JAN 10, 2024

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.j8nlkop56v5r/v1

Protocol Citation: REDI-NET Consortium 2024. L-2 LEECH PROCESSING. **protocols.io** https://dx.doi.org/10.17504/protocols.io.j8nlkop56v5r/v1

Protocol status: Working
We use this protocol and it's working

Created: Jan 10, 2024

Last Modified: Jan 10, 2024

L-2 LEECH PROCESSING

REDI-NET Consortium[1]

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DISCLAIMER

This work is supported by the US Army Medical Research and Development Command under Contract No.W81XWH-21-C-0001, W81XWH-22-C-0093 and HT9425-23-C-0059. The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army or Navy position, policy or decision unless so designated by other documentation.

ABSTRACT

This protocol details leech processing.

GUIDELINES

OBJECTIVE

To outline procedures to process leech samples for total nucleic acid extraction.

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 leech total nucleic acid extraction to allow downstream library preparation and sequencing for pathogen detection.

RESPONSIBLE PERSON

Principal Investigator, Study Coordinator, Entomology Component Lead, Managers

PROTOCOL integer ID:
93349

Keywords: Leech TNA extraction, KingFisher DNA/RNA extraction , Pathogen nucleic acid extration

Funders Acknowledgement:
USAMRAA
Grant ID: W81XWH-21-C-0001
USAMRAA
Grant ID: W81XWH-22-C-0093
USAMRAA
Grant ID: HT9425-23-C-0059

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.

- Use filter tips all the time.

- Storage of the buffers from IndiMag pathogen kit

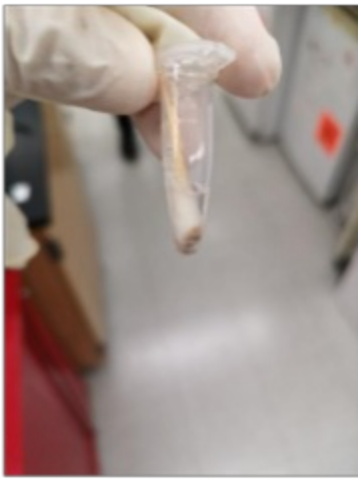
1. Proteinase K is stable for at least 1 year after delivery when stored at Room temperature (15-25°C). To store for more than 1 year or if ambient temperature often exceeds 25 °C, storage at 2-8 °C is recommended. Do not add Proteinase K directly to the Buffer VXL mixture! This can cause clogs or precipitates.
2. Precipitate 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. PHOTOS OF BLOOD MEAL AND COTTON SWAB

Figure 1: Blood meal

Figure 2: Cotton swab



APPENDIX 2. 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 Step 44 the preparation before tick homogenization. One spoon equals to 100 µL.

APPENDIX 3. EXPECTED OUTCOMES

Expected result

	A	B	C	D	E	F
	Sample	**Amount**	**Sample condition**	**Elution volume**	**DNA conc.** (ng/ul)	**RNA conc.** (ng/ul)
	Tick	1 unfed adult or 10 nymphs or 60 larvae	Frozen/live	75	20 - 30	10 - 20
	Leech	50 ul/ 3x3 mm/ 1 swab	Blood meal/ tissue/ swab	75	5 - 100	5 - 100
	Soil	0.25 - 0.3 g	Frozen/Fresh	75	<0.025 - 20	<0.01 - 20
	Water	750 ml	Half of the membrane	75	<0.025 - 20	<0.01 - 20

APPENDIX 4. DNA and RNA Measurement using QUBIT FLUOROMETER 4.0

DNA quantification:

According to the volume of sample used, add the 1xHS dsDNA QubitAssay 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:
2. 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.

	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

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 ● 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

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™ 4 Fluorometer, with WiFi NAME

Fluorometer TYPE

Invitrogen BRAND

Q33238 SKU

https://www.thermofisher.com/order/catalog/product/Q33238#/Q33238 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

	A	B	C
	Material	**Description**	**Mfg / Product #**
	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

	A	B	C
	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	Next Advance, ORANGER5-RNA
	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 oxidase beads	0.1 mm, 400 g	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
	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	ThermoFisher, 4306311
	Nonstick, RNase-Free Microfuge Tubes	1.5 mL	ThermoFisher, AM12450
	Nonstick, RNase-Free Microfuge Tubes	2.0 mL	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)	Locally sourced
	Isopropanol	100% (molecular biology grade)	Locally sourced
	Nuclease-free Water	For negative control	Locally sourced
	Dry ice	To maintain cold chain during sample handling using Bullet Blender	Locally sourced
	Ice bucket and ice	To keep sample cold	Locally sourced

	A	B	C
	Kimwipes	To dry material	Locally sourced
	Falcon tubes	15 mL and 50 mL	Locally sourced
	Cotton swabs	6 inches, sterile, plastic handle (consumable)	Fisher Scientific, 22-363-163 or equivalent
	Scalpels	Size 11 (consumable)	Fisher Scientific, 14-840-01 or equivalent
	Forceps	Straight and curved, fine point, Stainless steel, sterile	BioQuip, #4531, #4532
	Forceps	Straight, fine point	Bioquip, #4730
	Office scissors	8-10 inches, strong enough to cut the cotton swab handle	Locally sourced
	Sterile 1x PBS	To clean leech sample	Locally sourced
	Sterile petri dishes	100 mm diameter	Fisher Scientific, FB0875712 or equivalent

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

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

Buffer AL, Lysis buffer **Qiagen Catalog #19076**

Reagent DX **Qiagen Catalog #19088**

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

Orange RINO RNA Lysis Kit 250 pack (1.5 mL) **Next Advance Catalog #ORANGER5-RNA**

Sterile Microcentrifuge Tube 1.5 mL (RINO®) 500/case **Next Advance Catalog #TUBE1R5-S**

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

KingFisher™ Plastics for 96 deep-well format **Thermo Fisher Scientific Catalog #95040450**

KingFisher™ Flex™ Systems Consumables, KingFisher 96 tip comb fo 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, KingFish Duo Cap for elution strip **Thermo Fisher Catalog #97003540**

BRAND™ Self-adhesive Plate Sealing Film **Fisher Scientific Catalog #13-882-329**

MicroAmp Clear Adhesive Film **Applied Biosystems (ThermoFisher Scientific) Catalog #4306311**

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

Nonstick, RNase-free Microfuge Tubes, 2.0 mL **Thermo Fisher Catalog #AM12475**

Qubit assay tubes **Thermo Fisher Scientific Catalog #Q32856**

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

Qubit 1X dsDNA High Sensitivity Assay Kit **Thermo Fisher Scientific Catalog #Q33230**

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

SAFETY WARNINGS

RISKS 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, please, try to avoid contact with skin while preparing workbench for nucleic acid extraction.

BEFORE START INSTRUCTIONS

Note

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

1. Pre-cool the Bullet Blender by adding dry ice into the cooling compartment and running the cooling program.
2. Clean the work surfaces with RNaseZap, then wipe the surfaces with 70% molecular biology grade ethanol to remove additional contaminants.
3. Transfer 0.1 mm zirconium oxide beads (2 spoons, Appendix 2) to Clear RINO brand 1.5 ml screw-cap microcentrifuge tubes.*
4. 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 (Optional if using the MagMAX Microbiome Ultra Nucleic Acid Isolation Kit).
5. Buffer ATL may form precipitates upon storage. If necessary, warm to Temperature56 °C until the precipitates have fully dissolved. Prepare buffer ATL-DX: add Amount100 µL Reagent DX to Amount15 mL Buffer ATL. If smaller amounts are needed, transfer Amount1.5 mL of Buffer ATL into a sterile 2 ml vial and add Amount10 µL Reagent DX. Mix well, after addition of Reagent DX. After preparation, the mixture is stable for 6 months at TemperatureRoom temperature (15-25°C)**
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. When processing viable leeches, freeze at -20 °C for 01:00:00 or in dry ice for 00:20:00 to inactivate them and process them right away.
8. 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.

1. LEECH IDENTIFICATION

1

Leeches from each vial (either freshly from the field/animals or stored at −80°C) should be morphologically examined to identify its species. Only parasitic *Euhirudinea* species will be kept and other species will be discarded.

2 Leeches can be stored at -20℃ for up to 2 weeks and at -80℃ for up to 1 month. Long term storage at 4℃ is not recommended.

2. BIG LEECH BLOOD MEAL COLLECTION AND INNER ORGAN

3

Note

If leeches are about a pinky fingernail size, homogenize leech tissues as section 3.

Add 400 µL 1x PBS and 100 µL ATL-DX buffer in a Thermo Scientific Screw Cap 1.5 mL Micro tube containing 0.1 mm beads from the step 3 in Before You Start.

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

5 Use ice-cold 1x PBS to wash leech three times sequentially in petri dishes on ice to remove external contaminants.

6 Rinse leech with 70% ethanol.

7 Place the rinsed leech on Kimwipes to absorb the ethanol residuals. Place the leech in a petri dish on ice.

8 Use a pair of scissors to cut across the leech in the middle.

9 Drip the blood meal into the petri dish as much as possible.

10 Transfer the blood meal to a 1.5 ml tube by pipetting.

11 Place the leech back into the petri dish, use a scalpel to cut open the entire leech longitudinally.

12 Open the leech with forceps, use a sterile cotton swab to wipe the opened inner organs thoroughly.

13 Put the cotton tip of the swab in a 1.5 ml tube prepared in step 3, cut off the plastic handle from the cotton tip.

14 For processing bloodmeal, add 50 µL bloodmeal to a tube prepared in step 3.

> **Note**
>
> For those leeches without blood meal, only prepare the cotton swab. See Appendix 1 for the photos of blood meal and cotton swab collected from leeches.

15 The cotton swabs and blood meal in lysis buffer can be stored at -20 °C for a few days before processing.

3. SMALL LEECH TISSUE HOMOGENIZATION

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

17 Label orange RINO RNA lysis tubes on the cap, and add 60 µL cold, sterile 1x PBS into each orange RINO tube (avoid labeling on the side of the tubes due to potential damage during the beads beating process)

18 Use ice-cold1x PBS to wash leech three times sequentially in Petri dishes on ice to remove external contaminants.

19 Keep an empty Petri dish on ice. Cut the leech from head to tail, and trim the leech tissue into small pieces around 3mm x 3mm in the Petri dish.

20 Add one piece of 3mm x 3mm leech tissue into an orange RINO tube prepared in step 17.

21 Load the RINO tubes with leech tissue into the Bullet Blender. Add more dry ice into the cooling compartment of the Bullet Blender, if necessary.

22 Set the controls for Speed 10 and Time 00:03:00. Press Start. 3m

23 Repeat step 22.

24 After the run, remove the tubes from the instrument and visually inspect the samples. If homogenizati 3m

is incomplete, repeat the homogenization at speed 10 and Time 00:03:00.

25 Centrifuge the suspension at 100 x g, 00:01:00 to pellet debris. 1m

26 Without disturbing the tubes, carefully transfer the top 320 µL supernatant into the 1.5 ml tubes of Before You Start section step 3.

27 Add 80 µL ATL-DTX buffer into the tube.

4. MICROBE LYSIS

28 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 Screw Cap 1.5 mL MicroTube containing 0.1 mm beating beads. Add 162.5 µL 1x PBS and 100 µL ATL-DX buffer.

29 Include a negative control for each batch of samples: add 400 µL sterile 1xPBS and 100 µL ATL-DX Buffer to a Screw Cap 1.5 mL MicroTube containing 0.1 mm beating beads.

30 Load the tubes with blood meal and/or cotton tip or leech tissue lysate from step 13 and/or 14 and/or 27 into the fully cooled Bullet Blender (including samples and controls). Refill the dry ice compartment if necessary.

31 Set the speed at 12 and time at 00:05:00. Press Start. 5m

32 Let the samples settle for 00:01:00 and then repeat step 31. 1m

Note

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

5. INSTRUMENT SET UP (KingFisher Flex only, if using KingFis...

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

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

6. SET UP THE PROCESSING PLATES

35 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

	A	B	C	D	E
	Wash 1	2	Deep-Well	Buffer AW1	700 µL
	Sample	1	Sample Lysate	Lysate and lysis buffer	990 µL

7. EXTRACTION

36 Centrifuge the 1.5 mL tubes with lysate from step 32 for 12000 x g, 00:05:00 . 5m

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

38 Transfer 270 µL supernatant of step 36 without any particle carryover to the wells of the Deep-well plate containing proteinase K. This plate becomes the Sample Plate.

39 Add 135 µL Buffer VXL, 540 µL Buffer ACB, and 25 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 700 µL mixture to each sample.

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

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

8. QUANTIFICATION AND STORAGE

42 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.

43 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.

44 Proceed with sample testing following the REDI-NET SOP L-4 Leech 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 L-3 Leech Storage.

9. INSTRUMENT SET UP (KingFisher Duo Prime only, if using K...

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

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

10. SET UP THE SAMPLE PLATE AND ELUTION STRIP

47 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

	A	B	C	D
	Wash 3	D	80 % ethanol	750 µL
	Wash 4	E	100 % ethanol	750 µL
	Tip Comb	F	Tip comb	
		G	Empty	
		H		

48 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

11. EXTRACTION

49 Centrifuge the bead tubes with lysate from step 32 for 12000 x g, 00:05:00. 5m

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

51 Transfer 270 µL supernatant without any particle carryover to the wells of the sample row containing proteinase K. This plate becomes the Sample Plate.

52 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. Add 695 µL mixture to each sample.

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

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

12. QUANTIFICATION AND STORAGE

55 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.

56 Use 1 µL total nucleic acid for DNA and RNA concentration measurement using Qubit 4 Fluorometer.

Note

Kits needed: Qubit 1X dsDNA HS Assay Kit and Qubit RNA HS Assay Kit (see Appendix 4).

57 Proceed with sample testing following the REDI-NET SOP L-4 Leech 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 L-3 Leech Storage.