultra II RNA Library Prep, Part 3 (4.8 – 4.10) |  |
|  | Quality Control (need to check that Bioanalyzer available). Or plan to do this the following day if pooped. | 1.5h |
| Day 5 |  |  |
|  | repeat clean up with AMPure XP |  |
|  | Kapa Quant |  |
|  | Pool samples for submission to the IHG core |  |