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Using reverse transcription digital droplet PCR to count rare cytoplasmic incompatibility factor transcripts

Forked from Using digital droplet PCR to detect rare Wolbachia endosymbionts

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We use this protocol and it's working

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Disclaimer

Abstract

This protocol details four reverse transcription digital droplet polymerase chain reaction (RT-ddPCR) assays to count *cifA* and *cifB* mRNA from *w*Mel *Wolbachia* in *Drosophila melanogaster*. These assays count *cif* transcripts in duplex with either a synthetic spike-in RNA to normalize for technical variation or a *D. melanogaster* housekeeping gene to normalize for biological variation. With a limit of detection of about 1 *cifA* and 3 *cifB* copies per 20 µL reaction, the assays provide accurate and precise measurements across a wide dynamic range. We designed oligos with homology to *cifA* and *cifB* sequences from at least 33 *Wolbachia* strains, suggesting utility beyond *w*Mel. These methods will allow researchers to measure *cif* mRNA levels within individual insect tissues, enabling efforts to pair molecular and phenotypic data at unprecedented resolutions.

Materials

Materials

- Centrifuge tubes, 1.5 mL, safe-lock, PCR clean **Eppendorf Catalog #**22363212
- ddPCR Droplet Reader Oil **Bio-Rad Laboratories Catalog #**1863004
- ddPCR Supermix for Probes (no dUTP) **Bio-Rad Laboratories Catalog #**1863023
- DG8 cartridge **Bio-Rad Laboratories Catalog #**1864007
- DG8 cartridge holder **Bio-Rad Laboratories Catalog #**1863051
- DG8 gasket **Bio-Rad Laboratories Catalog #**1864007
- Droplet generation oil for probes **Bio-Rad Laboratories Catalog #**1863005
- Kimtech Science™ Kimwipes™ Delicate Task Wipes **Kimberly-Clark Catalog #**34155
- Microseal “B” Seal, Adhesive **Bio-Rad Laboratories Catalog #**MSB1001
- PCR plate, 96 well **Bio-Rad Laboratories Catalog #**1863005
- PCR plate, 96 well, ddPCR **Bio-Rad Laboratories Catalog #**12001925
- PCR plate heat seal, foil, pierceable **Bio-Rad Laboratories Catalog #**1814040
- 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
- Universal RNA Spike I **TATAA Biocenter Catalog #**RS25SI
- Water, molecular biology grade **MIDSCI Catalog #**KC248796

Note

Use high-quality plastics throughout this protocol. Low-quality pipette tips, for example, can result in droplet shearing, significantly reducing sample quality.

Oligos

- cifAwMelT1_dd forward primer: 5'-GGTCCTTGGAATAATTTGCGG-3'
- cifAwMelT1_dd reverse primer: 5'-TCAAACTCAGACTGTGGGC-3'
- cifAwMelT1_dd FAM-labelled probe: 5'-TTGCCACTTGATGGTTCTGGTGA-3'
- cifBwMelT1_dd forward primer: 5'-GCAAGGTACTAGAGCACAGG-3'
- cifBwMelT1_dd reverse primer: 5'-CACGAGCGTTGTTTCTACG-3'
- cifBwMelT1_dd FAM-labelled probe: 5'-AGGTGGTACTTCTACAGCACAAGG-3'
- B-Spectrin_dd forward primer: 5'-ATGACGACGGACATTTCGATTG-3'
- B-Spectrin_dd reverse primer: 5'-AACAGTCGGGAACTGGAGTTG-3'
- B-Spectrin_dd FAM-labelled probe: 5'-GGTCCTGGCAACGAGTACATCGAT-3'

- Universal RNA Spike I **TATAA Biocenter Catalog #**RS25SI

Note

The *cifA*- , *cifB*-, and *β-Spec*-ddPCR oligos should be used at primer:probe ratio of 900 nM:250 nM.
Spike oligos should be used at the provided concentration.

Equipment

- Centrifuge (Eppendorf, 5420)
- Centrifuge, for plates (Eppendorf, 5430R)
- Droplet generator (Bio-Rad, 1864002)
- Droplet reader (Bio-Rad, 1864003)
- Freezer (any)
- Pipette, 10 µL (Eppendorf, 4861000708)
- Pipette, 100 µL (Eppendorf, 4861000716)
- Pipette, 1000 µL (Eppendorf, 4861000732)
- QX Manager Software
- Rubber Brayer Roller, 4 inch (Amazon, B07YDNKSH6)
- Thermal PCR plate sealer (Bio-Rad, 1814000)
- Thermal Cycler (Bio-Rad, 1841100)
- Vortex mixer (MI0101002, Four E's Scientific)

Protocol materials

- Centrifuge tubes, 1.5 mL, safe-lock, PCR clean **Eppendorf** **Catalog #**22363212
- ddPCR Supermix for Probes (no dUTP) **Bio-Rad Laboratories** **Catalog #**1863023
- Water, molecular biology grade **MIDSCI** **Catalog #**KC248796
- ddPCR Droplet Reader Oil **Bio-Rad Laboratories** **Catalog #**1863004
- DG8 cartridge **Bio-Rad Laboratories** **Catalog #**1864007
- PCR plate, 96 well, ddPCR **Bio-Rad Laboratories** **Catalog #**12001925
- DG8 cartridge holder **Bio-Rad Laboratories** **Catalog #**1863051
- Universal RNA Spike I **TATAA Biocenter** **Catalog #**RS25SI
- DG8 gasket **Bio-Rad Laboratories** **Catalog #**1864007
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- Pipette tips, filtered, PCR clean and sterile, 0.1 - 10 uL **Eppendorf** **Catalog #**0030078519
- Pipette tips, filtered, PCR clean and sterile, 50 - 1,000 uL **Eppendorf** **Catalog #**0030078578
- Pipette tips, filtered, PCR clean and sterile, 2 - 100 uL **Eppendorf** **Catalog #**0030078543
- Microseal “B” Seal, Adhesive **Bio-Rad Laboratories** **Catalog #**MSB1001
- PCR plate, 96 well **Bio-Rad Laboratories** **Catalog #**1863005

Before start

Collect and process samples to be analyzed via RT-ddPCR. We follow the protocol below to collect *D. melanogaster* testes, extract and purify RNA, eliminate contaminating gDNA, and synthesize cDNA through reverse transcription.

Protocol

NAME

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

CREATED BY

J. Dylan Shropshire

Preview

Clean workstation with [M] 10 % (v/v) bleach and [M] 70 % (v/v) ethanol.

Assemble PCR reaction

1h

1 Remove the following reagents from the freezer and allow to thaw On ice for 00:30:00 : 30m

- ddPCR Supermix for Probes (no dUTP) **Bio-Rad Laboratories Catalog #**1863023
- Water, molecular biology grade **MIDSCI Catalog #**KC248796
- Primers and probes, as appropriate
- cDNA samples

2 Vortex reagents for 00:00:30 to mix. 30s

3 Centrifuge at 2000 rcf, Room temperature, 00:00:10 to bring contents to the bottom of each tube. 1m

4 Select one of the following assays.
Create a master mix in a Centrifuge tubes, 1.5 mL, safe-lock, PCR clean **Eppendorf Catalog #**22363212 based on the described composition.
The final volume of each assay will be 20 µL , including 2 µL of DNA template.
Calculate a 10% excess. 5m

4.1 *cifA/β-Spec and cifB/β-Spec* ddPCR assay reaction composition:

- 10 µL
  ddPCR Supermix for Probes (no dUTP) **Bio-Rad Laboratories Catalog #**1863023
- 6 µL Water, molecular biology grade **MIDSCI Catalog #**KC248796
- 1 µL *cifA* or *cifB* oligo mixture with FAM-labeled probe
- 1 µL *β-Spec* oligo mixture with HEX-labeled probe
- 2 µL cDNA template

Note

The *cifA/β-Spec and cifB/β-Spec* ddPCR assays are used to measure *cifA* and *cifB* transcript levels normalized to the transcription levels of a relatively stable host gene.
This assay is useful when:
- The number of *Wolbachia* transcripts per host transcription is more relevant than the total *Wolbachia* transcript count.
- Host cell numbers vary between treatment groups or samples.

4.2 *cifA/spike and cifB/spike* ddPCR assay reaction composition:

- 10 µL
  ddPCR Supermix for Probes (no dUTP) **Bio-Rad Laboratories Catalog #**1863023
- 5.5 µL Water, molecular biology grade **MIDSCI Catalog #**KC248796
- 1 µL *cifA* or *cifB* oligo mixture with FAM-labeled probe
- 1 µL RNA spike-in control primer mixture
- 0.5 µL RNA spike-in control HEX-labeled probe
- 2 µL cDNA template

Note

The *cifA/spike and cifB/spike* ddPCR assays are used to measure *cifA* and *cifB* absolute abundance and correct for extraction and processing efficiency.
This assay is useful when:
- Technical variations, such as RNA extraction efficiency, can significantly impact results.
- The biological effect size is small and can be masked by technical noise.
- There is a need to back-calculate transcript abundance in the original sample.

5 Vortex the master mix for 00:00:30 to mix. 30s

6 Dispense 18 µL of the master mix into each well in a PCR plate, 96 well **Bio-Rad Laboratories Catalog #**1863005. 10m

7 Add 2.0 µL DNA template to each reaction. 10m
Add 2.0 µL Water, molecular biology grade **MIDSCI Catalog #**KC248796 to no template control reactions (NTCs)

8 Use the Microseal “B” adhesive seal to seal the plate. 1m
Use a roller to ensure the seal is tight.
Mix thoroughly by vortexing the plate for 00:00:10 .

9 Centrifuge at 2204 rcf, Room temperature, 00:02:00 to bring contents to the bottom of each well. 2m

Note

After centrifugation, check that there are no bubbles across the wells.
If there are bubbles, repeat the centrifugation.

Generate droplets 9m 50s

10 Insert a DG8 cartridge **Bio-Rad Laboratories Catalog #**1864007 into a DG8 cartridge holder **Bio-Rad Laboratories Catalog #**1863051 . 15s

Note

Check that the orientation of the cartridge is aligned properly in the cartridge holder.
Slide the edges of the holder toward the center to snap close.

11 Peel away the adhesive seal to expose the sample. 1m
Be gentle while removing the seal to avoid aerosolizing the samples.
By pipetting, transfer 19.5 µL of each reaction mix to the sample wells of a DG8 cartridge.
Avoid pipetting air bubbles into the DG8 cartridge.

Note

Check each sample in the DG8 cartridge for air bubbles.
Remove air bubbles by pipetting slowly using a different tip.
Air bubbles can interfere with droplet generation.

12 By pipetting, add 70 µL Droplet generation oil for probes **Bio-Rad Laboratories Catalog #**1863005 to the oil wells in the DG8 cartridge **Bio-Rad Laboratories Catalog #**1864007 . 30s

13 Insert the DG8 cartridge holder **Bio-Rad Laboratories Catalog #**1863051 into the droplet generator. 2m
Close the lid.
Wait approximately 00:02:00 for droplets to be generated

14 While waiting, go to step #10 to prepare the next set of eight reactions. 4m

15 Open the droplet generator. 2m
Replace the cartridge holder containing droplets with the cartridge holder that was prepared while waiting.
Close the lid to start droplet generation.
Remove the cartridge from the first cartridge holder and check for droplets.
By pipetting, transfer the droplet well (40 µL) from the
DG8 cartridge **Bio-Rad Laboratories Catalog #**1864007 to a clean
PCR plate, 96 well, ddPCR **Bio-Rad Laboratories Catalog #**12001925 .

Note

If droplet generating is successful, the wells appear opaque.
Pipette slowly to avoid shearing the droplets.

16 Once all the samples have been transferred, seal the PCR plate with a 5s
PCR plate heat seal, foil, pierceable **Bio-Rad Laboratories Catalog #**1814040 using a thermal PCR plate sealer set to heat the foil at 180 °C for 00:00:05 .

Safety information

When using a PX1 plate sealer, ensure the PCR plate is correctly aligned with the heat sealer. Always position the foil with the red line facing upward to prevent it from sticking to the machine and causing damage.

Thermal Cycling 3h 1m

17 Transfer the sealed PCR plate containing droplets to a thermal cycler. 1m

18 Run the following program with a ramp rate of 1 °C per 00:00:01 and with a heated lid of 105 °C. 3h

1 cycle of:
- 95 °C for 00:10:00 to activate polymerase and denature DNA.

50 cycles of:
- 94 °C for 00:01:00 to denature DNA.
- 60 °C for 00:02:00 to anneal primers and replicate amplicons.

1 cycle of:
- 98 °C for 00:10:00 to inactivate polymerase.
- 12 °C forever to store amplicons until use.

Read droplets 1h

19 Transfer the assembled plate to the droplet reader, and secure it with the clamps in the machine.
Check that well A1 is in the top-left position and close the lid. 1m

Safety information

If the plate is not secured properly, the sampling needle can stick to the lid and damage the instrument.

20 Open the QX Manager software on the attached computer. 1m

21 If the droplet reader has not been used in the past week, it is important that the system be primed using the QX Manager software. 2m
- Select the "System Utilities" tab.
- Select "Prime."
- Select "Yes" to begin.
- Select "OK" after priming is complete.

22 Add a plate in the QX Manager software. 5m

22.1 Open the Plate Configuration window.
- Select the "Add Plate" tab.
- Select the plus icon beside "Add Plate."

- Select "Configure Plate" to open the plate configuration window with three tabs: "Plate Information," "Well Selection," and "Well Information."

22.2 Record general plate information.
- Select the "Plate Information" tab.
- Add a name for the plate and record it in your lab notebook.
- Define the supermix used in the reactions
- Define the file name and save the plate.

22.3 Identify the wells in the plate that contain droplets.
- Select the "Well Selection" tab.
- Select the wells that contain droplets.
- Click "Include Selected Wells."

22.4 Define the type of experiment and the contents of the wells.
- Select the "Well Information" tab.
- Click "Experiment Type" and select the appropriate type of experiment.
- Enter sample descriptions into the "Sample Description 1" through "Sample Description 4" fields.
- Select the appropriate "Sample Type" for each well.
- Click "Supermix" and select "Supermix for Probes."
- Click "Assay Type" and select "Single target per channel."
- Enter the target (e.g., *cifA*, *cifB*, *Bspec,* spike) in the "Target Name" field.
- Select the appropriate "Target Type" for each target.
- Click "Signal Ch1" and select "FAM."
- Click "Signal Ch2" and select "HEX" if reactions include *Bspec* or spike and "none" otherwise.

23 Select "Save" and "Start Run" to begin reading droplets

30m

Note

The software performs a prerequisites check to ensure that the instrument is ready. This will tell you if ddPCR Droplet Reader Oil **Bio-Rad Laboratories Catalog #**1863004 needs to be added or if waste needs to be emptied.
Always add 50 mL of [M] 10 % (v/v) bleach to the waste container after emptying.

24 After the run is complete, save files associated with the run.

20m

- Click "Save" to save the run.
- Select the "Data Analysis" tab.
- Click "Error Model" a select "Poisson" and "95%."
- Select the "Data Table" tab, ensure all columns are reported, and save it as a csv file.

- Select the "Run Information" tab and save the table.
- Select the "Reports" tab, click "Select All," and save the report as a PDF.
- Select the "1D Amplitude," "2D Amplitude," and "Concentration" tabs to save the plots associated with the run.