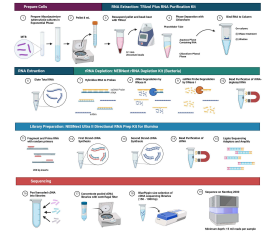


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RNA Extraction and RNA-Sequencing Method for Transcriptomic Analysis of *Mycobacterium tuberculosis*

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Morgan Hiebert^{1,2}, Meenu Sharma^{1,2}, Alwyn Go³, Christine Bonner³, Vanessa Laminman³, Morag Graham^{3,2}, Hafid Soualhine⁴

¹National Reference Centre for Mycobacteriology, National Microbiology Laboratory Branch, Public Health Agency of Canada;

²Department of Medical Microbiology and Infectious Diseases, University of Manitoba;

³Genomics Core Facility, National Microbiology Laboratory Branch, Public Health Agency of Canada;

⁴National Microbiology laboratory Branch, Public health Agency of Canada

BioTechniques



Morgan Hiebert

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We use this protocol and it's working

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Abstract

RNA-sequencing (RNA-seq) technologies have advanced exponentially in recent years, however application of RNA-seq to *M. tuberculosis* remains limited. We present a complete wet-lab and computational protocol for RNA-seq of *Mycobacterium tuberculosis* including RNA extraction, rRNA depletion, cDNA library preparation, high-throughput sequencing, and differential expression analysis. This research contributes to the literature by providing methodology for RNA-seq of *M. tuberculosis*, thereby bridging an important gap in mycobacterial transcriptomics.

Attachments



[Galaxy-Workflow-](#)

[Myco...](#)

25KB



Image Attribution

Graphical abstract created with BioRender.com

Guidelines

This protocol requires pure cultures of *Mycobacterium tuberculosis*, a Risk Group-3 (RG-3) organism, and should be carried out according to regional and institutional biosafety guidelines, such as the Biosafety Directive for *Mycobacterium tuberculosis* Complex [1]. The protocol contains variations on the manufacturer's protocols for the TRIzol Plus RNA Purification Kit and Phasemaker Tubes Complete System [2], NEBNext rRNA Depletion Kit (Bacteria) with RNA Sample Purification Beads and the NEBNext® Ultra™ II Directional RNA Library Prep kit with Sample Purification Beads [3].

Materials

Supplies

1. 0.9 McFarland Standard (E1039; Thermo Fisher Scientific, MA, USA)
2. 1.5% Agarose dye-free Gel Casette with internal standards, 250 bp – 1.5 kb (BDF1510; Sage Science, MA, USA)
3. Agilent RNA 6000 Nano Kit (5067-1511; Agilent Technologies, CA, USA)
4. Amicon® Ultra-0.5 Centrifugal Filter Units (UFC505096; MilliporeSigma, MA, USA)
5. BD BACTECTM MGITM 960 PZA Kit (245128; Becton Dickinson, NJ, USA), or BD BACTECTM MGITM 960 SIRE Kit (245128; Becton Dickinson), or BD BACTECTM MGITM 960 Supplement Kit (245124, Becton Dickinson)
6. BD BACTECTM MGITM 960 PZA Medium (245115; Becton Dickinson) or BD BACTECTM MGITM Mycobacterial Growth Indicator Tubes (245122, Becton Dickinson)
7. Chloroform (34854; MilliporeSigma)
8. Ethanol (P016EA95; Commercial Alcohols Incorporated, ON, CA)
9. FalconTM 15 mL Conical Centrifuge tubes (05-527-90; Thermo Fisher Scientific)
10. Flip cap Microcentrifuge Tubes: 2.0 mL (05-408-141; Thermo Fisher Scientific)
11. Genomic DNA ScreenTape and Reagents (5067-5366; 5067-5365; Agilent Technologies)
12. Microcentrifuge Tubes with Screw Caps: 2 mL (02-681-375; Thermo Fisher Scientific)
13. NEBNext® Multiplex Oligos for Illumina® (96 Unique Dual Index Primer Pairs) (E6440S; New England Biolabs, MA, USA)
14. NEBNext® rRNA Depletion Kit (Bacteria) with RNA Sample Purification Beads (E7860X; New England Biolabs)
15. NEBNext® UltraTM II Directional RNA Library Prep kit with Sample Purification Beads (E7765L; New England Biolabs)
16. NextSeqTM 1000/2000 P2 300M Reagents (600 cycles) (20075295; Illumina, CA, USA)
17. PureLinkTM DNase Set (12185010; Thermo Fisher Scientific)
18. QubitTM dsDNA High Sensitivity Assay kit (Q32854; Thermo Fisher Scientific)
19. QubitTM RNA High Sensitivity Assay kit (Q32852; Thermo Fisher Scientific)
20. RNaseZapTM RNase Decontamination Solution (AM9780; Invitrogen, MA, USA)
21. Triple-PureTM 0.1 mm zirconium beads (D1132-01TP; Benchmark Scientific, NJ, USA)
22. TRIzolTM Plus RNA Purification Kit and PhasemakerTM Tubes Complete System (A33254; Thermo Fisher Scientific)
23. UltraPureTM DNase/RNase-free Distilled Water (10977035; Invitrogen)

Equipment

1. 4200 TapeStation System (G2991BA; Agilent Technologies)
2. Agilent 2100 Bioanalyzer System (G2938A; Agilent Technologies)
3. BD BACTECTM MGITM 960 automated mycobacterial detection system (445885; Becton Dickinson)
4. BluePippin Size Selection System (BLU0001; Sage Science)
5. Fastprep-24TM Classic bead beating grinder and lysis system (116004500; MP Biomedicals, CA, USA)
6. Labnet OrbitTM 300 Multipurpose Digital Shaker (S2030-300-B; Mandel Scientific Company Inc, ON, CA).
7. MiniSpin® Mini Centrifuge (022620100; Eppendorf, Hamburg, Germany)
8. NanoDropTM 2000 spectrophotometer (ND-2000; Thermo Fisher Scientific)

9. NEBNext® Magnetic Separation Rack (S1515S; New England Biolabs)
10. NextSeq 2000 System (Illumina)
11. Qubit™ 4.0 Fluorometer (Q33238, Invitrogen)
12. Sorvall™ ST 16R Centrifuge (75004240; Thermo Fisher Scientific)
13. Thermocycler
14. VWR® Analog Vortex Mixer (10153-838; Avantor, PA, USA)

Safety warnings

! Biohazard Warnings: *Mycobacterium tuberculosis* is a risk group 3 (RG-3) organism and a Biosafety Level 3 (BSL-3) laboratory is required for higher risk research and diagnostics activities, as specified in the Biosafety Directive for *Mycobacterium tuberculosis* Complex [1]. All manipulations of *Mycobacterium tuberculosis* prior to inactivation must be performed in a Class II Type A2 Biosafety Cabinet (BSC), as described in the protocol. Personal protective equipment (PPE) required for this protocol may include, but is not limited to: scrub clothing, bouffant cap, powered air-purifying respirator (PAPR) or N100 respirator, BSC gown, disposable gloves worn with double-gloving technique. Accel TB Disinfectant (0.5% hydrogen peroxide) is used for disinfection of materials prior to removal from a Class II Type A2 BSC or the removal of non-viable materials from the BSL-3 laboratory.

Biohazardous Waste Disposal: Accel TB Disinfectant is used for disinfection of biohazardous waste prior to decontamination by autoclaving.

Chemical Warnings: This protocol uses TRIzol reagent (containing guanidine thiocyanate and phenol) and chloroform. TRIzol is a chaotropic agent with serious physical and health hazards including for exposure routes airborne (inhalation), ingestion (swallowing), contact (skin, eyes, etc.). Likewise, chloroform is an organic solvent with serious health hazards for exposure routes airborne (inhalation), ingestion (swallowing), contact (skin, eyes, etc.). Refer to chemical safety data sheets (SDS) for detailed hazard information. Chemical reagents should be handled in a fumehood before use as specified in the protocol with the PPE described above.

Chemical Waste Disposal: Chemical waste including TRIzol and chloroform should not be mixed with bleach, exposed to high temperatures, nor autoclaved. Do not allow TRIzol reagent to come into contact with Accel TB Disinfectant, as they may react to form toxic gases. Chemical waste containers should be surface disinfected with 70% ethanol before disposal in accordance with local requirements.

Ethics statement

Not applicable.

Before start

In a biosafety level 3 (BSL-3) laboratory, prepare small-volume in vivo liquid cultures of *Mycobacterium tuberculosis* by inoculating BD BACTECTM MGITM media prepared with BACTECTM MGITM 960 Supplement and incubating the cultures at 37°C in an orbital shaker for 2–4 weeks. Estimate the exponential phase by comparing culture turbidity to a 0.9 McFarland Standard.

Total RNA Extraction from low-volume, in vitro *Mycobacterium tuberculosis* cultures

- 1 RNA is extracted from low-volume, in vitro cultures of *Mycobacterium tuberculosis* using variations on the manufacturer's protocol for the TRIzol Plus RNA Purification Kit and Phasemaker Tubes Complete System [2].
- 2 Place exponential phase liquid cultures of *Mycobacterium tuberculosis*  On ice in a secondary container. 

Note

During all further manipulations prior to TRIzol treatment, keep samples on ice as much as possible.

- 3 Vortex the tubes containing liquid culture, then transfer contents ( 8 mL) into a 15 mL conical tube.
- 4 Pellet the cells by centrifugation  4000 x g, 4°C, 00:20:00 . Remove and discard the supernatant from each conical tube, careful not to disturb the pellet. 20m
- 5 Resuspend the pellet in  1 mL water (mix by pipetting, do not vortex) and transfer resuspended cells to a 2 mL screw capped tube containing 0.1 mm zirconium beads.
- 6 Using a microcentrifuge, spin cells at  5.000 x g, 4°C, 00:10:00 . Remove and discard the supernatant, careful not to disturb the cell pellet or zirconium beads. 10m
- 7 Resuspend the pellet in  500 µL of autoclaved water (mix by pipetting, do not vortex). Using a microcentrifuge, spin cells at  5000 x g, 4°C, 00:10:00 . Discard the supernatant, careful not to disturb the pellet. 10m
- 8 Add  1 mL of TRIzol reagent to the pellet and gently vortex to homogenize.

Note

If the cell pellet is larger than approximately 100 µL, add more TRIzol reagent to maintain a 10:1 ratio to the cells.



- 9 Incubate the cells in TRIzol at Room temperature for 00:05:00 . 5m
- 10 Using a FastPrep-24 homogenizer or equivalent, bead beat for 3 cycles of 4 m/s for 1 minute each and rest on ice for 1 minute between cycles.
- 11 Optional safe stopping point, store at 4 °C overnight or -20 °C for up to a year. * ||
- 12 Use microcentrifuge to spin the samples at 12.000 x g, 4°C, 00:05:00 to pellet cell debris. 5m
- 13 Transfer the supernatant (approximately 900-950 µL) from the 2 mL tube to Phasemaker tube, careful not to touch the pellet or zirconium beads.
- 14 Incubate the samples for 00:05:00 at Room temperature to allow complete dissociation of the nucleoprotein complexes. 5m
- 15 Add 200 µL of chloroform to Phasemaker Tube (or proportional if >1 mL Trizol was added). Shake vigorously by hand for 00:00:30 to mix. 30s
- 16 Incubate the samples at Room temperature for 00:03:00 and then centrifuge the sample for 12.000 x g, 4°C, 00:05:00 . 8m
- 17 Transfer the aqueous phase to a new 2 mL flip cap tube (will be approximately 50% of the volume of the starting lysate).

Note

Phasemaker tubes contain an inert, thick liquid polymer, which forms a gel-like layer isolating the colourless upper aqueous phase containing RNA and the lower red phenol-chloroform phase following centrifugation. Be careful not to touch the Phasemaker Gel when removing the aqueous phase.



- 18 Add an equal volume ( 450-500 μL) of 70% ethanol to the new tube containing aqueous phase, mix well by vortexing.
- 19 Transfer up to  700 μL of the sample to a spin cartridge with collection tube then centrifuge at  12.000 x g, Room temperature, 00:00:15 . 15s
- 20 Discard the column flow through, then re-insert the spin cartridge into the same collection tube.
- 21 Repeat Steps 18-19 until entire sample has processed.  [go to step #19](#)
- 22 Add  350 μL Wash Buffer I to the spin cartridge containing the bound RNA then centrifuge at  12.000 x g, Room temperature, 00:00:15 . 15s
- 23 Discard the flow through and collection tube, then insert the spin cartridge into a new collection tube.
- 24 Add  80 μL PureLink DNase mixture directly onto the surface of the spin cartridge membrane. Incubate at  Room temperature for  00:15:00 . 15m
- 25 Add  350 μL Wash Buffer I to the spin cartridge and centrifuge at  12.000 x g, Room temperature, 00:00:15 . 15s
- 26 Discard the flow through and collection tube, insert the spin cartridge into new collection tube.
- 27 Add  500 μL of Wash Buffer II to the spin cartridge then centrifuge at  12000 rpm, Room temperature, 00:00:15 . 15s
- 28 Discard the flow through, then re-insert the spin cartridge into the same collection tube.
- 29 Repeat Steps 27-28 once more.  [go to step #27](#) .



30 Centrifuge the spin cartridge at  12.000 x g, Room temperature, 00:02:00 to dry the membrane.

2m

31 Discard the collection tube, then insert the spin cartridge into a labelled recovery tube.

32 Add  33 μL of RNase-free water to the center of the spin cartridge.

33 Incubate for  00:01:00 at  Room temperature then centrifuge at  12.000 x g, Room temperature, 00:02:00 .

3m

34 Repeat Steps 32-33 twice more for a total of 3 sequential elutions.  [go to step #32](#) .

35 Discard the spin cartridge. The recovery tube contains the purified total RNA.

36 Store RNA extracts at  -80 °C



Total RNA Quantification and Quality Check

37 Quantify the extracted RNA a Qubit 4.0 fluorometer with a RNA High Sensitivity Assay kit.

Note

The rRNA depletion and cDNA library preparation protocol described below requires

 10 ng to  1 μg of total RNA for input.

38 Assess RNA purity using a NanoDrop™ 2000 spectrophotometer.

**Note**

RNA samples with an A260/280 ratio near 2 are generally considered "pure" RNA. The expected A260/230 ratio for pure RNA is 2.0-2.2.

Citation

Glase, J (1995)
. Validity of nucleic acid purities monitored by 260nm/280nm absorbance ratios.
Biotechniques.

[PMID: 7702855.](#)

[LINK](#)

- 39 Determine RNA quality by RNA Integrity Number (RIN) determined by the Agilent 2100 Bioanalyzer System and RNA 6000 Nano kit.

Note

RIN is calculated by the Bioanalyzer System using an algorithm which considers the electrophoretic pattern of sample RNA, including the 16S:23S rRNA ratio, to determine the "intactness" of RNA on a scale of one to ten. The rRNA depletion and cDNA library preparation protocol described below requires that input total RNA has a RIN > 7.

Citation

Schroeder A, Mueller O, Stocker S, Salowsky R, Leiber M, Gassmann M, Lightfoot S, Menzel W, Granzow M, Ragg T
(1970)
. The RIN: an RNA integrity number for assigning integrity values to RNA measurements.

<https://doi.org/>

[LINK](#)

Ribosomal RNA Depletion

- 40 Total RNA samples that met the requirements for integrity (RIN > 7) and quantity (10 ng – 1 µg) may proceed with rRNA depletion. Depletion of ribosomal RNA is performed using



the manufacturer's protocol for library preparation of intact RNA using NEBNext rRNA Depletion Kit (Bacteria) [3].

- 41 Dilute  10 ng -  1 µg of total RNA to a final volume of  11 µL in nuclease-free water, keeping  On ice .

Note

Keep RNA samples on ice as much as possible before conversion to cDNA.

- 42 Add  2 µL NEBNext Bacterial rRNA Depletion Solution and  2 µL NEBNext Probe Hybridization Buffer to the  11 µL of total RNA  On ice for a total volume of  15 µL .

- 43 Mix the reaction *thoroughly* by pipetting then briefly spin down the tube to collect the liquid from the sides of the tube.

- 44 Place the tube in a thermocycler and run the RNA/Probe Hybridization Reaction program below with the heated lid set to  105 °C :

Step 1:  95 °C for  00:02:00

Step 2: Ramp down to  22 °C at  0.1 °C per second

Step 3: Hold at  22 °C for  00:05:00

- 45 Briefly spin down the tube and place  On ice .

- 46 Add  2 µL of RNase H Reaction Buffer,  2 µL NEBNext Thermostable RNase H, and  1 µL Nuclease-free water to the  15 µL of hybridized RNA  On ice for a total volume of  20 µL .

- 47 Mix the reaction *thoroughly* by pipetting then briefly spin down the tube to collect the liquid from the sides of the tube.



- 48 Incubate the RNase H digestion reaction in a thermocycler for 00:30:00 at 55 °C with the lid set to 55 °C . 30m
- 49 Briefly spin down the tube and place On ice .
- 50 To the 20 µL of RNase H-treated RNA, add 5 µL of DNase I Reaction Buffer, 2.5 µL of NEBNext DNase I, 22.5 µL and Nuclease-free water on ice for a total volume of 50 µL .
- 51 Mix the reaction *thoroughly* by pipetting then briefly spin down the tube to collect the liquid from the sides of the tube.
- 52 Incubate the DNase I digestion reaction in a thermocycler for 00:30:00 at 37 °C with the lid set to 40 °C (or off). 30m
- 53 Briefly spin down the tube and place On ice .
- 54 Purify the rRNA-depleted RNA using NEBNext RNA Sample Purification Beads in a 1.8X ratio: add 90 µL of NEBNext RNA Sample Purification Beads to the 50 µL RNA suspension.
- Note

Vortex the NEBNext RNA Sample Purification Beads well to resuspend before use.
- Note

Agencourt RNAClean XP Beads may be used as an alternative to NEBNext RNA Sample Purification Beads.
- 55 Mix the RNA sample and beads thoroughly by pipetting and incubate On ice for 00:15:00 to allow the RNA to bind to the beads. 15m



- 56 Place the tubes on a magnetic plate for ⌚ 00:05:00 to separate the beads from the supernatant. 5m
- 57 Remove and discard the clear supernatant ($\leq$ 🧪 150 μL) without touching the RNA-bound beads.
- 58 Add 🧪 200 μL of freshly prepared 80% ethanol to the RNA bound to the magnetic beads remaining in the tubes. Incubate for ⌚ 00:00:30 at 🌡 Room temperature then remove the supernatant. 30s
- 59 Repeat Step 58 once for a total of two washes. ➡ [go to step #58](#)
- 60 Remove the tubes from the magnetic plate and air-dry for $\leq$ ⌚ 00:05:00 at 🌡 Room temperature to remove residual ethanol. 5m
- 61 Add 🧪 7 μL of nuclease-free water to each tube and mix thoroughly by pipetting.
- 62 Incubate the sample at 🌡 Room temperature for ⌚ 00:02:00 . 2m
- 63 Place the tubes on the magnetic plate for ⌚ 00:02:00 to separate the magnetic beads from the RNA. 2m
- 64 Aspirate 🧪 5 μL of the supernatant the containing rRNA-depleted RNA and transfer to a clean nuclease-free tube 🌡 On ice .
- 65 Store rRNA-depleted RNA at 🌡 -80 °C . *



cDNA Library Preparation

15m



66 The cDNA library preparation from rRNA-depleted RNA is performed using the manufacturer's instructions for the NEBNext Ultra II Directional RNA Library Prep kit with Sample Purification Beads [3].

67 Add  4 μL of NEBNext First Strand Synthesis Reaction Buffer and  1 μL of Random Primers to the  5 μL of rRNA-depleted samples on ice for a total volume of  10 μL .

68 Fragment the RNA by placing the tubes on a thermocycler and incubating at  94 °C for  00:15:00 .

15m

Note

Fragmentation conditions are optimized for generating 200 nucleotide inserts from intact RNA (RIN > 7).

69 Immediately transfer the tube to ice.



70 Add  8 μL of NEBNext Strand Specificity Reagent and  2 μL of NEBNext First Strand Synthesis Enzyme Mix to the  10 μL of fragmented and primed RNA  On ice for a total volume of  20 μL .

71 Keeping the solution on ice, mix the reaction *thoroughly* by pipetting.

72 Place the tube in a thermocycler with the heated lid set to  80 °C and run the following program for first strand cDNA synthesis:

40m

Step 1:  00:10:00 at  25 °C

Step 2:  00:15:00 at  42 °C

Step 3:  00:15:00 at  70 °C

Step 4: Hold at  4 °C

73 Add  8 μL of 10X NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix,  4 μL NEBNext Second Strand Synthesis Enzyme Mix, and  48 μL of Nuclease-



free water to the  20 μL First-Strand Synthesis Product  On ice for a total volume of  80 μL .

74 Keeping the solution on ice, mix the reaction *thoroughly* by pipetting.

75 Place the tube in a thermocycler for  01:00:00 at  16 °C with the heated lid set to  40 °C (or off) for second strand cDNA synthesis.

1h

76 Purify the double stranded cDNA with NEBNext Sample Purification Beads in a 1.8X ratio: add  144 μL of NEBNext Sample Purification Beads to the  80 μL cDNA suspension.

Note

SPRIselect Beads may be used as an alternative to NEBNext Sample Purification Beads.

77 Mix the cDNA sample and beads thoroughly by pipetting and incubate at  Room temperature for  00:05:00 to allow the DNA to bind to the beads.

5m

78 Place the tubes on a magnetic plate for  00:05:00 to separate the beads from the supernatant.

5m

79 Remove and discard the clear supernatant ($\leq$  230 μL) without touching the DNA-bound beads.

80 Add  200 μL of freshly prepared 80% ethanol to the RNA bound to the magnetic beads remaining in the tubes. Incubate for  00:00:30 at  Room temperature then remove the supernatant.

30s

81 Repeat Step 80 for a total of two washes.  [go to step #116](#)



- 82 Remove the tubes from the magnetic plate and air-dry for ≤ 00:05:00 at Room temperature to remove residual ethanol. 5m
- 83 Add 53 µL of 0.1X TE Buffer to each tube and mix thoroughly by pipetting.
- 84 Incubate the sample at Room temperature for 00:02:00 . 2m
- 85 Place the tubes on the magnetic plate for 00:02:00 to separate the magnetic beads from the DNA. 2m
- 86 Aspirate 50 µL of the supernatant the containing cDNA and transfer to a clean nuclease-free tube .
- 87 Store the purified double stranded cDNA at -20 °C . * ||
- 88 Add 7 µL of NEBNext Ultra II End Prep Reaction Buffer and 3 µL of NEBNext Ultra II End Prep Enzyme Mix to the 50 µL of purified double stranded cDNA On ice for a total volume of 60 µL .
- 89 Mix the reaction *thoroughly* by pipetting then briefly spin down the tube to collect the liquid from the sides of the tube. !
- 90 Place the tubes in a thermocycler with the heated lid set to 75C and run the following program for end-prep of the cDNA library: 1h
Step 1: 00:30:00 at 20 °C
Step 2: 00:30:00 at 65 °C
Step 3: Hold at 4 °C
- 91 Dilute the NEBNext Adaptor 5-fold in Adaptor Dilution Buffer before use (2.5 µL required per sample) and keep On ice .
- 92 Add 2.5 µL of diluted adaptor, 1 µL of NEBNext Ligation Enhancer, and 30 µL of NEBNext Ultra II Ligation Master Mix to the 60 µL of end prepped



cDNA On ice for a total volume of 93.5 μL .

93 Mix the reaction *thoroughly* by pipetting then briefly spin down the tube to collect the liquid from the sides of the tube.

94 Place the tubes in a thermocycler and incubate at 20 °C with the heated lid off for

15m

00:15:00

95 Add 3 μL of USER Enzyme to the ligation mixture and mix thoroughly by pipetting. and incubate the tubes at 37°C Program 8B (Table 4) was run to complete the Ligation Reaction.

96 Place the tubes in a thermocycler at incubate at 37 °C with the heated lid set to

15m

45 °C for 00:15:00 .

97 Purify the ligation reaction with NEBNext Sample Purification Beads in a 0.9X ratio: add 87 μL of NEBNext Sample Purification Beads to the 96.5 μL DNA suspension.

Note

Vortex the NEBNext Sample Purification Beads well to resuspend before use.

Note

SPRIselect Beads may be used as an alternative to NEBNext Sample Purification Beads.

98 Mix the sample and beads thoroughly by pipetting and incubate at Room temperature for 00:10:00 to allow the DNA to bind to the beads.

10m

99 Place the tubes on a magnetic plate for 00:05:00 to separate the beads from the supernatant.



- 100 Remove and discard the clear supernatant ($\leq$ 190 μL) without touching the DNA-bound beads.
- 101 Add 200 μL of freshly prepared 80% ethanol to the RNA bound to the magnetic beads remaining in the tubes. Incubate for 00:00:30 at Room temperature then remove the supernatant.
- 102 Repeat Step 101 for a total of two washes. [go to step #116](#)
- 103 Remove the tubes from the magnetic plate and air-dry for $\leq$ 00:05:00 at Room temperature to remove residual ethanol.
- 104 Add 17 μL of 0.1X TE Buffer to each tube and mix thoroughly by pipetting.
- 105 Incubate the sample at Room temperature for 00:02:00 .
- 106 Place the tubes on the magnetic plate for 00:02:00 to separate the magnetic beads from the DNA.
- 107 Aspirate 15 μL of the supernatant the containing the ligated DNA and transfer to a clean nuclease-free tube .
- 108 Store adaptor-ligated cDNA samples at -20 $^{\circ}\text{C}$
- 109 Perform PCR enrichment of adaptor ligated DNA with the unique dual index primer pairs supplied in the NEBNext Multiplex Oligos for Illumina kit: add 25 μL of NEBNext Ultra II Q5 Master Mix and 10 μL of Index Primer/i7 Primer Mix to the 15 μL of adaptor-ligated DNA (15 μL) On ice for a total volume of 50 μL .
- 110 Mix the reaction *thoroughly* by pipetting then briefly spin down the tube to collect the liquid from the sides of the tube.
- 111 Place the tube in a thermocycler with the heated lid set to 105C and run the following program for PCR enrichment of adaptor-ligated cDNA: 6m 55s

**Step 1: 1 cycle**

98 °C for 00:00:30

Step 2: 12 cycles

98 °C for 00:00:10

65 °C for 00:01:15

Step 3: 1 cycle

65 °C for 00:05:00

Step 4:

Hold at 4 °C

Note

PCR-enrichment of cDNA is optimized based on ~100 ng of RNA input.

- 112 Purify the ligation reaction with NEBNext Sample Purification Beads in a 0.9X ratio: add 45 µL of NEBNext Sample Purification Beads to the 50 µL DNA suspension.

Note

Vortex the NEBNext Sample Purification Beads well to resuspend before use.

Note

SPRIselect Beads may be used as an alternative to NEBNext Sample Purification Beads.

- 113 Mix the sample and beads thoroughly by pipetting and incubate at Room temperature for 00:05:00 to allow the DNA to bind to the beads.

5m

- 114 Place the tubes on a magnetic plate for 00:05:00 to separate the beads from the supernatant.



- 115 Remove and discard the clear supernatant ($\leq$ 100 μL) without touching the DNA-bound beads.
- 116 Add 200 μL of freshly prepared 80% ethanol to the RNA bound to the magnetic beads remaining in the tubes. Incubate for 00:00:30 at Room temperature then remove the supernatant.
- 117 Repeat Step 116 for a total of two washes. [go to step #116](#)
- 118 Remove the tubes from the magnetic plate and air-dry for $\leq$ 00:05:00 at Room temperature to remove residual ethanol.
- 119 Add 23 μL of 0.1X TE Buffer to each tube and mix thoroughly by pipetting.
- 120 Incubate the sample at Room temperature for 00:02:00 .
- 121 Place the tubes on the magnetic plate for 00:02:00 to separate the magnetic beads from the DNA.
- 122 Aspirate 20 μL of the supernatant the containing the ligated DNA and transfer to a clean nuclease-free tube .
- 123 Store the cDNA libraries at -20 $^{\circ}\text{C}$.

II

cDNA Library Pooling, Quantification & Quality Check

- 124 Quantify the cDNA libraries using a Qubit 4.0 fluorometer with a dsDNA High Sensitivity Assay kit.
- 125 Assess DNA purity using a NanoDrop™ 2000 spectrophotometer.

Note

DNA samples with an A260/280 ratio near 1.8 are generally considered "pure" DNA. The expected A260/230 ratio for pure DNA is 2.0-2.2.

126 To achieve a minimum of 15 million paired-end reads per sample using a NextSeq 2000, pool  1 ng of cDNA from each of 20 samples to yield library concentrations greater than  750 picomolar (pM) .

127 Concentrate the pooled libraries using an Amicon Ultra-0.5 centrifugal filter unit according to the manufacturer's instructions:

127.1 Insert an Amicon Ultra 0.5 filter into the supplied microcentrifuge tube.

127.2 Transfer up to  500 μL of the library pool onto the filter and place the cap onto the filter/tube.

Note

Optional: if the volume is less than 500 μl , bring the volume up to 500 μl with nuclease-free water.

127.3 Place the filter/tube into a centrifuge, counter-balance, and spin at  14000 x g for 10 minutes.

127.4 If there is more than  500 μL of library pool to concentrate, discard the flow-through and repeat Steps 127.2 and 127.3 with the remaining volume in the same filter/tube.

127.5 Once the library pool has run through the filter (approximately  20 μL remaining), remove the filter from the existing microcentrifuge tube and place upside down into a clean microcentrifuge tube.

127.6 Place in a centrifuge with the cap open and cap facing inward into the centrifuge and spin at  1000 x g for two minutes to collect the filtered library pool.



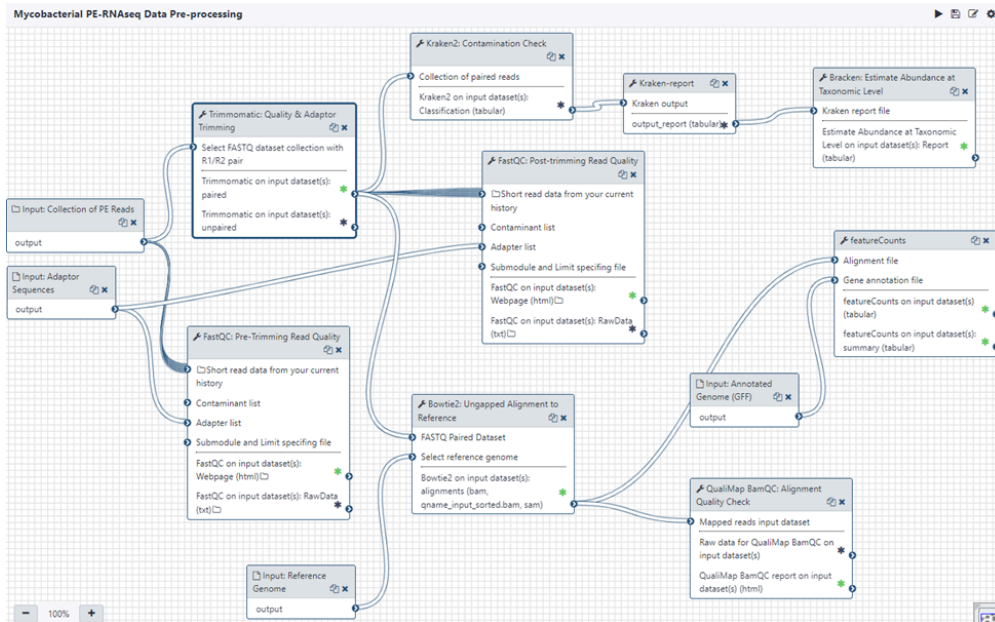
- 127.7 Remove from the centrifuge and discard the filter. Retain the filtered library and cap the tube.
- 128 Size-select the pooled cDNA libraries at a range of 150-1000 bp using a BluePippin according to manufacturer's instructions.
- 129 Quantify the pooled cDNA libraries using a Qubit 4.0 fluorometer with a dsDNA High Sensitivity Assay kit.
- 130 Determine library size and quality using an Agilent 4200 TapeStation System with Genomic DNA ScreenTape Analysis reagents according to manufacturer's instructions.

RNA Sequencing

- 131 Sequence pooled cDNA libraries using the Illumina NextSeq 2000 platform with NextSeq 1000/2000 P2 (600 cycles) Reagents at a loading concentration of 1M 700 picomolar (pM) according to manufacturer's instructions. 1d 18h
- 132 De-multiplex forward (R1) and reverse (R2) paired-end sequencing files according to sample indexing.

RNA-sequencing Data Pre-processing

- 133 The RNA-sequencing data pre-processing workflow is performed in Galaxy, an open-source environment for data analysis. The workflow is described in sequential steps below, and it may be downloaded in its entirety using the attached document: *Galaxy-Workflow-Mycobacterial_PE-RNAseq_Data_Pre-processing.ga* 



Overview of the Mycobacterial PE-RNAseq Data Pre-Processing Workflow In Galaxy.

Citation

Galaxy Community (2024)

. The Galaxy platform for accessible, reproducible, and collaborative data analyses: 2024 update.

<https://doi.org/10.1093/nar/gkae410>

LINK

134 Assess pre-trimming quality of sequencing reads using FastQC (version 0.72).

Input: Paired-end reads as a dataset collection of paired R1/R2 FASTQ files

Output: Web FastQC Report for each FASTQ file



Expected result

- Expect >15 million reads per sample based on pooling and sequencing parameters.
- Expect a guanosine/cytosine content (GC%) greater than 60% as *M. tuberculosis* has a GC-rich genome.



Basic Statistics

Measure	Value
Filename	reverse
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	24038318
Sequences flagged as poor quality	0
Sequence length	35-151
%GC	64

Example of FastQC basic statistics table showing > 15 million reads and 60% GC content before trimming and filtering.

Software

FastQC

NAME

Simon Andrews

DEVELOPER

<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>

SOURCE LINK

- 135 Use Trimmomatic for adaptor trimming (ILLUMINACLIP), quality trimming (SLIDINGWINDOW:4:20), and filtering out reads below 20 nt in length (MINLEN:20)

Input: (1) Paired-end reads as a dataset collection of paired R1/R2 FASTQ files; **(2)** Adaptor Sequences in FASTA format

Output: (1) Paired-end reads in which both reads in the pair survived filtering and trimming as a dataset collection of paired R1/R2 FASTQ files; **(2)** Reads in which one of the pair failed the filtering and trimming steps as a dataset collection of unpaired R1/R2 FASTQ files.





In future steps, move forward only with paired-end reads in which both pairs survived trimming.

Citation

Bolger AM, Lohse M, Usadel B (2014)
. Genome analysis Trimmomatic: a flexible trimmer for Illumina sequence data.
Bioinformatics.

<https://doi.org/10.1093/bioinformatics/btu170>

LINK

136 Assess post-trimming read quality using FastQC (version 0.72).

Input: Paired-end reads in which both reads in the pair survived filtering and trimming as a dataset collection of paired R1/R2 FASTQ files

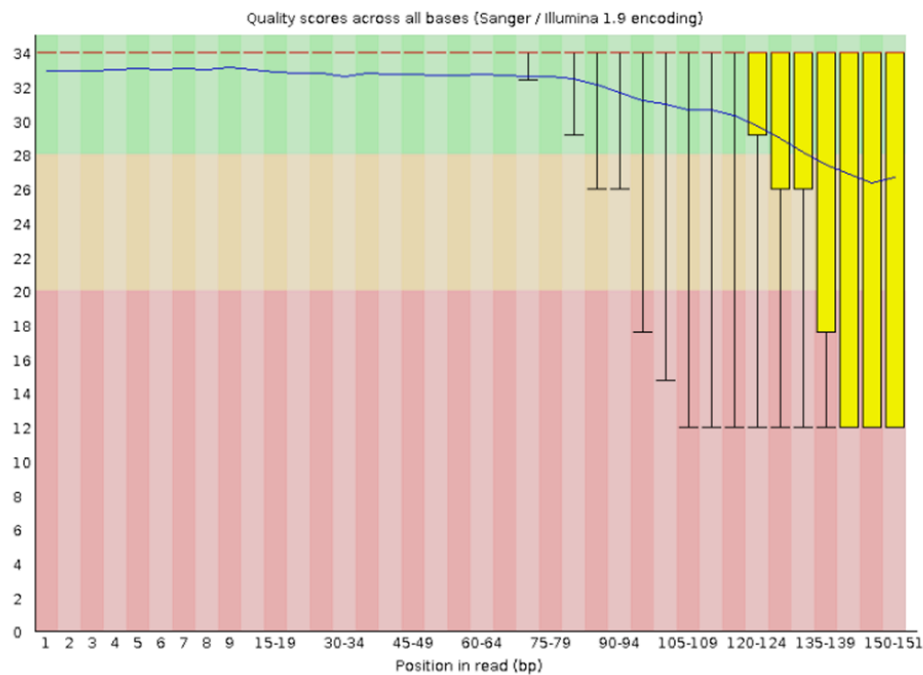
Output: Web FastQC Report for each FASTQ file

Expected result

- A threshold Phred score of 20 was used to ensure that high quality sequences are used for differential expression analysis.
- Desire >15 million reads to survive the trimming and quality filtering steps.
- Expect a guanosine/cytosine content (GC%) greater than 60% as *M. tuberculosis* has a GC-rich genome.
- Expect high sequence duplication levels and overrepresented sequences, due to sequencing of highly expressed transcripts.

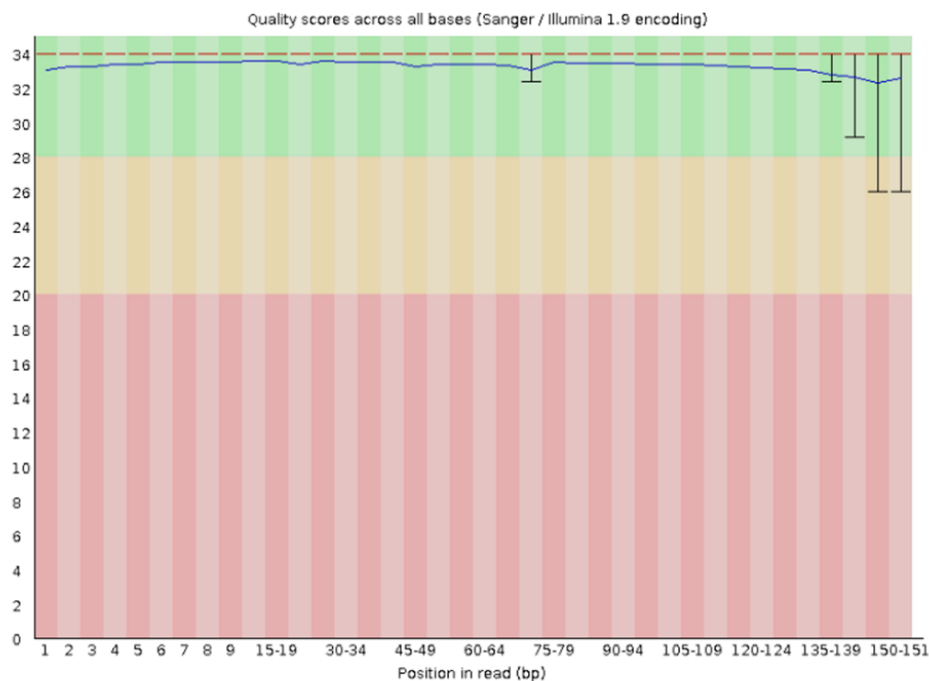


Per base sequence quality



Example of FastQC per base sequence quality graph and Phred scores before trimming and filtering.

✓ Per base sequence quality



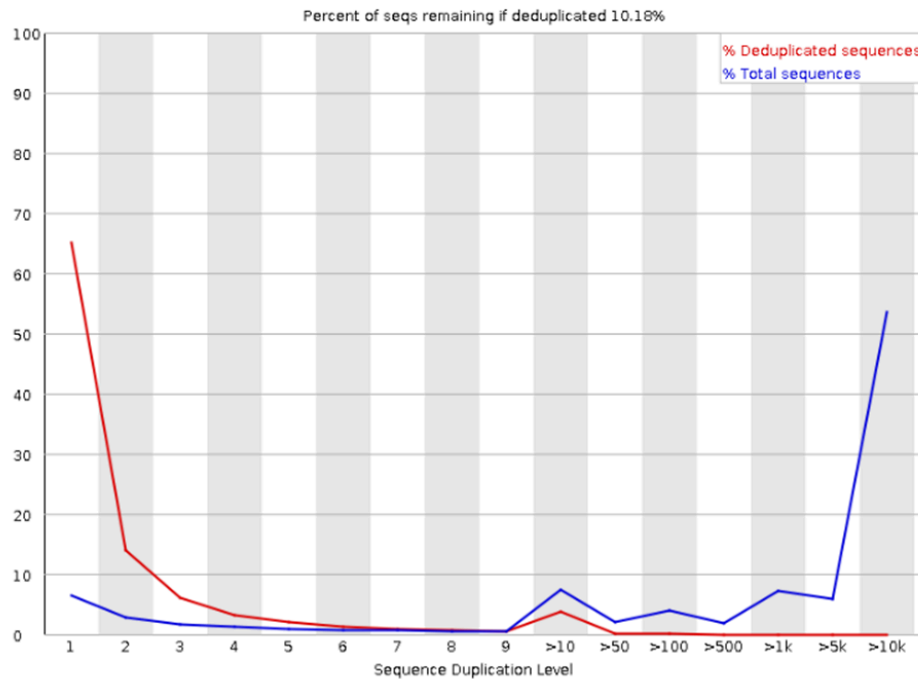
Example of FastQC per base sequence quality graph and Phred scores after trimming and filtering.

✓ Basic Statistics

Measure	Value
Filename	reverse
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	20054965
Sequences flagged as poor quality	0
Sequence length	20-151
%GC	60

Example of FastQC basic statistics table showing >15 million reads and 60% GC content after trimming and filtering.

Sequence Duplication Levels



Example FastQC Sequence Duplication Level graph showing high levels of sequence duplication.

137 Use Kraken to detect potential contamination in three steps:

Citation

Wood DE, Lu J, Langmead B (2019). Improved metagenomic analysis with Kraken 2.

<https://doi.org/10.1186/s13059-019-1891-0>

LINK

137.1 Use Kraken2 to assign taxonomic labels to each sequencing read. Use standard-ful Kraken database with prebuilt Refseq indexes including archaea, bacteria, viral, plasmid, human, UniVec_Core sequences



Input: Paired-end reads in which both reads in the pair survived filtering and trimming as a dataset collection of paired R1/R2 FASTQ files

Output: Collection of Kraken2 read classification files in tabular format

- 137.2 Run Kraken-report to obtain a summary of taxonomic classification for sequencing reads. This step determines the absolute number of reads assigned to each taxonomic level using the Kraken2 classification files generated in the previous step.

Input: Collection of Kraken2 read classification files in tabular format

Output: Collection of Kraken-report files in tabular format

- 137.3 Use Bracken to estimate the relative abundance of reads assigned at the genus level.

Input: Collection of Kraken-report files in tabular format

Output: Collection of tabular reports detailing number and fraction of reads assigned to given genera

Expected result

name	taxonomy_id	taxonomy_lvl	kraken_assigned_reads	added_reads	new_est_reads	fraction_total_reads
Mycobacterium	1763	G	23576488	101839	23678327	0.99982
Corynebacterium	1716	G	427	5	432	0.00002
Rhodococcus	1827	G	90	6	96	0.00000
Nocardia	1817	G	11	0	11	0.00000

Example Bracken report showing >99% of reads assigned to Mycobacterium genus.

- 138 Align paired-end reads to the *M. tuberculosis* H37Rv reference genome (accession NC_000962.3) using Bowtie2.

Input: (1) Paired-end reads in which both reads in the pair survived filtering and trimming as a dataset collection of paired R1/R2 FASTQ files; **(2)** reference genome in FASTA format (accession NC_000962.3)

Output: Collection of alignments in BAM format



Citation

Langmead B, Salzberg SL (2012). Fast gapped-read alignment with Bowtie 2.

<https://doi.org/10.1038/nmeth.1923>

[LINK](#)

139 Determine alignment quality using Qualimap2 BamQC.

Input: Collection of alignments in BAM format

Output: Web QualiMap Report for each BAM alignment file



Expected result

- Expect >95% of reads to map to the reference genome.
- Expect duplication rate and overlapping read pairs to be high due to sequencing of highly expressed transcripts.
- Expect GC percentage to be >60%.

Globals

Reference size	4,411,532
Number of reads	40,109,930
Mapped reads	39,797,350 / 99.22%
Unmapped reads	312,580 / 0.78%
Mapped paired reads	39,797,350 / 99.22%
Mapped reads, first in pair	19,915,097 / 49.65%
Mapped reads, second in pair	19,882,253 / 49.57%
Mapped reads, both in pair	39,748,552 / 99.1%
Mapped reads, singletons	48,798 / 0.12%
Secondary alignments	0
Read min/max/mean length	20 / 151 / 117.8
Overlapping read pairs	19,477,962 / 97.12%
Duplicated reads (flagged)	0 / 0%
Duplicated reads (estimated)	38,188,416 / 95.21%
Duplication rate	71.45%
Clipped reads	0 / 0%

ACGT Content

Number/percentage of A's	905,892,488 / 19.3%
Number/percentage of C's	1,425,474,104 / 30.37%
Number/percentage of T's	919,074,072 / 19.58%
Number/percentage of G's	1,442,364,206 / 30.73%
Number/percentage of N's	986,997 / 0.02%
GC Percentage	61.1%

Example excerpt from QualiMap Report.

Citation

Okonechnikov K, Conesa A, García-Alcalde F (2016)
. Qualimap 2: advanced multi-sample quality control for high-throughput sequencing data.

<https://doi.org/10.1093/bioinformatics/btv566>

LINK



- 140 Use featureCounts (version 1.6.0.2) to determine read counts per gene using an annotated *M. tuberculosis* H37Rv reference genome (AL_123456.3).

Input: (1) Collection of alignments in BAM format; **(2)** annotated reference genome in GFF3 format (AL_123456.3)

Output: (1) featureCount tables listing the read count assigned to each geneid in tabular format; **(2)** featureCounts Summary showing the number of assigned and unassigned reads in tabular format.



Expected result

Geneid	1401498-12
dnaA	992
dnaN	737
recF	176
Rv0004	80
gyrB	2296
gyrA	1914
Rv0007	328
ileT	37
alaT	28
Rv0008c	46
ppiA	1045
Rv0010c	291
Rv0011c	374

Example of first 14 lines of an Output featureCounts Table

Status	600452-4
Assigned	38543150
Unassigned_Unmapped	312580
Unassigned_MappingQuality	228094
Unassigned_Chimera	0
Unassigned_FragmentLength	0
Unassigned_Duplicate	0
Unassigned_MultiMapping	0
Unassigned_Secondary	0
Unassigned_Nonjunction	0
Unassigned_NoFeatures	412773
Unassigned_Overlapping_Length	68485
Unassigned_Ambiguity	544848

Example of Output featureCounts Summary



Citation

Liao Y, Smyth GK, Shi W (2014)

. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features.

<https://doi.org/10.1093/bioinformatics/btt656>

LINK

- 141 Export/download the collection of featureCount tables in tabular format to be used as input for differential expression analysis.

Differential Expression Analysis

- 142 Normalization of read counts and differential expression analysis as described below is performed using DESeq2 (version 1.42.1), operating within R Studio. Before beginning, load the following libraries:

```
library(tidyverse)
library(janitor)
library(openxlsx)
library(DESeq2)
```

Citation

Love MI, Huber W, Anders S (2014)

. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2.

<https://doi.org/>

LINK

- 143 Create a metadata file in CSV format that includes the name of each featureCounts file downloaded in Step 141 as well as sample information that will be used for differential expression analysis.

	A	B	C	D	E
	id	strain	tx	pool	phenotype
	1800686_1	1800686	drug	1	R
	1800686_2	1800686	drug	1	R
	1800686_3	1800686	drug	7	R
	1800686_7	1800686	control	5	R
	1800686_8	1800686	control	6	R
	1800686_9	1800686	control	6	R
	1800713_1	1800713	drug	1	S
	1800713_2	1800713	drug	1	S
	1800713_3	1800713	drug	2	S
	1800713_7	1800713	control	5	S
	1800713_8	1800713	control	6	S
	1800713_9	1800713	control	6	S

Example Metadata file in which columns show id (file name), strain (sample name), tx (treatment condition), pool (number of sequencing pool), and phenotype (sensitive or resistant).

144 Compile all featureCounts data into a single dataframe:

144.1 Create a log of files used for analysis called file_list:

Example Command:

```
file_list <- tibble(filename = list.files(path = "C:/Project_folder/", pattern = "*.tabular"))
%>%
  mutate(filepath = paste0("C:/Project_folder/", filename))
```

Note: all featureCount files must be in a single folder with the location specified in the command above.

144.2 Import all featureCount tables specified in file_list into a collection of dataframes called files:

Example Command:

```
files = lapply(file_list$filename, read.delim)
```

- 144.3 Compile the collection of featureCount dataframes into a single dataframe and tidy the data:

Example Commands:

```
raw_counts <- bind_cols(files, .name_repair = "universal") # bind dataframes
clean_counts <- dplyr::rename(raw_counts, "gene" = "Geneid...1") # rename column 1 to "gene"
clean_counts = clean_counts[,!grepl("*Geneid",names(clean_counts))] # remove redundant columns
clean_counts <- clean_names(clean_counts) # clean sample names
clean_counts <- rename_with(clean_counts, ~str_remove(.x, "x")) # clean sample names
```

- 145 Read metadata into a dataframe, convert all variables to factors, and set reference levels for each factor.

Example Commands:

```
metadata <- read.csv('metadata.csv', header = TRUE, sep = ",") # Import
```

```
metadata <- as.data.frame(unclass(metadata), stringsAsFactors = TRUE)
metadata[sapply(metadata, is.numeric)] <- lapply(metadata[sapply(metadata, is.numeric)], as.factor)
# Convert character and numeric variables to factors
```

```
metadata$tx <- relevel(metadata$tx, ref = "control")
metadata$phenotype <- relevel(metadata$phenotype, ref = "S")
# Set reference levels for each factor
```

- 146 Construct DESeqDataSet Object named dds from count data and metadata.

Example Command:

```
dds <- DESeqDataSetFromMatrix(countData=clean_counts,
                              colData=metadata,
                              design=~phenotype+ tx + phenotype:tx, tidy = TRUE)
```

- 147 Perform DESeq analysis on DESeqDataSet Object, generating large DESeqDataSet called analysis.

Example Commands:

```
analysis <- DESeq(dds,
                  test = "Wald",
```

```
fitType = "parametric",  
sfType = "ratio")
```

resultsNames(analysis) # Returns the names of estimated effects of the model

Note

Consult the DESeq vignette to determine the most appropriate test, fitType, and sfType for your dataset.

- 148 Generate a results table (formal class DESeqResults) for each desired contrast.

Example commands:

Example 1: Investigate effect of treatment (tx) on strains with a sensitive phenotype

```
result_table <- results(analysis,  
  contrast = c("tx", "control", "drug"),  
  alpha = 0.05)
```

Example 2: Investigate the effect of treatment (tx) on strains with a resistant phenotype

```
result_table <- results(analysis, list(c("tx_drug_vs_control", "phenotypeR.txdrug")),  
  alpha = 0.05)
```

Example 3: Investigate the difference between phenotypes with treatment

```
result_table <- results(analysis, list(c("phenotype_R_vs_S", "phenotypeR.txdrug")),  
  alpha = 0.05)
```

Example 4: Investigate if the effect of treatment is different between phenotypes

```
interaction <- results(analysis, name = "phenotypeR.tx1drug",  
  alpha = 0.05)
```

- 149 Convert results tables from a formal class DESeqResults to a dataframe and export to an excel file.

Example Commands:

```
df_result <- as.data.frame(result_table) # convert formal class of DESeqResults into a  
dataframe  
write.xlsx(df_result, file = "DESeq_results.xlsx", colNames = TRUE, rowNames = TRUE) #  
export as an excel file
```



Protocol references

Citation

PHAC (2017). Biosafety Directive for Mycobacterium tuberculosis Complex (MTBC).
Public Health Agency of Canada (PHAC).

<https://www.canada.ca/en/public-health/services/laboratory-biosafety-biosecurity/biosafety-directive-mycobacterium-tuberculosis->
LINK

Citation

Invitrogen, ThermoFisher Scientific (2016)
. User Guide: TRIzol Plus RNA Purification Kit and Phasemaker Tubes Complete System [Internet].

[https://www.thermofisher.com/document-connect/document-connect.html?
url=https://assets.thermofisher.com/TFS-Assets%2FMSG%2Fmanual](https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FMSG%2Fmanual)
LINK

Citation

New England Biolabs (2024)
. Protocol for Library Preparation of Intact RNA using NEBNext rRNA Depletion Kit (Bacteria) (NEB #E7850, NEB #E7860) and NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB #E7760, NEB #E7765) [Internet].

<https://www.neb.com/en/protocols/2019/09/18/protocol-for-library-preparation-of-intact-rna-using-nebnext-rrna-depletion-kit-bact>
LINK

Citations

PHAC. Biosafety Directive for Mycobacterium tuberculosis Complex (MTBC)

<https://www.canada.ca/en/public-health/services/laboratory-biosafety-biosecurity/biosafety-directive-mycobacterium-tuberculosis->

Invitrogen, ThermoFisher Scientific. User Guide: TRIzol Plus RNA Purification Kit and Phasemaker Tubes Complete System [Internet]

[https://www.thermofisher.com/document-connect/document-connect.html?](https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FMSG%2Fmanuals)

[url=https://assets.thermofisher.com/TFS-Assets%2FMSG%2Fmanuals](https://assets.thermofisher.com/TFS-Assets%2FMSG%2Fmanuals)

New England Biolabs. Protocol for Library Preparation of Intact RNA using NEBNext rRNA Depletion Kit (Bacteria) (NEB #E7850, NEB #E7860) and NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB #E7760, NEB #E7765) [Internet]

<https://www.neb.com/en/protocols/2019/09/18/protocol-for-library-preparation-of-intact-rna-using-nebnext-rrna-depletion-kit-bact>

Step 133

Galaxy Community. The Galaxy platform for accessible, reproducible, and collaborative data analyses: 2024 update.

<https://doi.org/10.1093/nar/gkae410>

Step 135

Bolger AM, Lohse M, Usadel B. Genome analysis Trimmomatic: a flexible trimmer for Illumina sequence data.

<https://doi.org/10.1093/bioinformatics/btu170>

Step 137

Wood DE, Lu J, Langmead B. Improved metagenomic analysis with Kraken 2.

<https://doi.org/10.1186/s13059-019-1891-0>

Step 138

Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2.

<https://doi.org/10.1038/nmeth.1923>

Step 139

Okonechnikov K, Conesa A, García-Alcalde F. Qualimap 2: advanced multi-sample quality control for high-throughput sequencing data.

<https://doi.org/10.1093/bioinformatics/btv566>

Step 140



Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features.

<https://doi.org/10.1093/bioinformatics/btt656>

Step 142

Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2.

<https://doi.org/>

Step 38

Glase, J. Validity of nucleic acid purities monitored by 260nm/280nm absorbance ratios

PMID: 7702855.

Step 39

Schroeder A, Mueller O, Stocker S, Salowsky R, Leiber M, Gassmann M, Lightfoot S, Menzel W, Granzow M, Ragg T. The RIN: an RNA integrity number for assigning integrity values to RNA measurements.

<https://doi.org/>