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REDI-NET FF-2 FILTH FLY PROCESSING

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Remote Emerging Disea...

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We use this protocol and it's working

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Disclaimer

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Abstract

The overarching aim of the REDI-NET is to develop a collaborative laboratory network between domestic and international partnering institutions to address disease surveillance needs in order to effectively detect, predict and contain potentially emergent zoonosis. This SOP provides guidance on filth fly total nucleic acid extraction to allow downstream library preparation and sequencing for pathogen detection.

Guidelines

APPENDIX 1. BASIC FLY IDENTIFICATION

APPENDIX 3. POOLING STRATEGY

APPENDIX 4. MEASURING SPOON FOR 0.1 mm BEATING BEADS

The spoon (Next Advance, MSP01-RNA) is used for 0.1 mm beating beads measurement. The step is described on 6.6.4 the preparation before Fly homogenization. One spoon equals to 100

APPENDIX 5. QUBIT

DNA and RNA Measurement using QUBIT FLUOROMETER 4.0

DNA quantification:

According to the volume of sample used, add the 1xHS dsDNA Qubit Assay for a final volume of 200 µL (i.e., if using 1 µL of sample, add 199 µL of 1x HS dsDNA Qubit Assay.

RNA Quantification:

1. In a new microcentrifuge tube/falcon tube (depending on the number of samples processed), prepare a working solution of the Qubit HS RNA Assay:

	A	B	C
	Reagents	Volume/sample	Volume for n+1 sample
	Qubit RNA HS Assay buffer	199 µL	.... µL
	Qubit RNA HS Assay Dye	1 µL	.... µL

1. In a new 0.6 ml tube, mix 199 µL of Qubit HS RNA Assay working solution and 1 µL of the sample. Incubate for 1 minute at room temperature before reading.

MAINTENANCE OF EQUIPMENT

Caution on RNA handling:

1. RNases are very stable and difficult to inactivate and only minute amounts are sufficient to destroy RNA.
2. Care should be taken to avoid inadvertently introducing RNases into the samples during or after the purification procedure.
3. Sample handling and extraction should be performed under an extraction hood and respecting Good Laboratory Practices.
4. Use filter tips all the time.

Storage of the buffers from IndiMag pathogen kit

1. Proteinase K is stable for at least 1 year after delivery when stored at Room temperature (15 °C — 25 °C). To store for more than 1 year or if ambient temperature often exceeds 25 °C , storage at 2 °C — 8 °C is recommended. Do not add Proteinase K directly to the Buffer VXL mixture! This can cause clogs or precipitates.
2. Precipitates may form after storage at low temperature or prolonged storage. To dissolve precipitate, incubate Buffer VXL or ACB for 00:30:00 at 37 °C , with occasional shaking.
3. Reconstituted Buffer AW1 can be stored at Room temperature (15 °C — 25 °C) for up to 1 year. Mix well after adding Ethanol.
4. Buffer AVE is RNase-free upon delivery. It contains sodium azide, an antimicrobial agent that prevents growth of RNase-producing organisms. However, as this buffer does not contain any RNase degrading chemicals, it will not actively inhibit RNases introduced by inappropriate handling. When handling Buffer AVE, take extreme care to avoid contamination with RNases. Follow general precautions for working with RNA, such as frequent change of gloves and keeping tubes closed whenever possible.

Materials

Note

NOTE: If product number is listed, please ensure use of this or equivalent product.

	A	B
	Equipment	Mfg / Product #
	Dino-Lite 5MP Edge	AM7115MZT
	Adjustable DinoScope Stand	MS35B
	Magnetic light source (x2)	IMAGE Grill Lights Magnetic BBQ Grill Light
	AAA batteries	EBL Pack of 8 AAA Batteries 1,100mAh AAA
	Dino-Lite Driver Software	REDI-NET Program
	BioQuip Portable Chill Table	BioQuip, 1429
	AC power converted to US standard	120 V and 60Hz AC
	KingFisher Flex Magnetic Particle Processor with 96 Deep-Well Head or KingFisher Duo Prime Magnetic Particle Processor	ThermoFisher, 5400630 (Flex) or ThermoFisher, 5400110 (Duo Prime)
	Bullet Blender 24 Gold	Next Advance, BB24-AU
	Adjustable micropipettes	Locally sourced
	Multi-channel micropipettes	Locally sourced
	Vortex	Locally sourced
	Tube centrifuge	Locally sourced
	Plate centrifuge	Locally sourced
	Qubit 4 Fluorometer	ThermoFisher, Q33238
	Thermo Heater Mixer	Locally sourced

	A	B	C
	Material	Description	Mfg / Product #

	A	B	C
	Posi-Click 5 mL Microcentrifuge Tubes	For storage of individual specimen	Thomas Scientific, 1149Y05
	96 well Eppendorf tube racks	To hold specimen on chill plate during imaging	1164M62
	Porcelain chill table stage or white paper note cards	To better visualize fly during imaging	Locally sourced
	Petri dishes, disposable	To hold flies under DinoScope (consumable)	Bioquip, 4787
	Color lab tape	For labeling and sealing boxes of flies (consumable)	Thomas Scientific, 1184X64
	ZymoBIOMICS Microbial Community Standard Material	For TNA extraction positive control	Zymo Research, D6300
	AcroMetrix HIV-1 Controls	For TNA extraction positive control; BSL-2	ThermoFisher, CLS430320-12EA
	Human gammaherpesvirus (EBV) positive control	For TNA extraction positive control	Naval Medical Research Center
	IndiMag Pathogen Kit	w/o plastics, 384 reactions	Indical Bioscience, SP947257
	Buffer ATL	200 mL, Tissue Lysis Buffer	Qiagen, 19076
	Reagent DX	1 mL, Antifoaming Reagent	Qiagen, 19088
	Measuring Spoon 100 µL	RNase Free, pack of 10, reusable	Next Advance, MSP01-RNA
	Orange RINO RNA lysis kit	Bead lysis kits (consumable)	Next Advance, ORANGER5-RNA
	Clear RINO brand microcentrifuge tubes	1.5 mL, screw-cap (consumable)	Next Advance, TUBE1R5-S
	Thermo Scientific Screw Cap Micro Tubes	1.5 mL Screw Cap Tube, NonKnurl, NonSkirted, Natural, E-Beam Sterile tube w/ attached cap	Fisher Scientific, 14-755-208
	Zirconium oxide beads	0.1 mm, 400 g (consumable)	Fisher Scientific, 50-154-2950
	KingFisher Deepwell 96 Plate	KingFisher	ThermoFisher, 95040450
	KingFisher 96 tip comb for DW magnets	KingFisher Flex ONLY	ThermoFisher, 97002534
	KingFisher Duo Prime 12-tip comb	KingFisher Duo Prime ONLY	ThermoFisher, 97003500

	A	B	C
	Elution Strip	KingFisher Duo Prime ONLY	ThermoFisher, 97003520
	KingFisher Duo Cap for Elution Strip	KingFisher Duo Prime ONLY	ThermoFisher, 97003540
	BRAND Self-adhesive Plate Sealing Film	Aluminum (consumable)	Fisher Scientific, 13-882-329 or equivalent
	MicroAmp Clear Adhesive Film	KingFisher (consumable)	ThermoFisher, 4306311
	Nonstick, RNase-Free Microfuge Tubes	1.5 mL (consumable)	ThermoFisher, AM12450
	Nonstick, RNase-Free Microfuge Tubes	2.0 mL (consumable)	ThermoFisher, AM12475
	Qubit assays tubes	For Qubit‰ DNA/RNA measuring (consumable)	Thermo Fisher, Q32856
	RNaseZap RNase Decontamination Solution	To remove RNase from the working area	ThermoFisher, AM9780
	Qubit 1X dsDNA HS Assay Kit	(consumable)	ThermoFisher, Q33230
	Qubit RNA HS Assay Kit	(consumable)	ThermoFisher, Q32852
	Ethanol	100% (molecular biology grade) (consumable)	Locally sourced
	Isopropanol	100% (molecular biology grade) (consumable)	Locally sourced
	Nuclease-free Water	For negative control	Locally sourced
	Dry ice	To maintain cold chain during sample handling using Bullet Blender	Locally sourced

Safety warnings

RISK AND PERSONAL PROTECTION

1. Caution should be taken while processing samples as some chemicals may be harmful. Please use a fume-hood when required to avoid inhaling harmful chemicals.
2. Gloves should be worn all the time when handling samples.
3. Decontaminants such as DNA/RNaZap could irritate the skin, avoid contact with skin while preparing the workbench for nucleic acid extractions.

SORT FLY SAMPLES

1 Flies can be sorted one of three ways:

1.1 Flies are separated into groups of biting flies and non-biting flies based on their mouthparts.

1.2 Flies can be identified and sorted by genera (e.g., Musca sp.).

1.3 Flies can be identified to species level using appropriate morphological keys. Morphological keys present in this SOP (Appendix 1) should aid identification.

2 Following sampling, flies can be stored at -20 °C for up to 2 months and at -80 °C for long term storage.

3 Flies can be pooled for processing by mouthpart type, genus, or species. Do not pool flies from different cartons. The maximum recommended pool sizes are noted in the table below.

	A	B	C	D	E
	Size	Example	SS Beads	ZrO2 Beads	Fly Wash
	Small Flies	Horn Fly (Haematobia irritans)	15	10	20
	Medium Flies	Housefly (Musca domestica)	5	3	10
	Large Flies	Flesh Fly (Sarcophagidae)	3	2	5

STEPS BEFORE HOMOGENIZATION

4

Note

NOTE: To prevent contamination samples nucleic acid extraction and amplification (PCR) should be performed in separate rooms.

5 Pre-cool the Bullet Blender by adding dry ice into the cooling compartment and running the cooling program.

6 Clean the work surfaces with RNaseZap, then wipe the surfaces with 70% molecular biology grade ethanol to remove additional contaminants.

7 Transfer 0.1 mm zirconium oxide beads (two spoons, Appendix 4) to Thermo Scientific Screw Cap 1.5 mL Micro Tubes.

8 For the 1st time use of the IndiMag pathogen kit, add 100% ethanol to Buffer AW1 and AW2, and add 100% Isopropanol to ACB as indicated on the bottles.

9 Buffer ATL may form precipitates upon storage. If necessary, warm to 56 °C until the precipitates have fully dissolved. Prepare buffer ATL-DX: add 100 µL Reagent DX to 15 mL Buffer ATL. If smaller amounts are needed, transfer 1.5 mL of Buffer ATL into a sterile 2 mL vial and add 10 µL Reagent DX. Mix well, after addition of Reagent DX. After preparation, the mixture is stable for 6 months at room temperature (15-25 °C).

10 MagAttract Suspension G from the IndiMag pathogen kit needs to be vortexed thoroughly for 3 mins (before first use) or 1 minute (before subsequent uses) to ensure that the magnetic silica particles are fully resuspended.

11 Prepare a few 15 mL or 50 mL conical centrifuge tubes with nuclease-free water for preparing TNA elution in KingFisher Flex or KingFisher Duo Prime to avoid cross-contamination.

12 Select a processing strategy for the samples using the guidance in the figure below:

FLY HOMOGENIZATION - STAINLESS STEEL BEADS

13 Clean forceps with 70% ethanol and Kimwipes before use and between samples

14 Label orange RINO® tubes on the caps to avoid the label rubbing off during homogenization.

15 Add the flies to the tubes and note the number of flies being pooled in each tube.

16 Add 400 µL ice cold 1x PBS to each tube.

17 Ensure the Bullet Blender is fully cooled down. Add more dry ice into the cooling compartment of the Bullet Blender, if necessary, then load the RINO® tubes with flies and PBS.

18 Set the controls for Speed 10 and Time 3. Press start.

19 Inspect the tubes and repeat Step 18 if the flies are not fully homogenized.

20 Remove the tubes and centrifuge the samples at 100 x g, 4°C, 00:01:00 to pellet debris. 1m

21 Meanwhile, add 80 µL of ATL-DX Lysis Buffer to 1.5 mL tubes containing 0.1 mm ZrO2 beads from Step 7.

22 Without disturbing the orange RINO® tubes, carefully transfer the top (clear) 320 µL homogenate into 1.5 mL tubes from the preceding step.

23 Include a positive control for each batch of samples: transfer 37.5 µL ZymoBIOMICS Microbial Community Standard Material and 100 µL EBV, and 100 µL HIV standard into a tube from step 7. Add 82.5 µL 1x PBS.

24 Include a negative control for each batch of samples: a bead tube with 320 µL cold sterile 1xPBS only.

25 Add more dry ice into the cooling compartment of Bullet Blender, if necessary, then load the bead tubes.

26 Set the speed at 12 and time at 3. Press Start.

27 Let the samples settle for 1 minute and then repeat step 26.

28 Centrifuge the tube at 100 x g, 4°C, 00:01:00 . 1m

29 Carefully transfer the 350 µL supernatant from the RINO tube to a new snap-cap 1.5 mL microcentrifuge tube, avoiding bead carryover (slight bead contamination is tolerated).

Note

STOPPING POINT: lysed samples can be stored at 4°C overnight.

FLY HOMOGENIZATION - ZIRCONIUM OXIDE BEADS

30 Clean forceps with 70% ethanol and Kimwipes before use and between samples

31 Label clear RINO® tubes on the caps to avoid the label rubbing off during homogenization.

32 Add the flies to the tubes and note the number of flies being pooled in each tube.

33 Add 320 µL 1x PBS and 80 µL of ATL-DX to each tube containing flies. A master mix can be made ahead for this step.

34 Include a positive control for each batch of samples: transfer 37.5 µL ZymoBIOMICS Microbial Community Standard Material and 100 µL EBV, and 100 µL HIV standard into a tube from step 7. Add 82.5 µL 1x PBS.

35 Include a negative control for each batch of samples: a bead tube with 320 µL cold sterile 1xPBS only.

36 Add more dry ice into the cooling compartment of Bullet Blender, if necessary and then load the all bead tubes.

37 Set the Bullet Blender to Speed 12 and Time 3. Press Start.

38 Let the samples settle for 1 minute and then repeat step 37.

39 Centrifuge the tube at 100 x g, 4°C, 00:01:00 . 1m

40 Carefully transfer the 350 µL supernatant from the RINO tube to a new snap-cap 1.5 mL microcentrifuge tube, avoiding bead carryover (slight bead contamination is tolerated).

Note

STOPPING POINT: lysed samples can be stored at 4°C overnight.

FLY WASH HOMOGENIZATION

41 Clean forceps with 70% ethanol and Kimwipes before use and between samples.

42 Label 1.5 mL or 2.0 microcentrifuge tubes.

43 Add the flies to the tubes and note the number of flies being pooled in each tube.

44 Add 320 µL 1x PBS and 80 µL ATL-DX to each tube containing flies. A master mix can be made ahead for this step.

45 Close the lids and vortex the samples on high for at least 30 seconds or up to 1 minute.

Note

NOTE: This step will continue to be optimized as more fly types from various environments and pool sizes can be tested. An alternate approach may include a longer mixing step on the Hula mixer.

46 Briefly centrifuge the tubes to collect liquid and flies at the bottom of the tube.

47 Remove the liquid portion (< 400 µL) from these tubes and transfer to the prepared clear RINO® tubes containing ZrO2 beads from step 7.

48 Include a positive control for each batch of samples: transfer 37.5 µL ZymoBIOMICS Microbial Community Standard Material and 100 µL EBV, and 100 µL HIV standard into a tube from step 7. Add 82.5 µL 1x PBS.

49 Include a negative control for each batch of samples: a bead tube with 320 µL cold sterile 1xPBS only.

50 Add more dry ice into the cooling compartment of Bullet Blender, if necessary and then load the all bead tubes.

51 Set the Bullet Blender to Speed 12 and Time 3. Press Start.

52 Let the samples settle for 1 minute and then repeat step 51.

53 Centrifuge the tube at 100 x g, 4°C, 00:01:00 . 1m

54 Carefully transfer the 350 µL supernatant from the RINO tube to a new snap-cap 1.5 mL microcentrifuge tube, avoiding bead carryover (slight bead contamination is tolerated).

Note

STOPPING POINT: lysed samples can be stored at 4°C overnight.

INSTRUMENT SET UP (KingFisher Flex only, if using KingFisher Duo Prime, go to step 67)

55 Confirm 96 deep-well magnetic heads and 96 well deep-well heat blocks are being used.

56 Ensure the program **IndiMag_Pathogen_KF_Flex_4wash** has been downloaded and loaded onto the KingFisher Flex instrument.

SET UP THE PROCESSING PLATES

57 Set up the Wash, Elution, and Tip Comb Plates outside the instrument according to the following table:

Note

NOTE: **DO NOT** use the elution buffer provided by the kit for TNA elution. The ingredients in the elution buffer inhibit the downstream DNA sequencing efficiency.

	A	B	C	D	E
	Plate ID	Plate position	Plate type	Reagent	Volume per well
	Tip comb	7	Place a 96 Deep-well Tip comb in a deep-well plate		
	Elution	6	Deep-Well	Nuclease-free water	75 µL
	Wash 4	5	Deep-Well	100 % ethanol	750 µL
	Wash 3	4	Deep-Well	80% ethanol	750 µL
	Wash 2	3	Deep-Well	Buffer AW2	700 µL
	Wash 1	2	Deep-Well	Buffer AW1	700 µL
	Sample	1	Sample Lysate	Lysate and lysis buffer	985 µL

EXTRACTION

58 Centrifuge the 1.5 mL tubes with lysate from step 54 for 12000 x g, 4°C, 00:05:00 . 5m

59 Add 20 µL of Proteinase K into wells (based on number of samples) of a new Deep-well plate.

60 Transfer 270 µL supernatant of step 58 without any particle carryover to the wells of the Deep-well plate containing proteinase K. This plate becomes the Sample Plate.

61 Add 135 µL Buffer VXL, 540 µL Buffer ACB, and 20 µL MagAttract Suspension G to each sample in the sample plate. For multiple samples, make a master mix with 10% overage. Invert slowly to mix the master mix, avoid foaming (can be mixed on Hula mixer for 2 min). Add 695 µL mixture to each sample.

62 Select the program IndiMag_Pathogen_KF_Flex_4wash on the instrument.

63 Start the run, then load the prepared plates into position when prompted by the instrument.

QUANTIFICATION AND STORAGE

64 After the running protocol is completed (~35 minutes), immediately remove the elution plate from the instrument and cover the plate or transfer the eluate to the final tube or plate of choice for final storage.

65 In a 0.6 mL microcentrifuge tube, use 1 µL total nucleic acid for DNA and RNA concentration measurement using Qubit 4 Fluorometer following manufacturer instructions (Kits needed: Qubit 1X dsDNA HS Assay Kit and Qubit RNA HS Assay Kit). (see Appendix 5)

66 Proceed with sample testing following the REDI-NET SOP FF-4 Filth Fly Testing or store at -20 °C for less than 2 weeks (for long-term storage the sample needs to be stored at -80 °C following the REDI-NET SOP FF-3 Filth Fly Storage).

INSTRUMENT SET UP (KingFisher Duo Prime only, if using KingFisher Flex, go to step 55)

67 Confirm 12-tip magnetic head and 12 well deep-well heat blocks are being used.

68 Ensure the program **IndiMag_Pathogen_KF_Duo_4wash** has been downloaded and loaded onto the KingFisher Duo Prime instrument.

SET UP THE SAMPLE PLATE AND ELUTION STRIP

69 Set up the Sample Plate according to the table below:
1.

	A	B	C	D
	Row ID	Plate Row	Reagent	Volume per well
	Sample row	A	Lysate and lysis buffer	985 µL
	Wash 1	B	Buffer AW1	700 µL
	Wash 2	C	Buffer AW2	700 µL
	Wash 3	D	80 % ethanol	750 µL
	Wash 4	E	100 % ethanol	750 µL
	Tip Comb	F	Tip comb	700 µL
		G	Empty	
		H		

70 Set up the Elution Strip according to the table below:

Note

***Note: DO NOT** use the elution buffer provided by the kit for TNA elution. The ingredients in the elution buffer inhibit the downstream DNA sequencing efficiency*

	A	B	C	D
	Row ID	Plate Row	Reagent	Volume per well
	Elution	A	Nuclease-free water	75 µL

EXTRACTION

71 Centrifuge the bead tubes with lysate from step 54 for 12000 x g, 4°C, 00:05:00 5m

72 Add 20 µL of Proteinase K into wells (based on number of samples) of a sample row.

73 Transfer 270 µL supernatant from step 71 without any particle carryover to the wells of the sample row containing proteinase K.

74 Add 135 µL Buffer VXL, 540 µL Buffer ACB, and 20 µL MagAttract Suspension G to each sample in the sample row. For multiple samples, make a master mix with 10% overage. Invert slowly to mix the master mix, avoid foaming (can be mixed on Hula mixer for 2 min). Add 695 µL mixture to each sample.

75 Select program IndiMag_Pathogen_KF_Duo_4wash on the instrument.

76 Start the run, then load the prepared plate/strip into position when prompted by the instrument.

77 Keep the door open while extraction. The chamber of the KingFisher Duo Prime is small. Closing the door makes the ethanol vapor restrained inside the chamber and increases the ethanol contamination.

QUANTIFICATION AND STORAGE

78 After the protocol is completed (~35 minutes), immediately remove the elution strip from the instrument and transfer the eluate to the final tube or plate of choice for final storage.

79 Use 1 µL total nucleic acid for DNA and RNA concentration measurement using Qubit 4 Fluorometer (Kits needed: Qubit 1X dsDNA HS Assay Kit and Qubit RNA HS Assay Kit). (see Appendix 5)

80 Proceed with sample testing following the REDI-NET SOP FF-4 Filth Fly Testing or store at -20 °C for less than 2 weeks (for long-term storage the sample needs to be stored at -80 °C following the REDI-NET SOP FF-4 Filth Fly Storage).

Protocol references

REDI-NET Overview Summary

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