Protocol for:

**Directional high-throughput sequencing of RNAs without gene-specific primers**

Reagents:

* Direct-zol RNA MicroPrep isolation kit (Zymo Research, Irvine, CA, USA)
* High Sensitivity RNA ScreenTape Assay (Agilent Technologies, Germany)
* 1% agarose E-Gel EX gel (Invitrogen, USA)
* Zymoclean Gel RNA Recovery Kit (Zymo Research, Irvine, CA, USA)
* T4 RNA ligase (Promega, USA)
* Recombinant RNasin Ribonuclease Inhibitor (Promega, USA)
* PEG 8000, Molecular Biology Grade Polyethylene Glycol 8000 (Promega, USA)
* Agencourt RNAClean XP (Beckman Coulter)
* RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA)
* Maxima SYBR Green/Fluorescein qPCR Master Mix (Thermo Scientific, USA)
* ProNex Size-Selective Purification System (Promega, USA)
* High Sensitivity D1000 ScreenTape System (Agilent Technologies, Germany)
* Qubit Fluorometer with high-sensitive dsDNA kit (Thermo Fisher Scientific, USA)
* Ion PGM Hi-Q View OT2 400 Kit (Thermo Fisher Scientific, USA)
* Ion Sphere Quality Control Kit (Thermo Fisher Scientific, USA)
* Ion PGM Hi-Q View Sequencing Kit (Thermo Fisher Scientific, USA)

Oligos:

* 100 µM M13-RNA oligo (5'-UGUAAAACGACGGCCAGU-3' )
* 100 µM P1-6N tailed random primer (5’-CCTCTCTATGGGCAGTCGGTGATNNNNNN-3’)
* a set of 10 µM forward primers IonA-barcode with M13 tail (5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGX10TGTAAAACGACGGCCAGT-3’), where X10 refers to Ion Torrent barcode sequences
* 10 µM reverse primer P1 (5’-CCTCTCTATGGGCAGTCGGTGAT-3’)

Protocol:

1. RNA isolation from the frozen cells following Tough-to-Lyse instructions of Direct-

zol RNA Micro Prep kit and proper aseptic RNA handling techniques

* + add 300-500 µL of TRI Reagent to the frozen cells before they have thawed
  + beat beating in 2 mL tubes with 0.1 mm Glass Beads (MoBio Laboratories, USA) using Power Lyse 24 homogenizer at 3400 RPM for 40 seconds
  + proceed with Direct-zol instructions for sample purification

1. High Sensitivity RNA ScreenTape Assay to check RNA isolation



1. Cutting and purification of rRNA 16S/18S fragments from precast 1% agarose E-Gel EX gel
   * purification using instructions of Zymoclean Gel RNA Recovery Kit
2. High Sensitivity RNA ScreenTape Assay from purified 16S/18S rRNA fragments
   * see an example figure in point 7.
3. Ligation of M13-RNA to purified 16S/18S rRNA fragments with Promega’s T4 RNA ligase
   * prepare 40 % PEG solution in advance
   * now 20 000-fold concentration of M13-RNA compared to rRNA
     + an example of ligation ingredients in the table below: 5.9 nM rRNA sample and 100 µM M13-RNA adapter
   * incubate the reaction at 37 °C for 40 minutes in thermocycler
   * no **heat activation** after the reaction, but apply directly for purification

|  |  |
| --- | --- |
| Components | 20.4 µL reaction |
| 0.02 pmol 16S/18S rRNA | 3.4 µL |
| 400 pmol M13-RNA | 4 µL |
| T4 RNA Ligase 10X buffer | 2 µL |
| RNasin Ribonuclease Inhibitor (40u/µL) | 0.5 µL |
| PEG, 40 % | 10 µL |
| T4 RNA Ligase | 0.5 µL |

1. Agencourt RNAClean XP cleaning of the ligation products
   * add 20 µL nuclease-free water to the sample to dilute the viscous solution before purification
   * purification in accordance with the manufacturer's instructions
   * 1.8 X sample volume: 40 µL ligation product (20 µL ligation solution + 20 µL H2O) and 72 µL RNAClean XP solution
   * elution to 20 µL of nuclease-free water
2. High Sensitivity RNA ScreenTape Assay from ligation products



1. cDNA synthesis using RevertAid First Strand cDNA Synthesis Kit
   * use 100 pmol of P1-random primers in 20 µL reaction
   * 8 µL of purified ligation product as a template
   * incubation at 25 °C for 5 min, 45 °C for 60 min, and termination of reaction by heating at 70 °C for 5 min
2. Agencourt RNAClean XP cleaning of the cDNA products
   * purification in accordance with the manufacturer's instructions
   * 1.6 X sample volume
   * elution to 18 µL of nuclease-free water
3. Amplification of the cDNA using Maxima SYBR Green/Fluorescein qPCR Master Mix

|  |  |  |
| --- | --- | --- |
| Components | 37.5 µl reaction | Final concentration |
| Maxima SYBR Green Master Mix | 18.75 µL |  |
| 10 µM forward primer: IonA-barcode with M13-tail | 1.5 µL | 0.4 µM |
| 10 µM reverse primer: P1 | 1.5 µL | 0.4 µM |
| Template cDNA | 6 µL |  |
| Nuclease-free water | 9.75 µL |  |

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| --- | --- | --- | --- |
| Cycle step | Temperature | Time | Cycles |
| Initial denaturation | 95 °C | 5 min | 1 |
| Denaturation  Annealing  Extension | 95 °C  52 °C  72 °C | 15 s  30 s  30 s | 30 |
| Final extension | 72 °C | 5 min | 1 |

1. Purification, dual size-selection and concentration of PCR products using ProNex Size-Selective Purification System and dual size-selection instructions

* to eliminate too long fragments: mix 1:1 (v/v ratio) of PCR product and ProNext (here 35 µL + 35 µL) and after placing sample on a magnetic stand, transfer the supernatant to a clean tube (too long fragments stay to the beads)
* to eliminate too short fragment: mix additional 0.28:1 (v/v) ratio of ProNex (here 9.8 µL) into the supernatant and after placing sample on a magnetic stand short fragments are in the supernatant and desired fragment are bound to the resin
* continue following the washing and elution steps
* now elution to 18 µL of elution buffer to concentrate the sample

1. High Sensitivity D1000 ScreenTape System Assay from purified products



1. Concentration measurement of purified products, pooling of samples (and purification of the pooled sample if needed), and final concentration measurement of the pool for OT2 emulsion PCR of Ion Torrent sequencing

* concentration measurement of each sample using Tape Station system or Qubit Fluorometer and pooling equal amounts of DNA (now 20 ng)

1. OT2 emulsion PCR (Ion sphere quality control included), bead washing, bead enrichment, and Ion Torrent sequencing with PGM
   * performed in accordance with the manufacturer's instructions using Life Technologies reagents
2. Data analysis (see supplementary material)