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Nanopore Transcriptomic Sequencing with C. elegans

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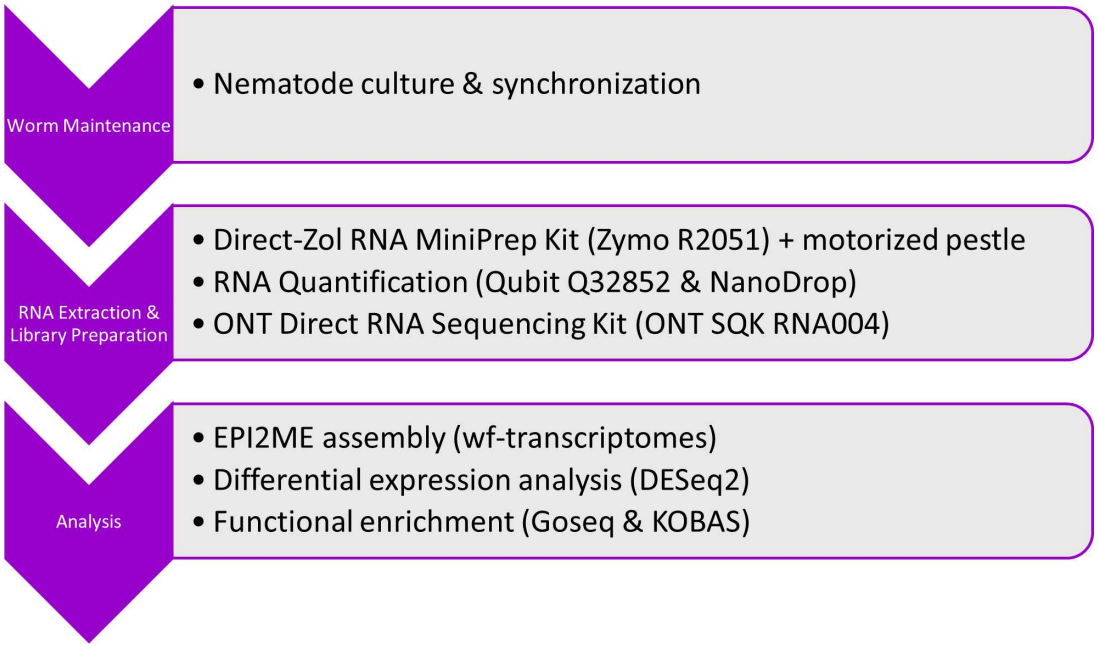
Disclaimer

This protocol was created as an assignment for BIT 495/595: Portable Genome Sequencing (Fall 2024) at NC State University.

Abstract

This protocol entails sequencing native RNA of young adult *C. elegans* using NanoPore sequencing (Oxford NanoPore Technologies, ONT). RNA extraction will be conducted using modified versions of Zymo's "**Direct-zol‰ RNA Miniprep**" (Zymo R2050) and ONT's "**Worm (Caenorhabditis elegans) RNA**" protocols. Library preparation will be conducted according to ONT's **Direct RNA Sequencing Kit** (ONT SQK-RNA004) protocols and sequencing will be conducted on a MinION Mk1C device (Figure 1). Subsequent analysis should be conducted via alignment and assembly by the wf-transciptomes workflow in EPI2ME followed by followed by differential expression analysis with DESeq2 and functional enrichment with Goseq and KOBAS 2.0 in R Studio.



Protocol Workflow

Materials

- Direct-zol RNA Miniprep Plus **Zymo Research Catalog #**R2070
- Qubit RNA HS (High Sensitivity) assay **Thermo Fisher Scientific Catalog #**Q32852
- Oxford Nanopore Direct RNA sequencing (SQK-RNA002) **Oxford Nanopore Technologies Catalog #**SQK-RNA002
- T4 DNA Ligase - 20,000 units **New England Biolabs Catalog #**M0202S
- NEBNext Quick Ligation Reaction Buffer (5X) - 2.0 ml **New England Biolabs Catalog #**B6058S
- Qubit dsDNA HS Assay kit **Thermo Fisher Scientific Catalog #**Q32854
- RNA MinION Flow Cell **Oxford Nanopore Technologies Catalog #**FLO-MIN004RA

Protocol materials

- TRI Reagent **Zymo Research Catalog #**R2050-1-50 Step 18
- MilliQ Water Step 29
- Oxford Nanopore Direct RNA sequencing (SQK-RNA002) **Oxford Nanopore Technologies Catalog #**SQK-RNA002

Materials, Step 45

- NEBNext Quick Ligation Reaction Buffer (5X) - 2.0 ml **New England Biolabs Catalog #**B6058S Materials, Step 45.1
- T4 DNA Ligase - 20,000 units **New England Biolabs Catalog #**M0202S Materials, Step 45.1
- Freshly prepared 70% ethanol in nuclease free water Step 57
- RNA MinION Flow Cell **Oxford Nanopore Technologies Catalog #**FLO-MIN004RA Materials
- Nematode growth media agarose **Catalog #**substance_media_ngma Step 1
- M9 solution for nematode culture Step 3
- RNA wash buffer (Optional) **Zymo Research Catalog #**R1003-3-24 In 2 steps
- DNase/RNase-Free Water **Zymo Research Catalog #**W1001-1 In 4 steps
- Qubit dsDNA HS Assay kit **Thermo Fisher Scientific Catalog #**Q32854 Materials, Step 78
- Qubit RNA HS (High Sensitivity) assay **Thermo Fisher Scientific Catalog #**Q32852 Materials, Step 32
- Direct-zol RNA Miniprep Plus **Zymo Research Catalog #**R2070 Materials

Before start

Parent *C. elegans* worm cultures should be reared on Nematode Growth Medium (NGM) plates with OP50 lawns according to standard protocols (see WormBook's "**Maintenance of *C. elegans***"), roughly 2-4 days before Day 0. All reagents in the Direct-Zol RNA MiniPrep Kit (Zymo R2051) and ONT Direct RNA Sequencing Kit (ONT SQK-RNA004), including the MinION RNA flow cells (FLO-MIN004RA), should be brought to room temperature on Day 3.

Nematode Culture & Synchronization (Day 0)

20m

1 Culture *C. elegans* on

Nematode growth media agarose **Contributed by users** **Catalog #**substance_media_ngma

plates (NGM agar plates) until adulthood and/or they have begun laying eggs onto the *E. coli* lawn.

2 Prepare fresh **bleaching solution** in a sterile 1.5mL microcentrifuge tube:

	Volume (µl)
DI Water	250
Bleach	250
1M NaOH	500

3 Using a P1000, **add 1mL of M9+TX**

M9 solution for nematode culture **Contributed by users** + 0.1% Triton-X-100 (22mM KH2PO4, 42mM NA2HPO4, 89mM NaCl, 1mM MgSO4, 0.1% Triton X-100) **to a plate of worms**. With the same tip, pipette the solution across the plate multiple times to wash the worms off of the lawn into the solution.

4 Transfer the worms to a new 1.5mL microcentrifuge tube and **centrifuge for 10 sec to pellet** the worms.

5 Remove the supernatant and **add the bleaching solution**.

6 **Incubate for** 00:02:00 **(2-5 minutes)**, shaking occasionally, until the majority of the worm bodies have been dissolve. 2m

Note

IMPORTANT: Do not allow more than 5 minutes of incubation in the bleaching solution. The point of this step is to dissolve the bodies of the adult worms but leave the eggs unharmed, which is time-dependent. On the opposite side, if the bodies have not been sufficiently broken up then they could retain residual bleach, which would additionally harm future progeny.

7 **Centrifuge for 10-20 sec** to pellet. Remove the supernatant and **resuspend in M9+TX**.

8 **Repeat Step 7** two more times (for a total of **3 washes**). 5m

9 **Centrifuge for 10-20 sec** to pellet, then remove 990µl of supernatant, **leaving 50-100µl** in the tube.

10 Using a P100, **resuspend the pellet in the 50-100µl of M9+TX**, then **transfer to a fresh NGM plate** by pipetting droplets of eggs outside of the lawn.

11 **Wait** 00:05:00 **(5-10 minutes)** for the plate to dry. 5m

12 Store the plate upside-down in a **20°C incubator** until the eggs have hatched and reach young adult (roughly 60hr) or another desired age. 3d

RNA Extraction & Sequencing (Day 3) - Part 1, RNA Extraction 1h

13 Using a P1000, **add 1mL of M9+TX to the plate** of worms. With the same tip, pipette the solution across the plate multiple times to wash the worms off of the lawn into the solution.

14 **Transfer the worms** to a new 1.5mL microcentrifuge tube.

15 **Allow 2 min** for the worms to settle to the bottom of the tube. 2m

Note

NOTE: Large worms (young adult & Day 1 adult) should settle quickly (~60 sec). Smaller worms (L4 and below) will take more than 1 min to pellet and should be discarded with the supernatant.

16 **Remove the supernatant** and **resuspend in M9+TX**.

17 **Repeat Steps 2-3** two more times (for a total of **3 washe**s). 8m

18 Remove the supernatant and **add 600µl of**
TRI Reagent **Zymo Research Catalog #**R2050-1-50

19 **In a fume hood or BSC**, insert a **motorized pestle** into the tube and use **1-2 sec pulses for 60 sec** to break the worm cuticle. Visually inspect the tube under a microscope to confirm lysis. 1m

20 **Add 700µl of 95% ethanol** to the lysed sample and mix by gently pipetting.

21 Transfer the sample to a **Zymo-Spin IICR Column** in a Collection Tube and centrifuge for
16000 x g, 4°C, 00:01:00 . 1m

22 **Transfer the column to a new collection tube** and discard the flow-through.

23 Treat with DNase I to remove DNA

23.1 Add **500µl of** RNA wash buffer (Optional) **Zymo Research Catalog #**R1003-3-24 to the column, then centrifuge at 16000 x g, 4°C, 00:01:00 . Discard the flow-through. 1m

23.2 Combine 5µl **DNase I** (6 U/µl) with 75µl **DNA Digestion Buffer** in an RNase-free tube and mix by gentle inversion. Add the 80µl solution directly to the column matrix.

23.3 **Incubate** at room temperature for **15 minutes**.

15m

24 Add 400µl of **Direct-zol RNA PreWash** buffer to the column and centrifuge at 16000 x g, 4°C, 00:01:00 . Discard the flow-through. 1m

25 **Repeat Step 14**. 1m

26 Add 700µl of RNA wash buffer (Optional) **Zymo Research Catalog #**R1003-3-24 to the column and centrifuge at 16000 x g, 4°C, 00:01:00 . 1m

27 **Transfer the column** to a new RNAse-free tube.

27.1 Add 50µl of DNase/RNase-Free Water **Zymo Research Catalog #**W1001-1 directly to the column matrix and **incubate for 2-5 min**.

27.2 Centrifuge at 16000 x g, 4°C, 00:01:00 to elute the RNA. 1m

27.3 Keep the sample on ice or frozen at -80°C until use.

RNA Extraction & Sequencing (Day 3) - Part 2, RNA Quantification via NanoDrop 5m

28 Add DI Water to a Kimwipe and wipe down the **NanoDrop Spectrophotometer**.

Equipment	
NanoDrop Spectrophotometer	NAME
2000/2000c Spectrophotometers	TYPE
NanoDrop	BRAND
ND2000CLAPTOP	SKU
https://www.thermofisher.com/order/catalog/product/ND2000CLAPTOP	LINK

29 Add **2µl of DI Water** MilliQ Water **Contributed by users** to the pedestal, then open the NanoDrop software for “Nucleic Acids” and run the initial setup & calibration.

30 Wipe the pedestal and add **2µl of** DNase/RNase-Free Water **Zymo Research Catalog #**W1001-1 then run a **Blank** measurement.

31 Wipe the pedestal and add **2µl of sample**. Record the concentration (in ng/µl), A260/A280, and A260/A280.

RNA Extraction & Sequencing (Day 3) - Part 3, RNA Quantification via Qubit

10m

32 Prepare Qubit 1X dsRNA HS **working solution** by diluting **Qubit RNA HS reagent** 1:200 in **Qubit RNA HS buffer** from the Qubit RNA HS (High Sensitivity) assay **Thermo Fisher Scientific Catalog #**Q32852 in a clean, Nuclease-Free tube.

33 In a PCR tube, combine **190µl of working solution** with 10µl of **standard**. Label the top of the tube for Standard “1”.

34 **Repeat Step 2** for Standard “2”.

35 In a PCR tube, combine **199µl of working solution** with **1µl of sample**.

36 **Vortex** the standards and sample tubes for 5 sec, then briefly centrifuge.

37 Allow the standards and sample to **incubate at room temperature for 2 min**. 2m

38 On the Qubit Fluorometer, select 1x dsRNA High Sensitivity then select "**Read Standards**" and insert Standard #1 into the sample chamber. Select "**Run standards**".

Equipment

Qubit Fluorometer	NAME
Fluorometer	TYPE
Invitrogen	BRAND
Q33238	SKU
https://www.thermofisher.com/order/catalog/product/Q33238#/Q33238	LINK

39 **Replace with Standard #2** and select "Run standards" again. Then select "Next" to proceed.

40 Replace with the sample tube and enter the sample volume added to the assay tube (1µl) then select "**Run sample**". Record the concentration (in ng/µl).

41 **Your RNA should have** a minimum concentration of **125 ng/µl** (1 ug in 8µl), an **A260/A280 ≥ 2.0** and an **A260/A230 of 2.0-2.3**.

Note

IMPORTANT: If you do not meet these QC criteria then you may need to perform an additional
clean & concentrate procedure prior to library preparation.

RNA Extraction & Sequencing (Day 3) - Part 4, Setup for Library Preparation & Sequencing

10m

42 Thaw MinION RNA flow cell (FLO-MIN004RA) and bring to room temperature

43 Power on the **Mk1C** and insert a **MinION RNA flow cell (FLO-MIN004RA)**.

Equipment

MinION Mk1C	NAME
Sequencer	TYPE
Oxford Nanopore Technology	BRAND
MIN-101C	SKU
https://store.nanoporetech.com/minion-mk1c.html	LINK

Note

IMPORTANT: The Direct RNA Sequencing Kit (SQK-RNA004) is only compatible with RNA flow cells (FLO-MIN004RA).

44 Log in to the MinKNOW software then **select Mk1C in the connection manager**. Navigate to the Start page and select "**Flow Cell Check**" and select "Start" after the flow cell type and ID has been recognized.

45 **Thaw and prepare the Direct RNA sequencing (SQK-RNA004) library reagents** while the flow cell check is running:

Oxford Nanopore Direct RNA sequencing (SQK-RNA002) **Oxford Nanopore Technologies Catalog #**SQK-RNA002

45.1 Prepare **1x NEBNext Quick Ligation Reaction Buffer**

NEBNext Quick Ligation Reaction Buffer (5X) - 2.0 ml **New England Biolabs Catalog #**B6058S

and **T4 DNA Ligase**

T4 DNA Ligase - 20,000 units **New England Biolabs Catalog #**M0202S according to the manufacturer's instructions and place on ice.

45.2 Spin down the **RT Adapter (RTA)**, **RNA CS (RCS)** (if using), and **RNA Ligation Adapter (RLA)**, pipette mix and **place on ice**.

45.3 Thaw the **Wash Buffer (WSB)** and **RNA Elution Buffer (REB)** at room temperature and mix by vortexing. Then spin down and **place on ice**.

45.4 *After the flow cell check has finished:* **Check the number of active pores** (a minimum of 800 are needed) and the flow cell health indicators after the check has been completed to ensure there are no issues.

RNA Extraction & Sequencing (Day 3) - Part 5, Library Preparation

2h 10m

46 Prepare **1ug of RNA sample in 8µl** of

DNase/RNase-Free Water **Zymo Research Catalog #**W1001-1 in a PCR tube. Mix gently by pipetting then briefly spin down.

47 In the same PCR tube, combine the reagents in the following order (pipetting gently to mix in between):

Reagent	Volume (µl)
RNA	8
NEBNext Quick Ligation Reaction Buffer	3
RNA CS (RCS)	0.5
Murine RNAse Inhibitor	1
RT Adapter (RTA)	1

T4 Ligase	1.5
Total	**15**

48 Spin down then incubate for 00:10:00 at room temperature. 10m

49 In a clean 1.5 mL DNA LoBind Eppendorf tube, combine the following reagents together to make the **reverse transcription master mix (RTMM)**:

Reagent	**Volume (µl)**
Nuclease-Free Water	13
10 mM dNTPs	2
5x Induro RT Reaction Buffer	8
Total	**23**

50 **Transfer the RTMM to the PCR tube** containing your **adapter-ligated RNA** and mix by pipetting.

51 Add **2µl of Induro Reverse Transcriptase** to the reaction and mix by pipetting.

52 Place the tube in a thermal cycler and incubate at 60 °C for 00:30:00, then 70 °C for 00:10:00, and bring the sample to 4 °C before proceeding to the next step. 40m

53 **Transfer the sample** to a clean 1.5 ml Eppendorf DNA LoBind tube.

54 Resuspend the stock of **Agencourt RNAClean XP** beads by vortexing.

55 **Add 72 µl of resuspended Agencourt RNAClean XP beads** to the reverse transcription reaction and mix by pipetting.

56 **Incubate on a Hula mixer** (rotator mixer) for 00:05:00 at room temperature. 5m

Equipment	
Hula mixer	NAME
Mixer	TYPE
Invitrogen	BRAND
15920D	SKU
Any rotator mixer	SPECIFICATIONS

57 **Prepare 200µl** of
Freshly prepared 70% ethanol in nuclease free water **Contributed by users** .

58 **Spin down** the sample and **pellet on a magnet**. Keep the tube on the magnet, and **pipette off the supernatant** when clear and colorless.

59 Keep the tube on magnet until the supernatant is clear and colorless before **washing the beads with 150 µl of freshly prepared 70% ethanol**, as described below:

59.1 Keeping the magnetic rack on the benchtop, **rotate the tube by 180°**. **Wait for the beads** to migrate towards the magnet and to form a pellet.

59.2 **Rotate the tube 180° again** (back to the starting position), and **wait for the beads to pellet** again.

60 Carefully **remove the 70% ethanol** using a pipette and discard.

61 Spin down and place the tube back on the magnet until the eluate is clear and colourless. Keep the tubes on the magnet and **pipette off any residual ethanol**.

62 Remove the tube from the magnetic rack and resuspend the pellet in **23 µl** DNase/RNase-Free Water **Zymo Research Catalog #**W1001-1 . Incubate for 00:05:00 at room temperature. 5m

63 **Pellet the beads** on a magnet until the eluate is clear and colourless.

64 Remove and retain **23 µl** of eluate into a clean 5 ml Eppendorf DNA LoBind tube.

Note

NOTE: At this stage, the RT-RNA sample can be stored at -80°C for later use.

65 In the same 1.5 ml Eppendorf DNA LoBind tube, combine the reagents in the following order:

	Reagent	Volume (uL)
	RT-RNA Sample	23
	NEBNEXT Quick Ligation Reaction Buffer	8
	RNA Ligation Adapter (RLA)	6
	T4 DNA Ligase	3
	Total	**40**

66 Mix by pipetting.

67 Incubate the reaction for 00:10:00 at room temperature. 10m

68 Resuspend the stock of **Agencourt RNAClean XP beads** by vortexing.

69 **Add 16 µl** of resuspended Agencourt RNAClean XP beads to the reaction and mix by pipetting.

70 **Incubate on a Hula mixer** (rotator mixer) for 00:05:00 at room temperature. 5m

71 **Spin down the sample and pellet on a magnet**. Keep the tube on the magnet for 00:05:00 , and pipette off the supernatant when clear and colourless. 5m

72 **Add 150 µl** of the **Wash Buffer (WSB)** to the beads. 5m

Close the tube lid and resuspend the beads by flicking the tube.

Return the tube to the magnetic rack, allow the beads to pellet for 00:05:00 and pipette off the supernatant when clear and colourless.

73 **Repeat Step 72.**

74 **Spin down** the tube and replace onto the magnetic rack until the beads have pelleted to **pipette off any remaining Wash Buffer (WSB)**.

75 Remove the tube from the magnetic rack and **resuspend the pellet in 13 µl RNA Elution Buffer (REB)** by the gently flicking the tube. 10m

Incubate for 00:10:00 at room temperature.

76 **Pellet the beads** on a magnet for 00:05:00 until the eluate is clear and colourless. 5m

77 Remove and **retain 13 µl of eluate** into a clean 5 ml Eppendorf DNA LoBind tube.

78 Quantify **1 µl of reverse-transcribed and adapted RNA** using the Qubit dsDNA HS Assay kit **Thermo Fisher Scientific Catalog #**Q32854 .

Note

IMPORTANT: The final concentration should be ideally > 30 ng.

79 The reverse-transcribed and adapted RNA is now ready for loading into the flow cell.

Note

IMPORTANT: The RNA library must be sequenced immediately and cannot be stored for later use.

RNA Extraction & Sequencing (Day 3) - Part 5, Priming & Loading the Flow Cell

10m

80 Thaw the **Sequencing Buffer (SB)**, **Library Solution (LIS)**, **RNA Flush Tether (RFT),** and **Flow Cell Flush (FCF)** at room temperature.

Mix by vortexing and spin down.

81 To prepare the **flow cell priming mix** in a clean 1.5 ml Eppendorf DNA LoBind tube, combine the following reagents.

Mix by vortexing and spin down at room temperature.

Reagent	Volume (µL)
RNA Flush Tether (RFT)	30
Flow Cell Flush (FCF)	1,170
Total	**1,200**

82 Open the **MinION** or GridION device lid and slide the flow cell under the clip.

Press down firmly on the flow cell to ensure correct thermal and electrical contact.

83 Slide the flow cell priming port cover clockwise to open the **priming port**.

84 After opening the priming port, check for a small air bubble under the cover. **Draw back a small volume** to remove any bubbles according to the following:

84.1 Set a **P1000 pipette** to 200 µl

84.2 Insert the tip into the **priming port**

84.3 Turn the wheel until the dial shows 220-230 µl, to **draw back 20-30 µl**, or until you can **see a small volume of buffer entering the pipette tip**

85 **Load 800 µl of the priming mix** into the flow cell via the priming port, avoiding the introduction of air bubbles. 5m

Wait for 00:05:00 .

86 *During this time*, **prepare the library** for loading by combining the following in a new 1.5 ml Eppendorf DNA LoBind tube:

Reagent	Volume (µl)
Sequencing Buffer (SB)	37.5
Library Solution (LS)	25.5
RNA Library	12
Total	**75**

87 **Complete the flow cell priming acccording to the following**:

87.1 Gently lift the SpotON sample port cover to make the **SpotON sample port** accessible.

87.2 **Load 200 µl of the priming mix** into the flow cell priming port (not the SpotON sample port), avoiding the introduction of air bubbles.

88 **Mix the prepared library gently** by pipetting up and down just prior to loading.

89 **Add 75 µl of the prepared library** to the flow cell via the **SpotON sample port** in a **dropwise** fashion.

Ensure each drop flows into the port before adding the next.

90 Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port and close the priming port.

91 Place the **light shield** onto the flow cell, as follows:

91.1 Carefully place the leading edge of the light shield against the clip.

> Note
>
> **NOTE:** Do not force the light shield underneath the clip.

91.2 Gently lower the light shield onto the flow cell. The light shield should sit around the SpotON cover, covering the entire top section of the flow cell.

RNA Extraction & Sequencing (Day 3) - Part 5, Running the Sequencing Reaction

92 **Navigate to the Start page** on the Mk1C and click "**Start Sequencing**".

93 Fill in the experiment details, such as name and flow cell position and sample ID.

94 On the Kit page, select the **Direct RNA Sequencing Kit (ONT SQK-RNA004)**.

95 On the Run configuration tab, **select "super-accurate" base-calling**.

96 **Click "Start"** on the Review page to start the sequencing run.

Note

NOTE: The sequencing run may take 1-3 days to run to completion.

Sequencing Analysis (Day 3+)

20m

97 After the sequencing reaction has finished, concatenate the fastq files on a Linux or Max device using the command prompt. *

Note

NOTE: If using a Windows device, you may need 3rd party software to concatenate files.

Note

NOTE: It is not necessary to concatenate files for EPI2ME workflows

98 **Download the *C. elegans* reference genome**: 10m

98.1 Go to **NCBI's genome browser** and search *C. elegans*.

98.2 Select the Assembly with the green check-mark next to it (it will be the most recent RefSeq version).

Note

IMPORTANT: Make sure the reference genome matches the strain you sequenced (available in the "Modifier" column)

98.3 Click "Download"

98.4 When prompted, Select "Genome sequences (FASTA)", "Annotation features (GFF)", "Transcripts (FASTA)" as the file types

98.5 Name the zip file then click "Download". Make sure to extract the folder after downloading.

99 Open EPI2ME (on cloud or desktop) and run the concatenated file on the **wf-transcriptomes** workflow. 10m

99.1 Sign in to EPI2ME and Navigate to the "Launch" tab.

Note

NOTE: For large analysis projects like transcriptomes, it is recommended to use the Cloud capabilities, which requires the user to have an EPI2ME account.

99.2 Select the "Transcriptomes" workflow from the list.

99.3 Click "Run in Cloud" and "Launch".

99.4 Select the "Input Options" on the left side...

for "FASTQ" select the folder containing the fastq_pass files from your sequencing reaction

for "Reference genome" select the file for the worm reference genome (.fna or .fasta) from NCBI

for "Reference transcriptome" select the file for the worm reference transcriptome (.fna or .fasta) from NCBI

for "Reference annotation" select the file for the worm genome annotation features (.gff) from NCBI

for "direct RNA" set to "True" or click the checkbox

Note

NOTE: Diffeerential expression analysis can be conducted in EPI2ME whithin this workflow by navigating to "Differential Expression Options" on the left and editing the expression parameters.

99.5 Select "Launch workflow".

Note

NOTE: EPI2ME transcriptome alignment and assembly may take anywhere from 3-10 hours depending on the sample.

100 If conducting differential expression analysis, prepare a reference set and run the **DESeq2 workflow** in R Studio.

101 If conducting functional enrichment with a prepared reference set, run with **GOseq** and **KOBAS** 2.0 in R Studio.

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