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RNASeq in *C. elegans* following manganese exposure

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Abstract

Manganese is a metal that is required for optimal biological functioning of organisms. Absorption, cellular import and export, and excretion of manganese are all tightly regulated. While some genes involved in regulation, such as DMT-1 and ferroportin are known, it is presumed that many more are involved and as yet unknown. Excessive exposure to manganese, usually in industrial settings, such as mining or welding, can lead to neurotoxicity and a condition known as manganism that closely resembles Parkinson's disease. Elucidating transcriptional changes following manganese exposure could lead to the development of biomarkers for exposure. This unit presents a protocol for RNA sequencing in the worm *Caenorhabditis elegans* to assay for transcriptional changes following exposure to manganese. This protocol is adaptable to any environmental exposure in *C. elegans.* The protocol results in counts of gene transcripts in control versus exposed conditions, and a ranked list of differentially expressed genes for further study.

Keywords

manganese; *C. elegans*; RNASeq

Introduction

Manganese (Mn) is a metal required in trace amounts for the optimal health of biological organisms. It is the 12th most abundant element in the earth's crust, and so is found naturally in soil and water. Occupational exposure occurs in mining (Rodier, 1955), welding (Racette et al., 2001), and other industrial operations, such as battery manufacture and pesticide use. Also, Mn levels in infant formula and parenteral nutrition may have health consequences. Mn homeostasis is tightly regulated biologically, but excessive exposure results in toxicity, particularly neurotoxicity. In humans, manganism is a condition resembling Parkinson's disease that results from excessive manganese exposure. The mechanisms underlying Mn homeostasis and dysregulation are of interest in order to understand both the toxicity of the metal and the normal functioning of the cell in the presence of Mn. These protocols offer a means to test the effects of Mn exposure on mRNA levels from changes in transcription and/or mRNA stability in *C. elegans* using genome-wide RNA Sequencing (RNASeq). *C. elegans* is a genetically and environmentally tractable organism with a fully sequenced and

well-annotated genome. Studies using Mn in *C. elegans* have been used to characterize the role of several genes important to Mn homeostasis. In this unit, RNASeq is used to assess transcriptional changes genome-wide following exposure of larval stage 1 (L1) or larval stage 4 (L4) worms to Mn as compared to control unexposed worms.

Basic Protocol 1 - Synchronization of worms and Mn treatment

This protocol describes exposure of the worm to Mn. The N2 wildtype *C. elegans* strain or other strain of interest may be used for all experiments. Worms are maintained at 20C on 8P agar fed with the NA22 strain of *E. coli.* Mn lethality curves are performed by exposing N2 worms to hypochlorite and allowing eggs to develop to either L1 or L4 stage, followed by aqueous suspension Mn exposure. L1 worms are exposed to 0, 2.5mM, 5mM, 7.5mM, 10mM, 25mM, and 50mM of Mn to test for lethality. The LD50 for L1 worms is determined experimentally from these exposures. In our hands, experimental exposures were carried out at 10mM. L4 worms were exposed to 0, 10mM, 25mM, 50mM, 100mM, 200mM, and 400mM Mn to test for lethality. The LD50 for L4 worms is determined from these exposures. In our hands, experimental exposures were carried out at 100mM. A higher dose is required to penetrate the cuticle of the more developed worm. Mn is suspended in 85mM NaCl and exposures are followed by three washes with NaCl. For lethality testing, this is followed by plating of approximately 40 worms on NGM media with OP50 strain *E. coli* in triplicate for each dose. Surviving worms are counted 24 or 48 hours post-treatment. For experimental samples, washes are followed immediately by a high quality RNA isolation protocol (**Basic Protocol 2, next**).

Materials—8P agar (see Reagents and Solutions)

NGM agar (see Reagents and Solutions)

NA22 strain *E. coli*

OP50 strain *E. coli*

N2 strain *C. elegans* or other strain of interest

Manganese (MnCl2 tetra hydrate) Sigma Aldrich cat #203734-25G

Hypochlorite solution (see Reagents and Solutions)

85 mM NaCl in commercial grade water; Fisher cat #BP358-212

Commercial grade water; Fisher cat #BP24854

M9 buffer (see Reagents and Solutions)

30% sucrose solution in water; Fisher cat #S5-3

Sterile H2O (autoclaved)

150 mm Petri plates; Corning cat #351058

35 mm Petri plates; Corning cat #351008

Transfer pipets; Corning cat #357524

15 ml conical tubes; Crystalgen cat #23-2266

Siliconized microfuge tubes; Denville cat #C19035

Vortex

Nutator (Spectrum Biomixer)

Dissecting microscope (Zeiss Stemi 2000)

Autoclave

37C bacterial incubator

1. Grow worms according to previously published protocols (Brenner, 1974) on large (150 mm) 8P agar plates seeded with NA22 *E. coli* until 70-80% of the worms are gravid adults, recognizable by spherical eggs apparent within the body of the worm when observed by microscope.
2. Wash plates twice with M9 and transfer M9 containing suspended worms into 15 ml conical tube.

   All steps are carried out at room temperature
3. Vortex briefly to resuspend worms and centrifuge tubes at 1600 rpm for 1 minute.
4. Aspirate cloudy M9 and fill tube to the top with fresh M9. Vortex and spin at 1600 rpm. Repeat until wash solution is clear.
5. Aspirate wash solution. Add 5 ml hypocholorite (bleach) solution per 1 ml of worms.
6. Vortex for approximately 15 seconds and place on nutator for 8 minutes to break cuticle and release eggs from the adult worms.
7. After 8 minutes, observe the tubes under microscope to ensure that all eggs are released from the worms.
8. Add enough M9 to fill each tube to dilute the bleach solution. Vortex briefly and centrifuge for 1 minute at 1600 rpm.
9. Aspirate liquid, taking care not to aspirate egg pellet. Wash once with M9, vortex, and centrifuge 1 minute at 1600 rpm.
10. Add 10 ml of 30% sucrose. Vortex well. Centrifuge at 1600 for 8-10 minutes.

    If an insufficient quantity of eggs is recovered after this step, it may be necessary to reduce the deceleration rate on the centrifuge in order to avoid disrupting the egg layer. Seek advice from your centrifuge

manufacturer if you are unsure how to do this. [*Au: how do you know if you have an insufficient quantity?]

11. Eggs will form a layer floating on top of the sucrose solution. Using a transfer pipet, carefully aspirate eggs, taking no more than 3 ml of solution from the top of the tube. Place this solution in a fresh 15 ml conical tube. Dilute with as much sterile water as possible.

    Take care not to disrupt the egg layer before or during aspiration

12. Vortex briefly and centrifuge at 1600 rpm for 1 minute.

13. Aspirate liquid and wash once with M9. Vortex and centrifuge at 1600 for 1 minute.

14. Aspirate liquid, leaving approximately 500 ul containing the purified egg pellet.

15. Using a 1000 ul pipet, resuspend the egg pellet in the remaining solution and carefully transfer to an unseeded (without bacteria) NGM plate. Incubate at 20C to allow eggs to hatch and worms to grow to the desired stage for the next step.

16. Allow worms to grow to the stage of interest. For L1 worms, 18 hours. For L4 worms, 43-56 hours.

    Eggs that will be grown to L4 stage should be plated onto 150 mm plates seeded with NA22 E. coli following synchronization protocol

17. Make Mn solution fresh in 85 mM NaCl at the experimentally determined LD50 concentration.

18. Wash worms from plates using M9 buffer. For worms grown on bacteria, wash in M9 until all turbidity is clear from the media by vortexing and centrifuging at 1600 rpm.

19. Resuspend worm pellet in 5 ml of M9. Vortex well.

20. Place several 2 ul drops on a microscope slide. Count the number of worms in each drop and calculate average to determine the number of worms per ul.

    Use a large bore pipet tip or cut the end of a standard tip to prevent worms from clogging in the pipet tip

21. Centrifuge at 1600 rpm and aspirate liquid.

22. Wash once with 85 mM NaCl. Resuspend pellet in 5 ml NaCl.

23. To a siliconized microfuge tube, add the volume of worms needed to treat 2,500 worms for lethality testing, or 30,000-50,000 worms for RNA isolation. For L1 worms, add 25 ul of MnCl2 stock solution and fill with 85 mM NaCl to a final volume of 500 ul. For L4 worms, add 50 ul of MnCl2 stock solution and fill with 85 mM NaCl to a final volume of 1000 ul.

    MnCl2 solutions are made in 85 mM NaCl. The NaCl solution used to prepare these stocks, and used in subsequent washes, should be prepared

using commercial grade water to avoid contamination with other metals or chelating agents that may be present in other water sources

24. Rotate tubes on nutator for 1 hour at 25C.

25. Wash three times in 85 mM NaCl. For L1 worms, centrifuge at 7000 rpm for 2 minutes following each wash. For L4 worms, centrifuge at 1000 rpm for 2 minutes following each wash.

26. For lethality testing, plate 40-60 worms on 35 mm NGM agar plates seeded with OP50 *E. coli*. Record the exact number of worms on each plate. Record the number of surviving worms on each plate 48 hours (for L1) or 24 hours (for L4) later for percent survival.

27. For RNA isolation, proceed directly to the Trizol extraction steps described in Basic Protocol 2.

Basic Protocol 2 - RNA isolation and quantification

This protocol yields high quality RNA, sufficient for RNA sequencing as described in Basic Protocol 3 - Library preparation and sequencing. Care should be taken throughout to use RNase-free supplies and equipment to prevent degradation of RNA.

Materials—Trizol reagent; Life Technologies cat # 15596018

Chloroform; EMD cat # CX-1059-1

SureOne 100-1025 ul pipet tips; Fisher 02-707-408

RNaseZap; Life Technologies cat #AM9780

Nuclease free water; Life Technologies cat #AM9932

Isopropanol; EMD cat #PX-1834P-1

Glycogen; Life Technologies cat #AM9510

Ethanol 100%; Decon Labs cat #2705HC

Turbo DNA-free DNase kit; Life Technologies cat #AM1907

MinElute Cleanup kit; Qiagen cat #74204

Chemical Fume hood

NanoDrop 2000 spectrophotometer (NanoDrop, Thermo Scientific, Wilmington, Delaware)

Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California)

Each tube should now contain 30,000 to 50,000 worms as a pellet with most of the liquid aspirated. The worms have been treated with the desired environmental exposure, in this case Mn, and are ready for RNA isolation, described below.

1. Immediately following Mn treatment and NaCl washes, add 1 ml Trizol to each tube.

   Perform this step in a fume hood

2. Freeze tubes in liquid nitrogen and subsequently thaw in a water bath at 37C. Repeat the freeze-thaw cycle 3 times.

3. Add 200 ul of chloroform to each tube using SureOne pipet tips, which are compatible with chloroform, or other compatible tips. Vortex tubes for 15 seconds and allow tosit at room temperature for 3 minutes, followed by centrifugation at 14,000 rpm for 15 minutes at 4C.

   Add chloroform in a fume hood

4. Following centrifugation, remove the top aqueous layer (clear) into an RNase free eppendorf tube. All subsequent steps should be carried out on a bench top treated with RNaseZap to remove RNases, and using pipets and equipment treated to be RNase free.

5. To each tube, add 500 ul of pre-chilled (−20C) isopropanol and 2 ul of glycogen.

6. Mix tubes by inversion and incubate at −20C for at least 1 hour. The incubation step can be performed overnight.

7. Following incubation, centrifuge samples at 14,000 rpm for 10 minutes at 4C.

8. Wash the RNA pellet in 300 ul of 75% ethanol prepared with RNase-free water.

9. Centrifuge samples at 14,000 rpm for 10 minutes at 4C.

10. Remove supernatant and discard and allow the RNA pellet to air dry for approximately 10 minutes.

11. Dissolve the pellet in 25 ul RNase-free water.

12. Treat samples with Turbo DNA-free DNase kit following the manufacturer's instructions to remove contaminating genomic DNA. Briefly:

13. Add 0.1 volume 10 TURBO DNase buffer and 1 ul TURBO DNase to each sample and mix gently.

14. Incubate samples for 20-30 minutes at 37C.

15. Resuspend DNase inactivation reagent and add 0.1 volume to each sample. Mix well.

16. Incubate samples at room temperature for 5 minutes with occasional mixing. Centrifuge at 10,000 g for 1.5 minutes.

17. Transfer RNA to a new RNase-free tube.

18. Further purify samples using the RNeasy MinElute Cleanup kit according to the manufacturer's instructions. Elute in a final volume of 14 ul.

19. Measure RNA quantity, 260/280 ratio, and 260/230 ratio using 1 ul of RNA in a NanoDrop 2000 spectrophotometer or similar.

20. Measure RNA integrity using the Agilent 2100 Bioanalyzer or similar.

Basic Protocol 3 - Library preparation and sequencing

RNASeq libraries are prepared following the modified protocol for directional whole transcript profiling. Whole transcriptome sequencing by RNASeq offers high sensitivity, a large dynamic range to perform global gene expression, mutation, allele-specific expression and other genome-wide analyses. Traditionally RNASeq libraries are generated usually by the synthesis of double stranded cDNA followed by the addition of sequencing adapters. This method does not provide any information related to the DNA strand from which the RNA was transcribed.

Newly evolving directional RNASeq technology provides strand specific sequence information for both coding and non-coding RNAs with an insight into antisense transcripts and their potential role in regulation by accurately quantifying overlapping transcripts. This makes it a more attractive approach to study the transcriptome in organisms that have a more compact genome such as *C. elegans*. Several protocols and applications are available for directional RNASeq library preparation. The most effective is the “dUTP” based method in which the second strand is marked by replacing the dTTPs with dUTPs and later, just before library amplification, the second strand is eliminated using UNG digestion and thus only the first strand is copied during PCR cycles.

Materials—Ribo-Zero Magnetic Gold Kit (Human/Mouse/Rat); Epicenter-Illumina cat # MRZG12324

ERCC Ex fold Spike-In mix2; Life Technologies cat #4456739

ECM (Ethachinmate); Wako cat #312-01791

100 mM dNTP nucleotides; Invitrogen, cat #10297-018

Ribo-Zero rRNA Removal Kit (Human/Mouse/Rat) Low Input or Regular Epicentre, cat # RZH1086 or RZH110424 (Regular)

SuperScript® III First-Strand Synthesis System; Invitrogen, cat #18080-051

RNaseOUT; Invitrogen, cat #10777-019

Second Strand Buffer; Invitrogen, cat #10812-014

E. coli DNA ligase; Invitrogen cat #18052-019

E. coli DNA polymerase; Invitrogen, cat #18010-025

MinElute columns, MinElute PCR Purification Kit (50); Qiagen cat #28004

UNG (Uracil-N-Glycosylase); Fermentas, cat #EN0361

Actinomicyn D; Sigma, cat #A1410-2MG

Or

Fisher, Cat. #BP60610

Stock=10 mg

Working solution=125ng/ ul in H2O

Prepare a stock as 5X and dilute to 1:5 to make working solution.

Store at 4°C

dUTP set (10 μmol each); Promega, ca. #U-1335

100 mM dNTP nucleotides; Invitrogen, cat #10297-018

KAPA Hifi Hotstart DNA Polymerase (100 U 1 U/Ul); Kapa, cat #KK2501

High Sensitivity DNA Kit; Agilent cat #5067-4626)

Qubit RNA BR Assay Kit; Invitrogen cat #Q10210

Qubit dsDNA HS Assay Kit; Invitrogen cat #Q32851

LightCycler 480 Multiwell plate 384 white; Roche, cat #04729749001

Ethanol, Absolute (200 Proof), Molecular Biology Grade; Fisher cat #BP2818-500

Agilent High Sensitivity DNA Kit; Agilent cat #5067-4626

KAPA HTP or LTP Library Preparation Kit for Illumina

TruSeq® DNA HT or LTP Sample Prepration Kit for Illumina (FC-121-2003)

Agencourt® AMPure® XP reagent; Beckman Coulter Inc.cat #A63882

ERCC ExFold control with protocol; Applied Biosystem cat #4456739

Library Quantification Kit; KAPA cat #KK4854

Covaris S2 (High Performance Ultrasonicator); Covaris Inc.

Thermocycler; BioRad

Light Cycler 480; Roche

2100 Bioanalyzer; Agilent

Qubit; Invitrogen

1.5ml Eppendorf tubes RNAse free; Fisher

Benchtop microcentrifuge

Vortex

Heat Block

Pipet tip, Filter; ART tip: 10 Reach; Fisher cat #21402482

Pipette tips; Filter; ART tip: 200; Fisher cat #212361

Diamond grip Gloves (Medium); Fisher cat #11-462-67C

1. Ribosomal RNA (rRNA)-depletion

Ribosomal RNA constitutes up to 99% of the total RNA and the enrichment of whole transcriptome RNA by depleting rRNA species using the Ribo-Zero technology has the potential to enhance our understanding of the transcriptome in both normal physiological and pathological processes. Ribo-Zero kits remove ribosomal RNA (rRNA) using a hybridization/bead capture procedure that selectively binds rRNA species using biotinylated capture probes. The probe:rRNA hybrid is then captured by magnetic beads and removed using a magnet, leaving the desired rRNA-depleted RNA in solution.

1.1 Use 500 ng (Qubit quantified) high quality total RNA isolated and evaluated as above to deplete ribosomal RNA using the Ribo-Zero Magnetic Gold Kit (Human/Mouse/Rat; MRZG12324; Epicentre-Illumina) according to the manufacturer's instructions. This kit removes both cytoplasmic and mitochondrial rRNAs.

1.2 Purify the rRNA depleted-sample using ethanol precipitation method as described in the Ribo-Zero user guide, using 2µl of ECM..

1.3 Resuspend the pellet in 5 µl of RNAse-free water and quantify using Qubit and evaluate by using RNA Pico chip on bioanalyzer.

1.4 The rRNA-depleted RNA can be used immediately in the next step or stored at −80 °C until ready to use.

2. Conversion of RNA to double stranded DNA

Enzymatic conversion of RNA into double-stranded DNA can be accomplished by a number of different procedures, which involve the action of reverse transcriptase and oligonucleotide-primed synthesis of DNA. The purpose of these procedures is to construct DNA with a high yield of conversion of RNA into DNA that can be used for downstream applications successfully. This protocol describes a method for making blunt-ended double stranded DNA that can then be ligated to Illumina adapters for sequencing.

(Optional) In this step, use ERCC ExFold RNA spike-In Mixes to assess the accuracy of measurements of differential gene expression in the sequencing data. The ERCC RNA

Spike-In Control Mixes are pre-formulated sets of 92 polyadenylated transcripts from the ERCC plasmid reference library.

2.1. First strand synthesis—Synthesis of complementary DNA (cDNA) from an RNA template mediated by reverse transcriptases (RTs)-an RNA-dependent DNA polymerase, is called reverse transcription. The SuperScript family of RTs is a first choice for cDNA synthesis. The cDNA generated can be used as a template for amplification by PCR or to generate a cDNA library.

2.1.1 Set a first strand synthesis reaction using SuperScript® III First-Strand Synthesis system according to the manufacturer's instructions and as described here: To 3.375 µl of ribozero RNA (3.375 µl) from above add 5.125 µl of RT Mix (1 µl of 10× RT buffer, 0.8 µl of Random Hexamers (50 ng/µl); 0.5 µl of OligodT (50 µM); 0.85 µl of DTT (0.1 M), 1.7 µl of MgCl2 (25 mM), 0.425 µl of dNTP (10 mM)), mix and incubate at 98°C for 1 minute, ramp down (0.1°C/sec) to 70°C for 5 minutes and go to 15°C ramping down (0.1°C/sec) using a thermocycler (Total time 15 min).

2.1.2 Then add 0.5 µl of Actinomycin D (5 X stock: dilute to 1:5 to make working solution and then use in the above reaction), 0.5 µl of RNaseOUT and 0.5 µl of Superscript III. Incubate at 25°C for 10 min, ramp to (0.1°C/sec) 42°C for 45 min, ramp to (0.1°C/sec) 50°C for 25 min and 75°C for 15 min using a thermocycler. (Total time 1 hour and 35 min). The presence of Actinomycin D would inhibit DNA-templated DNA polymerase activity.

Perform an ethanol precipitation:

2.1.3 Adjust the volume of the reaction mix from above to 50 µl using RNase-Free Water and add 5 µl of 3 M Sodium Acetate and 1 µl of ECM and mix by gentle vortexing.

2.1.4 Then add 250µl of 100% ethanol to each tube and mix thoroughly by gentle vortexing, incubate at –20°C for at least 1 hour or overnight (stop point) or −80°C for 30 min.

2.1.5 Centrifuge the tubes at >10,000 × g in a microcentrifuge for 30 minutes at 4°C. Carefully remove and discard the supernatant.

2.1.6 Wash the pellet with ice-cold 70% ethanol and centrifuge at >10,000 × g for 5 minutes. Carefully remove and discard the supernatant.

2.1.7 Centrifuge briefly to collect any residual supernatant. Carefully remove and discard the supernatant and allow the pellet to air dry at room temperature for 5 minutes.

2.1.8 Dissolve the pellet in 10 µl of the buffer (1 µl of RT buffer (10X), 1 µl of DTT (0.1M), MgCl2 (25 mM), 6 µl RNAse-free water). Resuspended RNA can be stored at –20°C if needed, otherwise continue to the second strand synthesis.

2.2. Second Strand Synthesis—Reverse transcribed cDNA is required to be converted into double stranded DNA before it can be manipulated for downstream assays. This requires DNA polymerase I, which is a template-dependent DNA polymerase. To make the template available for the polymerase the RNA must be exhausted from the ss cDNA:mRNA hybrid generated in the previous step. This employs a ribonuclease (RNase H) which recognizes the RNA component of a DNA:RNA hybrid and cleaves the RNA at a number of non-specific sites leaving short oligoribonucleotides attached to the cDNA, which serve as primers for the polymerase to synthesize the second strand cDNA. DNA Pol I is the enzyme of choice due to its 5′-3′ exonuclease activity that is needed to remove RNA in front of the enzyme. The newly synthesized strands of cDNA are joined by ligation using T4 ligase enzyme. To retain strand specificity this step is modified by incorporating dUTP in place of dTTP in the dNTP mixture. The subsequent destruction of the uridine-containing strand in the sequencing library allows us to identify the orientation of transcript.

2.2.1 Add 45 µl of RNAse-free water to the cDNA from above and Set up the Second Strand Synthesis reaction as follows: To the 55 µl of cDNA add 20 µl of the Second Strand Synthesis reaction mix (15 µl 5× SS buffer; 2 µl of dU/NTP mix (10mM of dATP, dGTP, dUTP, dCTP), 0.5 µl E coli Ligase, 2 µl of *E. Coli* DNA polymerase I, 0.5 µl of RNAse H). Incubate at 16°C for 2 hours.

2.2.2 Purify with MinElute columns and elute in 15 µl of EB Buffer (10 mM Tris-HCl, pH 8.0)

3. Fragmentation

Long cDNA strands are required to be fragmented into the size range that provides more robust and accurate transcript identification after sequencing.

3.1 Dilute the purified double stranded cDNA from above to 50 µl using 1× Low TE-Buffer (10 mM Tris-HCl (pH 8.0 or 8.5) with 0.1 mM EDTA). Solutions containing high concentrations of EDTA and strong buffers may negatively affect the end repair reaction, and should be avoided.

3.2 Use Covaris, High Performance Ultrasonicator, with a fragmentation size of 300bp to fragment the dsDNA as optimized by evaluating the libraries after amplification on the bioanalyzer.

3.3 The fragmented cDNA can then be used directly to the library preparation as below.

4. Library preparation

A cDNA library is a combination of linker (sequencing adapters) ligated cDNA fragments which together constitute a rRNA depleted whole transcriptome (>100 bp) of the test organism generated through a series of reactions.

Use the fragmented cDNA to convert into Illumina compatible RNASeq library using KAPA LTP or HTP and illumina TruSeq® DNA LT or HT reagents according to the manufacturer's instructions with some modifications.

4.1 End Repair: To the 50 µl of Fragmented, double-stranded cDNA add 20 µl of End Repair Master Mix and Mix, and incubate at 20 °C for 30 min. Proceed immediately to the End Repair Cleanup using 120 ul of Ampure Beads following KAPA's End Repair Cleanup protocol.

4.2 A-Tailing: To the beads with end-repaired DNA, add 50 µl of the A-Tailing Master Mix and thoroughly resuspend the beads by pipetting up and down multiple times. Incubate at 30 °C for 30 min. Clean up following KAPA's A-Tailing Cleanup protocol.

Adapter Ligation

4.3 Adapter Ligation: Illumina TrueSeq adapters provide single or dual indexed sequences that can be used to identify each sample within a pool of multiplexed sequencing data. These adapters are provided as concentrates and should be diluted according to the cDNA amount in the ligation reaction. Otherwise, a high number of adapter dimers can be produced in the enriched library.

4.4 To the dried beads with A-tailed DNA, add 45 µl of the Ligation Master Mix and 5 µl of the Illumina TrueSeq adapter (diluted to 2.5µM).

4.5 Thoroughly resuspend the beads by pipetting up and down multiple times and Incubate at 20 °C for 15 min.

4.6 Proceed immediately to the Post-Ligation Cleanup using 40 µl of the PEG/NaCl SPRI® Solution (0.8 X volume of the ligation reaction mix to remove any leftover adapters).

4.7 Thoroughly resuspend the beads by pipetting up and down multiple times and follow KAPA's First Post-Ligation Cleanup.

4.8 Thoroughly resuspend the beads in 22.5 µl of EB and elute as 20 µl from the beads.

UNG Treatment

4.5 UNG treatment: The adapter-ligated dS sDNA is then treated with Uracil-DNA Glycosylase (UNG) to destroy the second strand specifically, which puts the heterologous adapters in a specific orientation relative to the original transcriptional direction.

Assemble library amplification reaction as follows:

4.5.1 To the 20 µl of the post ligated Uracil-containing dsDNA from previous step add 1 µl of UNG, 1.5 µl dNTPs, 1.5 µl of each PCR Oligo 1 and 2 (0.5uM each), 8 µl 5X KAPA HiFi Buffer, 6.5 µl PCR Grade Water to make final volume 40 µl.

4.5.2 Mix thoroughly by pipetting up and down multiple times and Incubate at 37 °C for 15 mins

4.5.3 follow by heat inactivation at 98 °C degrees for 10 mins, keep on ice and continue immediately to the next step.

Library Amplification—Library Amplification: Limited PCR of the resulting UNG treated single-stranded cDNA generates the library for sequencing.

4.6 To the 40 ul of UNG treated dsDNA from the previous step add 2ul of 5X KAPA HiFi Buffer, 7 µl PCR Grade Water and 1 µl of KAPA HiFi Hotstart Polymerase enzyme, Mix and

4.7 Perform PCR with the thermocycling parameters: initial denaturation at 98 °C for 45 sec, following 14 cycles of denaturation at 98 °C for 15, annealing at 60 °C for 30 sec and extension at 72 °C for 30 sec followed by final elongation at 72 °C for 60 sec end with hold at 4 °C to stop the reaction.

4.8 Clean up following KAPA's Library Amplification Cleanup protocol using 40 µl of Ampure Beads (0.8 X volume of the amplification reaction mix and Elute in 30 ul EB).

4.9 Quantify the amplified library using Qubit and analyze 1 µl of the sample using an Agilent 2100 Bioanalyzer DNA Hisensitive chip

4.10 quantify with the KAPA Library Quantification Kit (according to the manufacturer instructions) by QPCR using Light Cycler 480. For optimal results, the yield of amplified library should be >0.3 µg, and the library fragment size distribution should be 200-500 bp.

4.11 If the bioanalyzer indicates the presence of Adapter dimers (peak at 126 bp) in more than 10% of the RNA library , perform a gel extraction (2-3% agarose gel, staining with SYBR Gold and visualization under transilluminator; avoid contact with ethidium bromide and UV visualization) to purify the RNA library.

This assay is a directional RNA sequencing (RNASeq) assay. These libraries can be used for single end as well as paired end sequencing on Illumina platforms, either individually or multiplexed per lane according to the desired depth of coverage (number of reads) for each transcriptome under study.

5. Sequencing

Illumina sequencing by synthesis (SBS) technology, that detects single bases as they are incorporated into growing DNA strands, is the most successful and widely adopted massively parallel sequencing (MPS) technology worldwide. All Illumina platforms are capable of performing multiplex sequencing. In the multiplexed sequencing method, RNA libraries prepared with a unique trueseq indexed adapters (as above) can be pooled into a single lane on a flow cell and sequenced together in one run on HISEQ2500 or any other Illumina platform. An automated three-read sequencing strategy identifies each uniquely tagged sample for individual downstream analysis.

Multiplexed sequencing can be performed for time- and cost-effective whole-transcriptome sequencing according to the size of transcriptome, desired number of aligned reads to discover highly expressed or low expressed genes, desired analysis, and the total number of output sequence reads per lane of the selected read length.

RNASeq libraries can be sequenced as multiplexed (up to 12 samples per lane of a high output flow cell) as single end for 1× 100 bp read length or paired end 2×100 bp on HISEQ2500 (Illumina) following standard protocols.

Basic Protocol 4 - Alignment and analysis

Analysis of the data produced by the Illumina HiSeq 2500 involves initial steps of processing by the Illumina Sequence Control Software (SCS v2.2) and Real Time Analysis (RTA) Pipeline v1.18 software packages (Illumina) followed by WASP (The Wiki-based Automated Sequence Processo-V3.1.5). These software components perform the functions of image analysis, base-calling and alignment. First the output data is demultiplexed into their component samples by the Illumina CASAVA v1.8 software package and then gsnap software (V 2012-07-20 ; http://research-pub.gene.com/gmap/) is used for mapping to a reference genome and HTSeq-count (v0.5.3p3; http://www-huber.embl.de/users/anders/HTSeq/) to assign unique counts to ENSEMBL annotated transcripts.

Raw FASTQ files are trimmed for adapter sequences then alignment to the ce6 genome is performed using ***gsnap*** (Wu and Nacu, 2010). Assignment of reads to genes is performed by htseq-count, a component of the ***HTSeq*** python library (Anders et al., 2015). Assignments are made using known transcripts in ENSEMBL annotation file Caenorhabditis_elegans.WBcel235.72.gtf using the *union* strategy and alignments with a quality score lower than 10 are excluded. Analysis of this output is performed using the R/Bioconductor packages ***edgeR*** for statistical comparison of count data (Robinson et al., 2010).

Materials—gsnap - 2012-07-20 - Linux

samtools - 0.1.19 - Linux

HTseq - v0.5.3p3 - Linux

R - 3.1.0 - platform independent

edgeR - 3.6.8 - platform independent

Step 1 – 3 should be run for each sample, then htseq-count output for all samples were combined into one file before statistical analysis.

1. gsnap -B 4 -N 1 -d ce6 -A sam -s Caenorhabditis_elegans.WBcel235.72.splicesites --read-group-id=flowcell.lane_4_I1 --read-group-name=G1 --read-group-platform=ILLUMINA trimmed.sequene.fastq | samtools view -Sb - | samtools sort - gsnap-rna-alignment
2. samtools view gsnap-rna-alignment.bam | htseq-count -s reverse -a 10 -t exon -m union -ce6.gtf > htseq-union.out
3. R script:

```
require(edgeR)
```

```
dat<- read.table(file="combined.htseq-union.out",header=T, sep="\t", as.is=T, row.names=1))

g<-grep("EN*", rownames(dat3), invert=T) ## remove non-gene rows from htseq output

cds <- DGEList(counts = dat[-g, ], group = grp) ## grp defines group information for each sample

d.cpm <- cpm(cds)

cds$sample$lib.size <- colSums(cds$counts)

cds <- calcNormFactors(cds)

design<-model.matrix(~grp) colnames(design)<-gsub("grp", "", colnames(design))

cds <- estimateGLMCommonDisp(cds, design)

cds <- estimateGLMTrendedDisp(cds, design)

cds <- estimateGLMTagwiseDisp(cds, design)

fit <- glmFit(cds, design)

lrt <-glmLRT(fit, coef=2)

topGenes<-topTags(lrt, n=nrow(cds))$table
```

Reagents and Solutions—8P Agar (1 liter)

3 g NaCl; Fisher cat # BP-358-212

25 g agar; BD cat #214030

20 g peptone; BD cat #211820

975 ml deionized water

Autoclave for 45 minutes on liquid cycle. After cooling, add the following:

1 ml 1 M CaCl2 in water; Fisher cat #C79-500

1 ml 1 M MgSO4 in water; Fisher cat #BP213-1

25 ml KPO4 pH 6 (see below)

Pour into appropriate sized Petri plates using sterile technique.

NGM Agar (1 liter)

3 g NaCl; Fisher cat # BP-358-212

17 g agar BD cat #214030

2.5 g peptone; BD cat #211820

975 ml deionized water

Autoclave for 45 minutes on liquid cycle. After cooling, add the following:

1 ml 1 M CaCl2 in water; Fisher cat #C79-500

1 ml 1 M MgSO4 in water; Fisher cat #BP213-1

25 ml KPO4 pH 6 (see below)

1.25 ml nystatin; Sigma cat #N1638-100ML

0.5 ml streptomycin sulfate (from 100 mg/ml stock solution); Sigma cat #S9137-100G

Pour into appropriate sized Petri plates using sterile technique.

KPO4 pH6

KH2PO4 108.3 g; Fisher cat #P285-3

K2HPO4 35.6 g; Fisher cat #P288-500

1 L deionized water

Autoclave and pH to 6.0

M9 Buffer (2 liters)

6 g KH2PO4; Fisher cat #P285-3

12 g Na2HPO4; Fisher cat #BP332-500

10 g NaCl; Fisher cat # BP-358-212

2 L deionized water

Autoclave 45 minutes on liquid cycle. After cooling, add 2 ml 1 M MgSO4.

Hypochlorite solution (5 ml)

1 ml Clorox bleach

250 ul 10 M NaOH in water; Sigma Aldrich cat #484024-1KG

3.75 ml sterile (autoclaved) H2O

Commentary

Background Information

Next generation sequencing technologies have changed the landscape of biological inquiry, allowing high-throughput interrogation of entire genomes. RNA sequencing, or RNASeq, is a next generation method to catalog transcripts and compare expression of genes across experimental conditions (Morozova et al., 2009; Mortazavi et al., 2008). As the cost of RNASeq has come down and technology has improved, RNASeq has replaced expression arrays as the favored method for testing differential gene expression (Shendure, 2008). In RNASeq, RNA from the tissue of interest is isolated and then converted into a cDNA library. The library is deep sequenced, generating on the order of millions of reads per sample. These reads are then aligned to open reading frames of the reference sequence of the organism from which the sample tissue was derived. Counts are then made of each transcript across the genome. Statistical methods can then be used to identify genes that are differentially expressed across experimental conditions. This unit describes a method to test the effect of Mn exposure on gene expression in worms. This protocol can be adapted to test other substances by substituting an appropriate exposure protocol.

Mn is required as a co-factor for many enzymes, including hydrolases, lyases, glutamine synthetase, arginase, and superoxide dismutase (Friberg et al., 1979; Roholt and Greenberg, 1956; Wedler and Denman, 1984). Mn homeostasis is tightly regulated. In mammals, dietary intake is sufficient to provide the levels needed for biological functioning, and deficiency is rare (Aschner, 2000, 2002). Neurotoxicity due to overexposure to Mn was first described in a group of ore crushers in 1837 (Couper, 1837). Symptoms included muscle weakness, paraplegia, tremor, whispering speech, and a tendency to lean forward while walking. Manganism was later described in several other groups of miners (Mena et al., 1967; Rodier, 1955) and in a group of ferromanganese plant workers (Huang et al., 1989). T1 weighted magnetic resonance imagery (MRI) shows that Mn accumulates preferentially in the basal ganglia of the brain (Butterworth et al., 1995; Inoue et al., 1991; Kulisevsky et al., 1992; Lucchini et al., 2000; Pujol et al., 1993; Weissenborn et al., 1995).

Regulation of Mn homeostasis occurs at the levels of absorption, which occurs across the intestinal wall, import into the cell, sequestration into cell organelles, export from the cell, and excretion. The primary divalent Mn transporter is divalent metal transporter 1 (DMT-1), which localizes to the plasma membrane and imports Mn from the extracellular matrix into the cell (Aschner and Gannon, 1994; Au et al., 2009; Bell et al., 1989; Burdo et al., 2001; Erikson and Aschner, 2002; Garrick et al., 2003). Other transmembrane importers include the citrate transporter (Crossgrove et al., 2003), zinc transporter ZIP8 (Fujishiro et al., 2011; He et al., 2006; Himeno et al., 2009), choline transporter (Lockman et al., 2001), dopamine transporter (Ingersoll et al., 1999), and cation-transporting ATPase 12A2, also known as Park9 (Gitler et al., 2009; Tan et al., 2011). Ferroportin is a known exporter of both Mn and iron from the cytoplasm to the extracellular matrix (Madejczyk and Ballatori, 2012; Yin et al., 2010). The solute carrier SLC30A10 has recently been recognized as a likely exporter of Mn (Chen et al., 2014). While these gene products are known to play a role in Mn homeostasis, it is expected that many more transporters remain to be identified. RNASeq

provides a method to query the entire genome, without prior expectations, to identify transcripts of interest for further study.

Critical Parameters

Culturing *C. elegans*

General practices for worm handling have been described previously (Brenner, 1974). Care should be taken to time the staging of worms properly, such that worms are at the desired stage for investigation. Larval stage 1 worms are treated with Mn 16-18 hours after synchronization. Larval stage 4 worms are treated 43-56 hours post synchronization. At adulthood, egg formation occurs and any RNA isolated with contain a mix of adult and embryonic transcripts. This is to be avoided.

RNA isolation

RNA isolation should ideally be carried out in a dedicated area not used for DNA and other work. Whether this is possible or not, the work area should be decontaminated using RNase Away, RNaseZap, or similar to remove contaminating RNases. Filter pipet tips should be used, and if possible, a dedicated set of pipets for RNA work only. Following isolation of RNA, samples should be handled on ice, limiting freeze-thaw cycles to absolute necessity.

Sequencing and Analysis

The experimental design should include a minimum of three biological replicates per experimental condition to allow for within treatment group estimation of variance (Auer and Doerge, 2010). Each treatment condition should be identically replicated. The total number of expected reads per flowcell lane should be considered and compared to the total number of desired reads per sample to determine the number of samples that can be multiplexed per lane. For example, using the technology described in this unit, 100-120 million reads are expected per lane. If 12 samples are multiplexed per lane, the expected number of reads per sample will be 10-12 million. Using current techniques, the desired number of reads per sample is 30-50 million. Therefore, a minimum of 3 sequencing lanes on a flowcell would be required for 12 samples. The configuration of samples on lanes should utilize the concept of blocking. This means avoiding a situation in which samples from one experimental condition are run on one lane, and samples from another condition are run on a separate lane. In that configuration lane effects could potential introduce bias into the final result. Instead, for example, lane one might contain sample 1 condition 1, sample 1 condition 2, sample 1 condition 3, and sample 1 condition 4. Lane 2 would contain sample 2 from each condition, and lane 3 would contain sample 3 for each condition. This blocked design minimizes the potential for introduction of bias (Auer and Doerge, 2010).

Deposit of data into public repository

Funding or publication conditions may require the deposition of data into a public repository. One such repository is the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) maintained by the National Center for Biotechnology Information (NCBI). The GEO provides a free archive of raw data, assigns accession numbers for sequence data, makes the data searchable by including descriptors and

experimental conditions, and freely distributes raw data. Submitted data may be retained as private until after publication. GEO provides a means for reviewers to access data. Submitted data must be made public following publication.

Troubleshooting

RNA amount or quality

One of the main problems that may be encountered is too little RNA, or RNA of insufficient quality as measured by 260/280 ratio, 260/230 ratio, or Bioanalyzer RIN score. The problem of too little RNA is solved by increasing the amount of starting material. For L1 worms, 30,000 worms should be sufficient to provide approximately 2 ug of high quality RNA. L4 worms are far more difficult to count, because they are heavier and do not stay suspended in liquid media. However, an estimated 20,000-30,000 worms provides 40-60 ug of RNA.

RNA quality is influenced by the presence of RNases, temperature, and repeated freeze-thaw cycles. If RNA quality is low, care should be taken to decontaminate all work surfaces, and use RNase free tubes and pipet tips. Gloves should be worn at all times when handling samples, and gloved hands should be decontaminated with RNA Zap. Sample should be stored at −80C, with care taken to keep the number of freeze-thaw cycles to an absolute minimum.

Sequencing

It is important to generate a high quality library of an optimum size to obtain optimal reads from the sequencing run. The ribosomal depletion step should be done carefully and it should remove more than 90% of the rRNA sequences from the sample. A QPCR test would be wise to test the libraries before sequencing. Care should be taken to carefully dilute the adapters according to the amount of the cDNA to reduce adapter dimers during sequencing.

Analysis

Additional resources are available online for troubleshooting analysis steps. For gsnap, documentation is available at http://research-pub.gene.com/gmap/. For samtools, documentation is available at http://www.htslib.org/. For HTseq, documentation is available at https://pypi.python.org/pypi/HTSeq. For edgeR, documentation is available at http://www.bioconductor.org/packages/release/bioc/vignettes/edgeR/inst/doc/edgeRUsersGuide.pdf. Additionally, http://seqanswers.com/ is a community based forum where users can post questions or problems related to next generation sequencing and analysis. It is also useful to seek out core resources where expert programming help may be available.

Anticipated Results

RNA isolation and purification will yield approximately 2 ug of high quality RNA, suitable for sequencing. RNASeq will yield 30-50 million reads per sample. Differential expression analysis of these reads in edgeR will produce tables of all genes with mapped reads, the log2 fold change, p-value, and false discovery rate adjusted p-value. This ranked list of differentially expressed genes is suitable for analysis using tools such as Reactome

(reactome.org), Kegg pathways (http://www.genome.jp/kegg/) or Ingenuity Pathway Analysis (IPA, Qiagen), with the caveat that IPA is not optimized for *C. elegans*. Pathway analysis can reveal genes in common pathways or families that may be involved in regulating Mn, in this case, or other experimental conditions of interest.

Time Considerations

Worms will take 2-3 days on a 150 mm plate to grow to an appropriate density for synchronization. Synchronization takes approximately 1 hour. Worms are then left to grow to the appropriate stage: 16-18 hours for L1, or 43-56 hours for L4. Mn treatment takes approximately 2 hours, including time to prepare solutions and incubation. RNA isolation takes several hours, including the incubation step at -20C. Mn treatment and RNA isolation can be completed in one day. The library preparation takes from 2-4 days and sequencing takes one week to complete on a high output mode for a single end 100 bp run. RNASeq pipeline also takes 2-4 days depending upon the amount of data generated.

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