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Workflow to extract and process RNA from *Drosophila* testes with and without the endosymbiont *Wolbachia*

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We use this protocol and it's working

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Abstract

This protocol describes a complete workflow for extracting and processing RNA from *Drosophila* testes, including those harboring the endosymbiont *Wolbachia*. We begin by extracting and purifying RNA using TRIzol:chloroform phase separation, followed by a TURBO DNase Kit treatment to remove contaminating genomic DNA. cDNA is then synthesized using the SuperScript IV VILO Master Mix. Before DNase treatment, this method consistently yields approximately 52.5 ng of RNA per testes pair, with A260/A280 of ~1.94 and A260/A230 of ~1.45. Using digital droplet PCR (ddPCR) to quantify the *Wolbachia cifA* gene and the *Drosophila* β-spectrin gene, we confirmed that the DNase treatment completely eliminates genomic DNA contamination. This workflow is suitable for performing sensitive, accurate, and precise RT-ddPCR assays to measure both *Wolbachia* and *Drosophila* mRNA levels.

Guidelines

Important considerations:

- Unless otherwise stated, steps are at room temperature.
- Unless otherwise stated, use pipettes for liquid transfer.
- All reagents and materials should be certified RNase-free.
- Use only freshly opened consumables.
- Always wear a KN95 mask to protect samples from RNases.
- Speaking while performing extractions risks RNase contamination.

Materials

Materials

- Centrifuge tubes, safe-lock, PCR clean, 1.5 mL **Eppendorf Catalog #**022363212
- Centrifuge tubes, safe-lock, PCR clean, 2 mL **Eppendorf Catalog #**022363344
- Chloroform, 99.8+%, ACS grade **Thermo Fisher Scientific Catalog #**032614.K2
- Conical tube, 50 mL **Olympus Catalog #**28-101
- Ethanol, 200 proof **KOPTEC Catalog #**V1001
- Glycogen, UltraPure **Invitrogen - Thermo Fisher Catalog #**10814010
- Homogenizing beads, 2.8 mm, ceramic **VWR International (Avantor) Catalog #**10158-554
- Isopropanol, 99.5%, molecular biology grade **Thermo Fisher Scientific Catalog #**327272500
- Kimtech Kimwipe delicate task wipes **Kimberly-Clark Catalog #**34155
- Low EDTA TE (1X), pH 8.0 **Quality Biological Catalog #**351-324-721
- Molecular Grade Water **MIDSCI Catalog #**786-73C
- Phosphate Buffered Saline, 10X Solution **Fisher Scientific Catalog #**BP3994
- Pipette tips, filtered, PCR clean and sterile, 0.1 - 10 uL **Eppendorf Catalog #**0030078519
- Pipette tips, filtered, PCR clean and sterile, 2 - 100 uL **Eppendorf Catalog #**0030078543
- Pipette tips, filtered, PCR clean and sterile, 50 - 1,000 uL **Eppendorf Catalog #**0030078578
- Qubit RNA High Sensitivity (HS) Assay Kit **Invitrogen Catalog #**Q32855
- RNase Free, Surface Decontaminant **ApexBio Technology Catalog #**10-228
- SuperScript IV VILO Master Mix **Invitrogen Catalog #**11756050
- TRIzol Reagent **Thermo Fisher Scientific Catalog #**15596026
- Tubes, 0.2 mL, flat cap **Thermo Fisher Catalog #**AB0620
- TURBO DNA-free™ Kit **Thermo Fisher Catalog #**AM1907
- Universal RNA Spike I **TATAA Biocenter Catalog #**RS25SI
- Sodium Acetate Trihydrate **Fisher Scientific Catalog #**S609500

Equipment

- Autoclave (Any)
- Bead-mill homogenizer (Benchmark Scientific, Bead Blaster 96)
- Centrifuge (Eppendorf, 5420)
- Centrifuge, refrigerated (Eppendorf, 5430-R)
- Chemical hood (Any)
- Drosophila Flypad (Flystuff, 59-119)

- Forceps, fine-tipped (Fine Science Tools, 11295-10)
- Freezer, -80C (Haier Biomedical, DW-30L1280TR)
- Graduated cylinder, 1 L (Any)
- Ice bucket (Any)
- Media bottle, 1 L, glass (Any)
- Mini centrifuge (VWR, 76269-064)
- NanoDrop Spectrophotometer (Thermo Scientific, ND-ONE-W)
- Petri dish, 35 mm, glass (Any)
- Pipette, 10 µL (Eppendorf, 4861000708)
- Pipette, 100 µL (Eppendorf, 4861000716)
- Pipette, 1000 µL (Eppendorf, 4861000732)
- Qubit 4 Fluorometer (Thermo Scientific, Q33238)
- Stereomicroscope (Zeiss, Stemi 305)
- Thermocycler (BioRad, C1000/S1000
- Thermomixer (Eppendorf, 5387000013)
- Vacuum chamber (Any)
- Vortex mixer (Any)

Preparations

- Dissecting dish: Add a thin layer of Sylgard 184 **Electron Microscopy Sciences Catalog #**24236-10 to the bottom of a 35 mm glass petri dish. Remove bubbles using a vacuum chamber.
- 1x PBS: Using an autoclaved graduated cylinder, add to a 1 L media bottle: 100 mL Phosphate Buffered Saline, 10X Solution **Fisher Scientific Catalog #**BP3994 and 900 mL Molecular Grade Water **MIDSCI Catalog #**786-73C
- 75% ethanol: Using the graduations of a Conical tube, 50 mL **Olympus Catalog #**28-101, mix: 30 mL Ethanol, 200 proof **KOPTEC Catalog #**V1001 and 10 mL Molecular Grade Water **MIDSCI Catalog #**786-73C

Protocol materials

- Kimtech Kimwipe delicate task wipes **Kimberly-Clark Catalog #**34155
- RNase Free, Surface Decontaminant **ApexBio Technology Catalog #**10-228
- Universal RNA Spike I **TATAA Biocenter Catalog #**RS25SI
- Low EDTA TE (1X), pH 8.0 **Quality Biological Catalog #**351-324-721
- Centrifuge tubes, safe-lock, PCR clean, 1.5 mL **Eppendorf Catalog #**022363212
- Glycogen, UltraPure **Invitrogen - Thermo Fisher Catalog #**10814010
- Isopropanol, 99.5%, molecular biology grade **Thermo Fisher Scientific Catalog #**327272500
- Centrifuge tubes, safe-lock, PCR clean, 2 mL **Eppendorf Catalog #**022363344
- TRIzol Reagent **Thermo Fisher Scientific Catalog #**15596026
- Homogenizing beads, 2.8 mm, ceramic **VWR International (Avantor) Catalog #**10158-554
- Qubit RNA High Sensitivity (HS) Assay Kit **Invitrogen Catalog #**Q32855
- Tubes, 0.2 mL, flat cap **Thermo Fisher Catalog #**AB0620
- TURBO DNA-free™ Kit **Thermo Fisher Scientific Catalog #**AM1907
- Ethanol, 200 proof **KOPTEC Catalog #**V1001
- Sodium Acetate Trihydrate **Fisher Scientific Catalog #**S609500
- Pipette tips, filtered, PCR clean and sterile, 50 - 1,000 uL **Eppendorf Catalog #**0030078578
- SuperScript IV VILO Master Mix **Invitrogen Catalog #**11756050
- Molecular Grade Water **MIDSCI Catalog #**786-73C
- Chloroform, 99.8+%, ACS grade **Thermo Fisher Scientific Catalog #**032614.K2
- Sylgard 184 **Electron Microscopy Sciences Catalog #**24236-10
- Pipette tips, filtered, PCR clean and sterile, 2 - 100 uL **Eppendorf Catalog #**0030078543
- TURBO DNA-free™ Kit **Thermo Fisher Catalog #**AM1907
- Phosphate Buffered Saline, 10X Solution **Fisher Scientific Catalog #**BP3994
- Pipette tips, filtered, PCR clean and sterile, 0.1 - 10 uL **Eppendorf Catalog #**0030078519
- Conical tube, 50 mL **Olympus Catalog #**28-101

Safety warnings

This protocol involves the use of various chemicals and reagents that require careful handling and strict adherence to safety guidelines to ensure safe laboratory practices. Please review the Material Safety Data Sheets (MSDS) for each reagent before beginning the protocol and take appropriate precautions. All steps should be performed while wearing gloves, a lab coat, and eye protection.

Testes dissection and sample collection

10m 15s

1 Thoroughly clean the working surface, stereomicroscope base, and fly pad with [M] 10 % (v/v) bleach, [M] 70 % (v/v) ethanol, and RNase Free, Surface Decontaminant **ApexBio Technology Catalog #**10-228. — 2m

2 Position an ice bucket to your dominant side of the stereomicroscope. — 30s

3 Anesthetize males using CO_2 and transfer them to a fly pad attached to a CO_2 line.
Position the fly pad beside the stereomicroscope, on your non-dominant side. — 1m

4 Fill a dissecting dish halfway with 1x PBS.
Position the dissecting dish under the optics of the stereomicroscope. — 30s

5 Fold a Kimtech Kimwipe delicate task wipes **Kimberly-Clark Catalog #**34155 and position it behind the dissecting dish.
Saturate the Kimwipe with RNase Free, Surface Decontaminant **ApexBio Technology Catalog #**10-228. — 30s

6 Using fine-tipped forceps in your non-dominant hand, grasp a fly from the fly pad at the upper end of its abdomen.
Transfer the fly to the bottom of the dissecting dish, ensuring you do not release it. — 5s

7 Using fine-tipped forceps in your dominant hand, grasp and gently pull the very tip of the fly's abdomen.
This action should release the abdominal contents, revealing digestive tissue, lipids, and testes. — 15s

> Note
>
> If the testes are not visible after pulling the abdomen tip, they may still be retained within the abdomen. Gently squeeze the abdomen with the forceps in your dominant hand, starting from the anterior and moving toward the posterior.

8 Once the testes are clearly visible and separated from the fly, use the forceps in your non-dominant hand to transfer the fly to the RNase-Free-saturated Kimwipe positioned behind the dissection dish. — 5s

9 Using both sets of forceps, carefully remove digestive tissues and other debris, transferring them to the RNase-Free-saturated Kimwipe. — 10s

10 Using the forceps in your dominant hand, grasp the testes and transfer them to a Centrifuge tubes, safe-lock, PCR clean, 2 mL **Eppendorf Catalog #**022363344 containing 800 µL of chilled TRIzol Reagent **Thermo Fisher Scientific Catalog #**15596026 and three Homogenizing beads, 2.8 mm, ceramic **VWR International (Avantor) Catalog #**10158-554 . 10s

> Note
>
> Placing the tissue directly into the TRIzol will cause it to stick to the forceps. Instead, place the tissue on the side of the tube above the TRIzol and then tilt the tube to immerse the tissue in the TRIzol.

11 Cap the tube and store On ice .

12 Repeat steps go to step #6 to go to step #11 to collect more tissue for each sample or to collect additional samples. Repeat go to step #1 between sample groups.

> Note
>
> Dissection requires practice. It is reasonable to expect that a practiced user can reliably dissect a fly and transfer its testes to a sample tube within 45 seconds or less.

13 Transfer samples to a bead-mill homogenizer tube holder chilled to -20 °C . 1m

14 Homogenize the samples at 1500 rpm, 00:02:00 . 3m

15 Centrifuge the samples at approximately 15000 rpm, 00:00:30 to remove contents from the lid of the tube. 1m

16 Store samples at -80 °C until further processing.

RNA extraction and purification

2h 44m

17 Thoroughly clean the working surface and equipment surfaces with [M] 10 % (v/v) bleach, [M] 70 % (v/v) ethanol, and RNase Free, Surface Decontaminant **ApexBio Technology Catalog #**10-228. 2m

18 Remove samples from the -80 °C freezer and allow the samples to thaw completely at Room temperature. 5m

19 Transfer samples to a bead-mill homogenizer tube holder chilled to -20 °C. 1m

20 Homogenize the samples at 1500 rpm, 00:02:00. 3m

21 Centrifuge the samples at approximately 15000 rpm, 00:00:30 to remove contents from the lid of the tube. 1m

22 Allow samples to sit for 00:05:00 at Room temperature to ensure complete dissociation of nucleoprotein complexes. 5m

23 (Optional) Add 1 µL of Universal RNA Spike I **TATAA Biocenter Catalog #**RS25SI pre-diluted to an appropriate concentration. 2m

> Note
>
> Quantifying the abundance of synthetic spike-in RNA will enable the user to control for RNA processing variation between samples. We dilute the RNA Spike I 1:64 before adding to samples.

24 (Optional) Prepare Spike I control sample: 2m

1. Add 24 µL Low EDTA TE (1X), pH 8.0 **Quality Biological Catalog #**351-324-721 to a Centrifuge tubes, safe-lock, PCR clean, 1.5 mL **Eppendorf Catalog #**022363212 .
2. Add 1 µL Universal RNA Spike I **TATAA Biocenter Catalog #**RS25SI pre-diluted to an appropriate concentration.
3. Keep this control sample on ice throughout the extraction process.

25 Thoroughly clean the working surface of the chemical hood with [M] 10 % (v/v) bleach, [M] 70 % (v/v) ethanol, and RNase Free, Surface Decontaminant **ApexBio Technology Catalog #**10-228 . 2m

26 Add 160 µL of Chloroform, 99.8+%, ACS grade **Thermo Fisher Scientific Catalog #**032614.K2 to each sample. Cap the tubes securely and shake vigorously for 00:00:15 or until the color is uniform throughout the sample. 1m

Safety information

This step should be completed in a chemical hood.

Note

Shake immediately after adding chloroform. To process in bulk, place sample tubes in one rack and place another rack on top (sandwich formation) and shake.

27 Incubate for 00:05:00 at Room temperature . 5m

28 Centrifuge at 12000 x g, 4°C, 00:15:00 to separate into three layers: a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase containing the RNA. 16m

Note

The interphase is relatively small compared to the organic and aqueous phases and can be easily overlooked.

29 Thoroughly clean the working surface and equipment surfaces with RNase Free, Surface Decontaminant **ApexBio Technology Catalog #**10-228 . 2m

30 Carefully remove 300 µL of the upper aqueous phase by pipetting at a slow speed (setting 2 on an Eppendorf Xplorer p1000) and transfer it to a new tube. Discard the old tube containing the organic phase. 5m

Note

Be extremely careful not to pipette any of the organic phase (bottom red layer) or the interphase. Always keep the pipette tip at the top of the solution and do not aspirate the entire aqueous phase. Minimize tube movement after centrifugation to prevent phase disruption.

31 Add 3 µL of Glycogen, UltraPure **Invitrogen - Thermo Fisher Catalog #**10814010 to the aqueous phase. 1m

Note

Glycogen acts as a carrier for RNA, improving precipitation efficiency.

32 Add 400 µL of Isopropanol, 99.5%, molecular biology grade **Thermo Fisher Scientific Catalog #**327272500 to the aqueous phase and invert the samples 10 times to mix thoroughly. 2m

33 Incubate the sample for 00:10:00 at Room temperature . 10m

34 Centrifuge at 12000 x g, 4°C, 00:20:00 . 22m

Note

The RNA pellet may be visible as a gel-like substance on the side or bottom of the tube, especially after centrifugation.

35 (Optional): If the pellet is not visible or smaller than expected, invert the tubes and repeat go to step #34 to encourage pellet formation.

36 Thoroughly clean the working surface and equipment surfaces with RNase Free, Surface Decontaminant **ApexBio Technology Catalog #**10-228. 2m

37 Pipette at a slow speed (setting 2 on an Eppendorf Xplorer p1000) to carefully remove all supernatant from the tube, leaving only the RNA pellet. 2m

38 Add 500 µL 75% ethanol to the RNA pellet. 2m

39 Vortex the sample for 00:00:10. 1m

40 Centrifuge at 7500 x g, 4°C, 00:05:00. 6m

41 Pipette at a slow speed (setting 2 on an Eppendorf Xplorer p1000) to carefully remove all supernatant from the tube, leaving only the RNA pellet. 2m

42 Repeat go to step #52 to go to step #55 three more times, for a total of four 75% ethanol washes. 33m

43 Centrifuge the samples at approximately 3200 x g, 00:00:15 and discard any remaining liquid by pipetting. 3m

44 Thoroughly clean the working surface and equipment surfaces with RNase Free, Surface Decontaminant **ApexBio Technology Catalog #**10-228. 2m

45 Air-dry the RNA pellet for 00:10:00 by leaving the tube lid open. Avoid working directly over the samples to prevent contamination. 10m

> Note
>
> A white pellet indicates salt contamination. A transparent pellet, once evaporated, suggests pure RNA. Do not allow the RNA to dry completely, as this significantly reduces solubility. Partially dissolved RNA samples will have an A260/280 ratio <1.6.

46 Re-suspend the RNA pellet in 25 µL Low EDTA TE (1X), pH 8.0 **Quality Biological Catalog #**351-324-721 by pipetting the solution up and down several times at a slow speed (setting 2 on an Eppendorf Xplorer p1000). 4m

47 Incubate at 60 °C for 00:10:00 at 0 rpm using the Thermomixer to facilitate dissolution. 10m

RNA clean up 1h 55m

48 Add 62.5 µL Ethanol, 200 proof **KOPTEC Catalog #**V1001 and 2.5 µL 3M Sodium Acetate Trihydrate **Fisher Scientific Catalog #**S609500 pH 5.2 to each sample. 4m
Mix well by flicking the tubes and store the samples at -20°C overnight.

49 Centrifuge at 21000 x g, 4°C, 00:30:00 . 31m

50 Thoroughly clean the working surface and equipment surfaces with RNase Free, Surface Decontaminant **ApexBio Technology Catalog #**10-228 . 2m

51 Pipette at a slow speed (setting 2 on an Eppendorf Xplorer p1000) to carefully remove all supernatant from the tube, leaving only the RNA pellet. 2m

52 Add 500 µL ice-cold 75% ethanol to the RNA pellet. 2m

53 Vortex the sample for 00:00:10 . 1m

54 Centrifuge at 7500 x g, 4°C, 00:05:00 . 6m

55 Pipette at a slow speed (setting 2 on an Eppendorf Xplorer p1000) to carefully remove all supernatant from the tube, leaving only the RNA pellet. 2m

56 Repeat go to step #52 to go to step #55 two more times, for a total of three 75% ethanol washes. 22m

57 Centrifuge the samples at approximately 3200 x g, 00:00:15 in a mini centrifuge and discard any remaining liquid by pipetting. 2m

58 Thoroughly clean the working surface and equipment surfaces with RNase Free, Surface Decontaminant **ApexBio Technology Catalog #**10-228 . 2m

59 Air-dry the RNA pellet for 00:10:00 by leaving the tube lid open. Avoid working directly over the samples to prevent contamination. 10m

Note

A white pellet indicates salt contamination. A transparent pellet, once evaporated, suggests pure RNA. Do not allow the RNA to dry completely, as this significantly reduces solubility. Partially dissolved RNA samples will have an A260/280 ratio <1.6.

60 Re-suspend the RNA pellet in 25 µL Low EDTA TE (1X), pH 8.0 **Quality Biological Catalog #**351-324-721 by pipetting the solution up and down several times at a slow speed (setting 2 on an Eppendorf Xplorer p1000). 4m

61 Incubate at 60 °C for 00:10:00 at 0 rpm using the Thermomixer to facilitate dissolution. 10m

62 **Quality control:** Using a NanoDrop Spectrophotometer, measure nucleotide concentration (A260), protein contamination (A280/A260), and chemical contamination (A230/A260). Check the spectral curve to ensure that there is not a peak at A270, which would indicate TRIzol contamination. 15m

DNase treatment

52m 30s

63 Thoroughly clean the working surface and equipment surfaces with RNase Free, Surface Decontaminant **ApexBio Technology Catalog #**10-228 . 2m

64 Prepare a master mix containing 2 µL of TURBO DNase buffer and 1 µL TURBO DNase per sample from the TURBO DNA-free™ Kit **Thermo Fisher Scientific Catalog #**AM1907 kit. Prepare extra volume to account for pipetting errors. 4m

65 Mix the master mix by flicking the tube. Be gentle, but mix thoroughly. 30s

66 Centrifuge the master mix at approximately 3200 x g, 00:00:02 in a mini centrifuge to bring contents to the bottom of the tube. 1m

67 Distribute 3 µL of the DNase mixture into individual Tubes, 0.2 mL, flat cap **Thermo Fisher Catalog #**AB0620 . 1m

68 Add 20 µL RNA to each reaction. 1m

69 Mix by flicking the tubes. 30s

70 Centrifuge the samples at approximately 3200 x g, 00:00:02 in a mini centrifuge to bring contents to the bottom of the tube. 1m

71 Incubate 37 °C for 00:30:00 in a thermocycler. 31m

72 Add an additional 1 µL or TURBO DNase to each sample.

73 Incubate 37 °C for 00:30:00 in a thermocycler.

Hold at 4 °C after incubation.

74 Add 2 µL inactivation reagent from the TURBO DNA-free™ Kit **Thermo Fisher Scientific Catalog #**AM1907 kit to each tube. 1m

75 Incubate for 00:05:00 at Room temperature. The inactivation reagent will settle during the incubation. Resuspend the inactivation reagent 2 to 3 times during the incubation by vigorously flicking the tubes. 5m

> Note
>
> If room temperature is below 22 °C, incubate on a heat block at 25 °C.

76 Centrifuge at 10000 x g, 00:01:30 to pellet the inactivation reagent. 2m 30s

77 Transfer 20 µL of supernatant to a Centrifuge tubes, safe-lock, PCR clean, 1.5 mL **Eppendorf Catalog #**022363212. Take care to avoid the white precipitate. 2m

78 **Quality control:** Perform a PCR reaction for 28s using the DNase-treated sample and run a gel. If DNA has been successfully removed, you should not be able to amplify this abundant host target. Use extracted DNA as a positive control. Include a no-template (water) control.

More details are available starting at step 13 in the following protocol:

> Protocol
>
> NAME
>
> PCR-based cytotyping to detect *Wolbachia* in insect tissues
>
> CREATED BY
> J. Dylan Shropshire
>
> Preview

79 **Quality control:** Measure RNA abundance using a Qubit 4 Fluorometer and Qubit RNA High Sensitivity (HS) Assay Kit **Invitrogen Catalog #**Q32855 .

cDNA synthesis

30m

80 Thoroughly clean the working surface and equipment surfaces with RNase Free, Surface Decontaminant **ApexBio Technology Catalog #**10-228 . 2m

81 Add the following, in order, to a Tubes, 0.2 mL, flat cap **Thermo Fisher Catalog #**AB0620 : 2m

1. 4 µL SuperScript IV VILO from the SuperScript IV VILO Master Mix **Invitrogen Catalog #**11756050 .
2. 1 µL to 16 µL of RNA. Do not exceed 2500 ng per reaction.
3. Water from the SuperScript IV VILO Master Mix **Invitrogen Catalog #**11756050 to bring the final volume to 20 µL .

Mix by flicking the tube.

> Note
>
> Keep remaining RNA from each sample to use as controls for genomic DNA contamination.

82 Centrifuge the SuperScript solution at approximately 3200 x g, 00:00:02 in a mini centrifuge to bring contents to the bottom of the tube. 1m

83 Run the following program on a thermocycler: 25m

1. Anneal primers - 25 °C for 00:10:00 .
2. Reverse transcribe RNA - 50 °C for 00:10:00 .
3. Inactivate enzyme - 85 °C for 00:05:00 .
4. Hold at 4 °C .

84 Store the cDNA at 4 °C for use within the next few days, -20 °C for use within the next few weeks, or -80 °C for longer-term storage.