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Version 2

REDI-NET L-2 LEECH PROCESSING V.2

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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 leech processing for total nucleic acid extraction.

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

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
Dengue virus type 1 (DENV-1) positive control	For TNA extraction positive control; BSL-2	ATCC, VR-1856 or locally sourced
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
Navy RINO RNA lysis kit	Bead lysis kits	Next Advance, NAVYR5-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



A	B	C
KingFisher™ 96 tip comb for DW magnets	KingFisher Flex <i>ONLY</i>	ThermoFisher, 97002534
KingFisher™ Duo Prime 12-tip comb	KingFisher Duo Prime <i>ONLY</i>	ThermoFisher, 97003500
Elution Strip	KingFisher Duo Prime <i>ONLY</i>	ThermoFisher, 97003520
KingFisher™ Duo Cap for Elution Strip	KingFisher Duo Prime <i>ONLY</i>	ThermoFisher, 97003540
BRAND Self-adhesive Plate Sealing Film	Aluminum (<i>consumable</i>)	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 (<i>consumable</i>)	Thermo Fisher, Q32856
RNaseZap™ RNase Decontamination Solution	To remove RNase from the working area	ThermoFisher, AM9780
Qubit™ 1X dsDNA HS Assay Kit	(<i>consumable</i>)	ThermoFisher, Q33230
Qubit™ RNA HS Assay Kit	(<i>consumable</i>)	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
Kimwipes	To dry material	Locally sourced
Falcon tubes	15 mL and 50 mL	Locally sourced
Cotton swabs	6 inches, sterile, plastic handle (<i>consumable</i>)	Fisher Scientific, 22-363-163 or equivalent
Scalpels	Size 11 (<i>consumable</i>)	Fisher Scientific, 14-840-01 or equivalent
Forceps	Straight and curved, fine point, Stainless steel, sterile	BioQuip, #4531, #4532

	A	B	C
	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**

⊗ Navy RINO RNA Lysis Kit 250 pack (1.5 mL) **Next Advance Catalog #NAVYR5-RNA**

⊗ Reagent DX **Qiagen Catalog #19088**

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



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

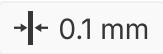
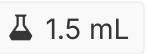
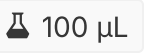
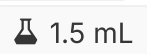
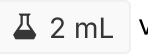
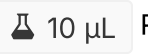
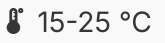
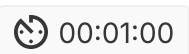
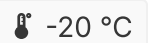
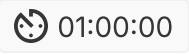
1. Caution should be taken while processing samples as some chemicals may be harmful. Please use a fume-hood when required to avoid inhaling harmful chemicals.
2. Gloves should be worn all the time when handling samples.
3. Decontaminants such as DNA/RNaseZap could irritate the skin, please, try to avoid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



Before start

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 Temperature  56 °C until the precipitates have fully dissolved. Prepare buffer ATL-DX: add Amount  100 µL Reagent DX to Amount  15 mL Buffer ATL. If smaller amounts are needed, transfer Amount  1.5 mL of Buffer ATL into a sterile  2 mL vial and add Amount  10 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 $-80\text{ }^{\circ}\text{C}$) 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 $-20\text{ }^{\circ}\text{C}$ for up to 2 weeks and at $-80\text{ }^{\circ}\text{C}$ for up to 1 month. Long term storage at $4\text{ }^{\circ}\text{C}$ 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 $400\text{ }\mu\text{L}$ 1x PBS and $100\text{ }\mu\text{L}$ 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 Start" section.
- 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 navy RINO RNA lysis tubes on the cap, and add  60 μL cold, sterile 1x PBS into each navy RINO tube (avoid labeling on the side of the tubes due to potential damage during the beads beating process)
- 18 Use ice-cold 1x 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  3 mm x  3 mm in the Petri dish.
- 20 Add one piece of  3 mm x  3 mm leech tissue into a navy 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 homogenization is incomplete, repeat the homogenization at speed 10 and Time  00:03:00 . 3m
- 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 Start" section step 3.
- 27 Add  80 μL ATL-DTX buffer into the tube.



4. SAMPLE 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 DENV-1 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
- 33 After beating, centrifuge the tubes with lysate for  12000 x g, 4°C, 00:05:00 5m

Note

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

5. INSTRUMENT SET UP (KingFisher Flex only, if using KingFisher Duo Prime, go to step 9)

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

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

6. SET UP THE PROCESSING PLATES

- 36 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	990 µL

7. EXTRACTION

- 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 $^{\circ}\text{C}$ for less than 2 weeks.

Note

For long-term storage the sample needs to be stored at  -80 $^{\circ}\text{C}$ following the REDI-NET SOP L-3 Leech Storage.

9. INSTRUMENT SET UP (KingFisher Duo Prime only, if using KingFisher Flex, go to section 5)

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

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

51 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. 

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

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

12. QUANTIFICATION AND STORAGE

54 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.

55 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).

56 Proceed with sample testing following the REDI-NET SOP L-4 Leech Testing or store at  -20 $^{\circ}\text{C}$ 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.

Protocol references

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REDI-NET Overview Summary

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