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F-2 FECES PROCESSING V.1

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REDI-NET Consortium[1]

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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

This protocol details feces processing.

Guidelines

OBJECTIVE

To outline procedures for total nucleic acid extraction from fecal samples.

SUMMARY/SCOPE

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 procedures for total nucleic acid extraction from fecal samples to provide materials for downstream library preparation and sequencing for pathogen detection.

RESPONSIBLE PERSON

Principal Investigator, Study Coordinator, Entomology Component Lead, Managers

Note

NOTE: *All study procedures must be conducted in compliance with national and local policies for prevention and control of COVID-19 infection.*

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 (15-25°C). To store for more than 1 year or if ambient temperature often exceeds 25 °C, storage at 2-8 °C is recommended. Do not add Proteinase K directly to the Buffer VXL mixture! This can cause clogs or precipitates.
2. Precipitation 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 (15-25°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.

APPENDICES

APPENDIX 1. MEASURING SPOON FOR 0.1 MM BEATING BEADS

The spoon (Next Advance, MSP01-RNA) is used for 0.1 mm beating beads measurement. One scoop equals to 100 µL .

	Sample	Amount	Sample condition	Elution volume	DNA conc. (ng/ul)	RNA conc.(ng/ul)
	Fecal	100 mg	Frozen/Fresh	75	5-50	5 - 100

Materials

EQUIPMENT AND MATERIALS

Note

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

	A	B
	Equipment	**Mfg / Product #**
	KingFisher™ Flex Magnetic Particle Processor with 96 Deep-Well Head or KingFisher™ Duo Prime Magnetic Particle Processor	ThermoFisher, 5400630 or ThermoFisher, 5400110
	Bullet Blender 24 Gold	Next Advance, BB24-AU
	Qubit 4 Fluorometer	ThermoFisher, Q33238
	Adjustable micropipettes	Locally sourced
	Multi-channel micropipettes	Locally sourced
	Vortex	Locally sourced
	Tube centrifuge	Locally sourced
	Plate centrifuge	Locally sourced
	Digital scale/balance	Locally sourced
	Thermo Heater Mixer	Locally sourced

	A	B	C
	Material	**Description**	**Mfg / Product #**
	IndiMag Pathogen Kit	w/o plastics, 384 reactions	Indical Bioscience, SP947257
	Buffer ASL	140 mL, Stool Lysis Buffer	Qiagen, 19082
	Measuring Spoon 100 µL	RNase Free, pack of 10. reusable	Next Advance, MSP01-RNA
	KingFisher™ Deepwell 96 Plate	KingFisher	ThermoFisher, 95040450

	A	B	C
	KingFisher™ 96 KF microplate	KingFisher Flex *ONLY*	ThermoFisher, 97002540
	KingFisher™ 96 tip comb for DW magnets	KingFisher Flex *ONLY*	ThermoFisher, 97002534
	KingFisher™ Duo Prime 12-tip comb	KingFisher Duo Prime *ONLY*	ThermoFisher, 97003500
	Elution Strip	KingFisher Duo Prime *ONLY*	ThermoFisher, 97003520
	KingFisher™ Duo Cap for Elution Strip	KingFisher Duo Prime *ONLY*	ThermoFisher, 97003540
	MicroAmp™ Clear Adhesive Film	KingFisher	ThermoFisher, 4306311
	RNase-Free Microfuge Tubes	Nonstick, 1.5 mL	ThermoFisher, AM12450
	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	Fisher Scientific, 50-154-2950
	Qubit™ 1X dsDNA HS Assay Kit	(*consumable)*	ThermoFisher, Q33230
	Qubit™ RNA HS Assay Kit	(*consumable)*	ThermoFisher, Q32852
	Qubit Assay Tubes	For Qubit DNA/RNA measurement (*consumable*)	Thermo Fisher, Q32856
	RNaseZap™ RNase Decontamination Solution	To remove RNase from working area	ThermoFisher, AM9780
	ZymoBIOMICS Microbial Community Standard Material	For positive controls	Zymo Research, D6300
	Zika virus (ZIKV) positive control	For TNA extraction positive control	NMRC made
	Human gammaherpesvirus (EBV) positive control	For TNA extraction positive control	NMRC made
	Forceps	For use with samples	Locally sourced
	Spatula	For use with samples	Locally sourced
	Ethanol	100% (molecular biology grade)	Locally sourced
	Isopropanol	100% (molecular biology grade)	Locally sourced
	Nuclease-free Water	To elute total nucleic acids	Locally sourced

	A	B	C
	Dry ice	To maintain cold chain during sample handling	Locally sourced
	Ice bucket	To contain the dry ice	Locally sourced
	Kimwipes	To dry material	Locally sourced
	Falcon tubes	15 mL and 50 mL	Locally sourced
	Data sheets	REDI-NET DCS SP-1 Sample Processing Form	REDI-NET Data Portal

Equipment

KingFisher™ Flex Purification System, KingFisher with 96 Deep-well Head NAME

Purification System TYPE

Thermo Scientific™ BRAND

5400630 SKU

https://www.thermofisher.com/order/catalog/product/5400630 LINK

Equipment

KingFisher™ Duo Prime Purification System NAME

Purification System TYPE

Thermo Scientific™ BRAND

5400110 SKU

https://www.thermofisher.com/order/catalog/product/5400110?SID=srch-srp-5400110 LINK

Equipment

Bullet Blender 24 Gold (1.5 mL snap and screw cap tubes, 4°C cooling)	NAME
Blender	TYPE
Next Advance	BRAND
BB24-AU	SKU
https://www.nextadvance.com/product/bullet-blender-24-gold/	LINK

Equipment

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

IndiMag Pathogen Kit w/o plastics (384 reactions) **INDICAL BIOSCIENCE Catalog #**SP947257

Buffer ASL (560 ml) **Qiagen Catalog #**19082

Measuring Spoon 100 uL RNase Free pack of 10 **Next Advance Catalog #**MSP01-RNA

KingFisher™ Flex™ Systems Consumables, KingFisher Flex Microtiter Deepwell 96 plate, V-bottom **Thermo Fisher Catalog #**95040450

KingFisher Microplate **Thermo Fisher Scientific** **Catalog #**97002540

KingFisher™ Flex™ Systems Consumables, KingFisher 96 tip comb for DW magnets **Thermo Fisher** **Catalog #**97002534

KingFisher™ Duo and KingFisher™ Duo Prime Consumables, 12-tip comb, for Microtiter 96 Deepwell plate **Thermo Fisher** **Catalog #**97003500

KingFisher™ Duo and KingFisher™ Duo Prime Consumables, Elution strip **Thermo Fisher** **Catalog #**97003520

KingFisher™ Duo and KingFisher™ Duo Prime Consumables, KingFisher Duo Cap for elution strip **Thermo Fisher** **Catalog #**97003540

MicroAmp™ Clear Adhesive Film **Thermo Fisher** **Catalog #**4306311

Nonstick, RNase-free Microfuge Tubes, 1.5 mL **Thermo Fisher** **Catalog #**AM12450

Thermo Scientific™ Screw Cap Micro Tubes **Fisher Scientific** **Catalog #**14-755-208

Bertin Corp 0.1mm Zirconium oxide beads (450g) (qty 500) **Fisher Scientific** **Catalog #**50-154-2950

Qubit™ 1X dsDNA High Sensitivity (HS) and Broad Range (BR) Assay Kits **Thermo Fisher Scientific** **Catalog #**Q33230

Qubit RNA HS (High Sensitivity) assay **Thermo Fisher Scientific** **Catalog #**Q32852

Qubit™ Assay Tubes **Invitrogen - Thermo Fisher** **Catalog #**Q32856

RNaseZap™ RNase Decontamination Solution **Thermo Fisher Scientific** **Catalog #**AM9780

ZymoBIOMICS Microbial Community Standard **Zymo Research** **Catalog #**D6300

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.

Before start

BEFORE START

1. Pre-cool the Bullet Blender by adding dry ice into the cooling compartment and running the cooling program.
2. Pre-heat the heater mixer at 95 °C .
3. Clean the work surfaces with RNaseZap, then wipe the surfaces with 70% /molecular biology grade ethanol to remove additional contaminants.
4. Transfer 0.1 mm zirconium oxide beads (2 spoons, Appendix 1) to Thermo Scientific Screw Cap Micro 1.5 ml Tubes.
5. For the first time use of IndiMag pathogen kit, add 100% ethanol to Buffer AW1 and AW2, and add 100% isopropanol to ACB as indicated on the bottles.
6. MagAttract Suspension G from IndiMag pathogen kit needs to be vortexed thoroughly for 00:03:00 (before first use) or 00:01:00 (before subsequent uses) to ensure that the magnetic silica particles are fully resuspended.
7. Aliquot nuclease-free water in big bottle into a few 15 ml tubes for preparing TNA elution in KingFisher Flex or KingFisher Duo Prime to avoid cross-contamination.

1. SAMPLE LYSIS

1 Add 700 µL of ASL buffer to the bead tubes prepared in step 4 from Before Start under the Guidelines & Warning tab.

2 Use a clean spatula or forceps to weigh about 100 mg fecal sample (roughly 5 drops of mouse feces) and place it into each prepared bead tube (If fecal sample is frozen, thaw and keep it On ice). Record the weight.

3 Include a positive control for each batch of samples: transfer 37.5 µL ZymoBIOMICS Microbial Community Standard Material, 100 µL EBV, and 100 µL ZIKV standard into a tube from step 4 from Before Start. Add 250 µL ASL buffer.

4 Include a negative control for each batch of samples: a bead tube from step 4 from Before Start with 500 µL ASL buffer only.

5 Refill the dry ice compartment of the Bullet Blender if necessary. Load all samples into the Bullet Blender.

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

7 After beating, incubate samples at 95 °C for 00:15:00 . 15m

8 After incubation, beat the samples in the Bullet Blender (Refill the dry ice compartment if necessary) at speed 12 and time at 3.

9 Let the samples settle for 00:01:00 in the Bullet Blender and then repeat step 8. 1m

2. INSTRUMENT SET UP

10

Note

KingFisher Flex only, if using KingFisher Duo Prime, go to 6. INSTRUMENT SET UP.

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

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

3. SET UP THE PROCESSING PLATES

12 Set up the Tip Comb, Wash, and Elution 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

4. EXTRACTION

13 Centrifuge the bead tubes with lysate from step 9 for 12000 x g, 00:05:00 . 5m

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

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

16 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 (it can be mixed on a Hula mixer for 00:02:00). Add 695 µL mixture to each sample. Each well including sample lysate should be 985 µL . 2m

17 Select the program **IndiMag_Pathogen_KF_Flex_4wash** on the instrument.

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

5. QUANTIFICATION AND STORAGE

19 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.

20 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.

Note

Kits needed: Qubit 1X dsDNA HS Assay Kit and Qubit RNA HS Assay Kit. (see Appendix 2 and Appendix 3).

NOTE: *If the DNA or RNA concentration measurement is too high, dilute* 2 µL *of sample in a new 1.5-mL tube with* 18 µL *nuclease-free water. Use* 3 µL *of the diluted sample for DNA or RNA quantification.*

21 Proceed with sample testing following the REDI-NET SOP F-4 Fecal Testing or store at -20 °C for less than 2 weeks.

> Note
>
> For long-term storage the sample needs to be stored at -80 °C following the REDI-NET SOP F-3 Fecal Storage.

6. INSTRUMENT SET UP

22

> Note
>
> KingFisher Duo Prime only, if using KingFisher Flex, go to 2. INSTRUMENT SET UP.

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

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

7. SET UP THE SAMPLE PLATE AND ELUTION STRIP

24 Set up the Sample Plate according to the table below:

	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
		F	Tip comb	
		G	Empty	
		H		

25 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
	Strip ID	**Row**	**Reagent**	**Volume per well**
	Elution	A	Nuclease-free water	75 µL

8. EXTRACTION

26 Centrifuge the bead tubes with lysate from step 9 for 12000 x g, 00:05:00 . 5m

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

28 Transfer 270 µL supernatant without any particle carryover to the wells of the Deep-well Row A containing proteinase K. This row becomes the Sample Row.

29 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. Add 695 µL mixture to each sample. Each well including sample lysate should be 985 µL .

30 Select the program **IndiMag_Pathogen_KF_Duo_4wash** on the instrument.

31 Start the run, then load the prepared plate and Elution Strip into position when prompted by the instrument.

Note

Keep the door open while extraction is in process. 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.

9. QUANTIFICATION AND STORAGE

32 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.

33 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.

Note

Kits needed: Qubit 1X dsDNA HS Assay Kit and Qubit RNA HS Assay Kit) (see Appendix 2 and Appendix 3.

NOTE: *If the DNA or RNA concentration measurement is too high, dilute* 2 µL *of sample in a new 1.5 mL tube with* 18 µL *nuclease-free water. Use* 3 µL *of the diluted sample for DNA or RNA quantification.*

34 Proceed with sample testing following the REDI-NET SOP F-4 Fecal Testing or store at -20 °C for less than 2 weeks.

Note

For long-term storage the sample needs to be stored at -80 °C following the REDI-NET SOP F-3 Fecal Storage.

APPENDIX 2. DNA and RNA Measurement using QUBIT FLUOROMETER 4.0 1m

35 DNA quantification:
2m
According to the volume of sample used, add the 1x HS 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). Vortex for 5 - 10 seconds, then incubate for 00:02:00 at Room temperature before reading.

36 RNA Quantification:

36.1 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

36.2 In a new 0.6 ml tube, mix 199 µL of Qubit HS RNA Assay working solution and 1 µL of the sample. Vortex for 5 - 10 seconds, then incubate for 00:02:00 at Room temperature before reading.
2m

Protocol references

REFERENCES

REDI-NET Overview Summary

1. User Guide: Indical IndiMag Pathogen Kit user's manual

2. Indical Sample Pretreatment F2 (HB-2514-EN-001): For isolation of bacterial and viral DNA from Feces.

3. International Human Microbiome Standards Consortium, IHMS - quality protocol sop for fecal samples DNA extraction protocol Q, Code: IHMS_SOP 06 V2.