T-2 TICK PROCESSING V.1

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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 tick processing.

GUIDELINES

OBJECTIVE

To clearly document the correct process for effective standardized field collections of ticks, and recommended personal protection measures.

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 field tick collections in the vicinity of watering holes by dragging.

RESPONSIBLE PERSON

Principal Investigator, Study Coordinator, Entomology Component Lead, Managers

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Keywords: SORT TICK SAMPLES, PREPARING TICK e-ID (IDX), IMAGING USING TICK E-ID, PREPARING DINOSCOPE, TICK HOMOGENIZATION

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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

BEFORE EACH COLLECTION

1. Clean forceps with 70%-ethanol.
2. Freeze and clean ice-packs.
3. Clean cool-boxes
4. Fully charge all equipment (e.g., GPS unit, tablets/phone). Make sure the tablet has enough free-space for field sampling pictures.

AFTER EACH COLLECTION

1. Clean all equipment thoroughly between sampling sites, including boots, cooler box (inside and outside), etc.
2. Tick drags should be sealed into trash bags for transport and cleaned *without detergents* (only use tap water and then air-dry) before the next collection.
3. Lint roller sheets should be disposed of in the waste bag after all ticks have been removed for testing.
4. Store sterile equipment separate from used equipment and samples.

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.

QUALITY CONTROL

This SOP is reviewed by the applicable supervisor annually or as required in order to maintain its relevance

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. The step is described on 6.6.4 the preparation before tick homogenization. One spoon equals to 100 uL.

APPENDIX 4 EXPECTED OUTCOMES

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

MATERIALS

EQUIPMENT AND MATERIALS

Note

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

	A	B

	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
	Wifi or ethernet internet connexion	(WPA-2 security only. WPA-e not yet supported)
	Laptop or desktop computer with Google Chrome	(Setup requires Ethernet or laptop with Bluetooth LE capabilities); PC preferred for DinoScope
	Dino-Lite Driver Software	REDI-NET Program
	BioQuip Portable Chill Table	BioQuip, 1429
	Multiplex imaging system Tick e-ID device	Vectech
	AC power converted to US standard	120 V and 60Hz AC
	● 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

Dino-Lite 5MP Edge AM7115MZT NAME

Microscope TYPE

Dino-Lite BRAND

AM7115MZT SKU

https://www.dinolite.us/products/usb-microscopes/usb-edge/am7115mzt/ LINK

magnification range of this microscope is 20x to 220x SPECIFICATIONS

Equipment

Adjustable Vertical Mount stand NAME

vertical stand TYPE

Dino-Lite BRAND

MS35B SKU

https://www.dinolite.us/products/accessories/stands/ms35b/ 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™ 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 #**
	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 tick during imaging	Locally sourced
	Petri dishes, disposable	To hold ticks under DinoScope	Bioquip, 4787
	Color lab tape	For labeling and sealing boxes of ticks	Thomas Scientific, 1184X64

	A	B	C
	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	Next Advance, ORANGER5-RNA
	Clear RINO brand microcentrifuge tubes	1.5 mL, screw-cap	Next Advance, TUBE1R5-S
	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

	A	B	C
	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
	Forceps	Fine point, straight tip, stainless, sterile; For sample handling	BioQuip, 4731
	Sterile 1x PBS	To clean tick sample	Locally sourced
	Sterile petri dishes	To hold tick sample during cleaning	Locally sourced
	Data sheets	REDI-NET DCS T-2	REDI-NET Data Portal

Posi-Lock™ 5 mL Microcentrifuge Tubes **Thomas Scientific Catalog #1149Y05**

96 Place Reversible Racks with Covers **Thomas Scientific Catalog #1164M62**

Color LAB-TAPE™ 0.94 **Thomas Scientific Catalog #1184X64**

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**

APPENDIX 12: Tray Cleaning Materials

1. Vectech IDX tray
2. Lens cleaner, glass cleaner or ethanol
3. Kimwipe or microfiber cloth
4. Phillips head screwdriver

APPENDIX 14. SET-UP INSTRUCTIONS FOR BARCODE PRINTING

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

BEFORE START

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 1) to Thermo Scientific Screw Cap Micro 1.5 ml 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.
5. 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.
6. 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. SORT TICK SAMPLES

1

Note

***NOTE**: The sections 1 to 6 in this SOP are for imaging adult, non-engorged ticks. The sections 7 to 18 in this SOP are for total nucleic extraction from tick samples previously imaged and identified.*

Sort larvae (pooled and stored by the same genus), nymph (individual), and adults (individual) into separate vials in the lab.

Note

***NOTE:** Do not leave larvae (or anything) on the lint roller. Freezing larvae on lint roller paper in -80 °C freezer will facilitate the removal of individual larval ticks for genus level identification and subsequent pooling. For preparing the larvae for TNA extraction, transfer 60 larvae directly into orange RINO tubes for storage to minimize transfer steps and avoid potential loss of the small larvae. The pooled larvae can be subjected to TNA extraction directly, or stored at -80 °C until extraction. See step 48.3.*

2. PREPARING TICK e-ID (IDX): Device setup using Bluetooth

2

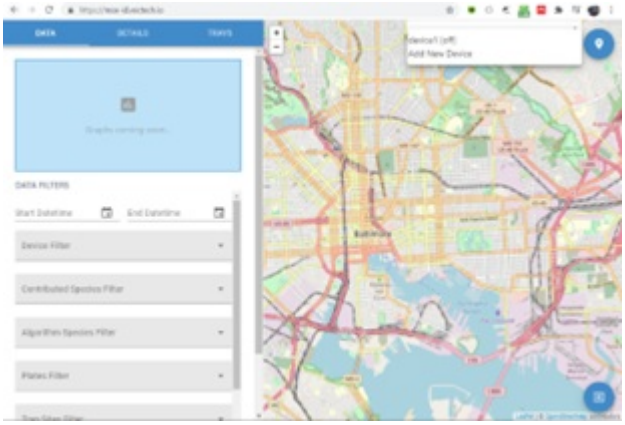
Note

***NOTE**: If the Tick e-ID is not recognizing the device, please, logout from google and your institution, and reboot the computer. Then login to the Tick e-ID platform using google account. Current device is not supporting larval ticks, which will only be imaged using DinoScope in section 5 and 6.*

Plug in the device. On a phone or computer which has both Google Chrome and Bluetooth capabilities, visit https://mos-id.vectech.io (if on mobile, use the desktop site option) and log in. For first time use, click the **"Sign Up"** tab, enter in a username (email), and a password. We will approve your account, and then you can continue.

3 If set-up with ethernet is desired, skip to Section 3.

4 Click the drop-down arrow at the top-center of the page (see figure). Click **"Add New Device,"** click on the bluetooth icon button. When a device with a name **"Agamotto"** appears, select the device and click **"Pair"**. When given the opportunity, select and enter your Wi-Fi credentials [user name, passcode] (the website may throw an error,but ignore this error). Return to the **"Data"** tab.



5 Click the drop-down arrow at the top-center of the page, and you should see a device with status **[booting]** or **[Idle]**. If [booting], wait for status to change to [idle] and stay. Then select "**Device**." Setup is complete. If your device soon changes to [Idle] under the device drop down menu, continue to 4. Imaging Protocol.

3. PREPARING TICK e-ID (IDX): Device setup using Ethernet

6 If setting up with ethernet instead of Bluetooth, follow these steps. Select the user menu at the bottom right corner of the screen. Select **"Connect Device."**

7 Select **“Device.”**If the device name is not known, contact Vectech for support. Then select your Wi-Fi name from the “**Wi-Fi SSD”** drop down menu. If you do not see your Wi-Fi in the drop down, verify the network is functioning on other devices. Then enter your Wi-Fi password. Select connect.

8 If a successful Wi-Fi connection is established, disconnect the device from power and ethernet, then reconnect power. If your device soon changes to [**Idle**] under the device drop down menu, continue to Section 4. Imaging Using Tick e-Id.

9 If you have trouble connecting to the device via Wi-Fi, try to connect an Ethernet cable directly from your device to your modem. You should soon see your device status change to [**Idle**] under the device drop down menu.

4. IMAGING USING TICK E-ID

10 On https://mos-id.vectech.io, navigate to the **“Trays”** tab. Verify that the device is plugged in. The device should flash lights when booting up.

11 Organize the specimens to be multi-imaged in sample tubes laid out in tubes in a 96-well sample rack (8x12). **MAINTAIN THE COLD CHAIN.** Keep samples in dry ice or in polystyrene boxes filled with cool

beans until ready to use. Place a glass tray on the chill table as you fill the rows before imaging and immediately return to storage tubes once imaged. Sequentially lay out the sequential sample across the row, with each row of 12 equaling one tray of multiplex photos.

Note

Tip: If you run samples in sequential order from top left to bottom right through the tray (A1- A4; A5- 8, A9-12), the system will auto-fill your sample numbers in the associated data fields.

Figure 1: *Placement of samples in the tray for identification.*

12 Wipe forceps using bleach wipes between each sample and rinse each RNA-later preserved sample in water before pulling tick sample on Kimwipes on chiller table to allow absorption of excess fluid before slotting into the imaging wells.

Note

Note: *It is primordial to clean the ticks and well-dry them prior imaging in order to maximize the capture of key features for morphological identification.*

13 Repeat step 12, until all 12 specimens are placed in the tray, dorsal side facing up.

14 Close the tray by placing the lid on the well-side of the tray until you hear the gentle click as the magnets connect to keep the tray closed.

***Figure 2**: Insertion of the tray.*

15 Insert tray into device tray slot. Tray will “click” into place when fully inserted.

16 To capture the dorsal view, go to the **“Trays”** tab in the website, click, **“Capture New Tray.”**

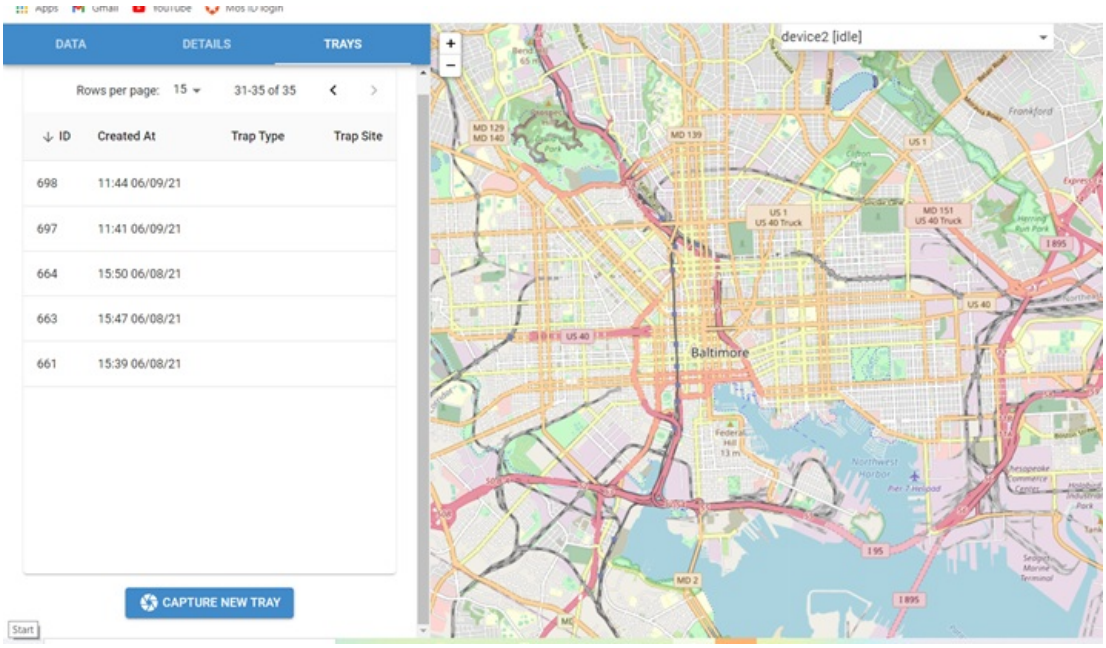


***Figure 3**: Screenshot of the “Capture New Tray” window.*

17 Images auto upload.

Note

NOTE:

- *Writing the tray number and the well-ID on the side of the tube after the tick e-ID imaging process could be helpful for double-checking and labeling Dinoscope images when done several days apart and for TNA extraction information.*

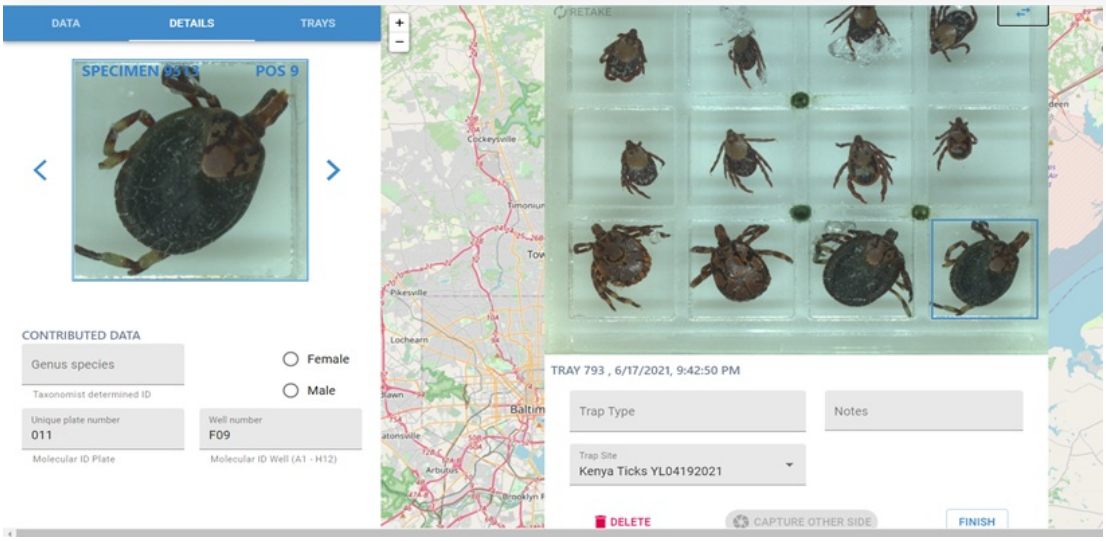


Figure 4*: Screenshot of the e-Tick ID while uploading images.*

18 Enter the following information:

18.1 Genus and species (if known).

Note

This field will autofill by clicking the arrow to view the next specimen. Autofill for this field is: previous specimen=current specimen. Once a field has data saved, autofill is no longer active.

18.2 Plate and well number.

Note

This field will autofill by clicking the arrow to view the next specimen. Autofill for this field is: for well, A1-H12, consecutive; for plate, previous specimen=current specimen. Once a field has data saved, autofill is no longer active.

19 Remove tray, flip upside down (i.e. dorsal to ventral), and insert tray again to capture the ventral image of the tick.

20 Click **“Capture Other Side”.**

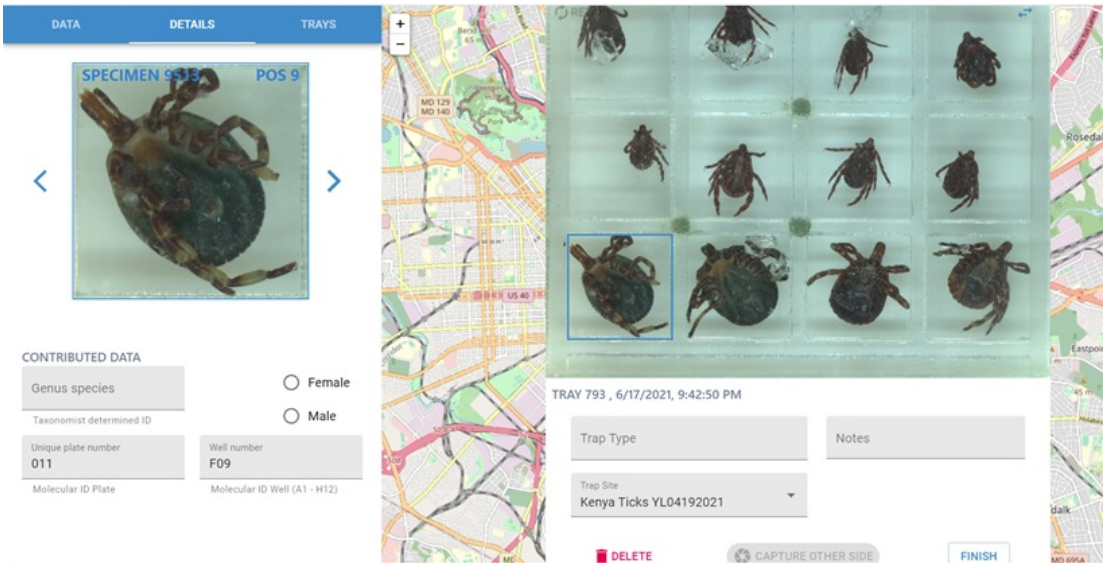


Figure 5: *Screenshot of the capture of ventral side of samples.*

21 Click “**Finish**,” and return specimens to original storage. Make a note of the tray number that corresponds to your sample row.

5. PREPARING DINOSCOPE

22 Place a portable chill table on the adjustable stand.

23 Place white porcelain stage directly on top of the chill table surface. If a porcelain stage is not available, cut a 5cm x 5cm square from a paper note card and use color lab tape to attach to the bottom of a petri dish to make a stage.

24 Once in place, secure the stage by taping either porcelain or petri dish stands to sides of the chill table.

25 Adjust the DinoScope holster on the stand so it is 4.5 cm above the surface of the stage.

26 Turn on the chill table (skip if not maintaining a cold chain) and wait until the surface temperature monitor reads -20 °C .

27 Ensure all specimens slated for imaging are arranged in individual Eppendorf tubes and stored in 96-well racks labeled with a plate number.

28 Use a Kimwipe and 75% ethanol to wipe clean the stage surface. If using a petri dish stage, replace paper cards.

6. IMAGING USING DINOSCOPE

29 Remove a specimen from tube with forceps and place in the center of the stage with the dorsal side facing up.

30 Using the DinoScope interface and stand fine adjustment, bring the specimen into focus (*magnification requirements will differ between larvae/ nymphs and adults*). Specimens should be centered in the field of view. Make sure all important characters are visible.

31 Annotate with the file naming convention: Sample ID-View (D = Dorsal, V = Ventral) Example: NMNH2020-D.

32 Ensure the correct magnification is entered into the Magnification field and that all metadata is included in the image output (magnification, magnification profile, scale, username and annotation) but does not

overlap with specimen in the image.

33 Just before taking an image readjust the white balance for each individual image to standardize the background color for all images.

34 Take a single image and review the photo to ensure quality (focus, frame, taxonomic characters). If the image is not satisfactory delete and repeat steps 31 to 35.

35 Use forceps to flip the specimen so that the ventral side is facing up.

36 Repeat steps 31 to 35 for ventral view.

37 Return specimen to tube and repeat steps 30 to 38 until a dorsal and ventral image are captured for all specimens.

38 When the imaging session is completed, copy photos from the dated folder and save.

7. BEFORE TICK HOMOGENIZATION

39

Note

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

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

program.

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

41 Transfer 0.1 mm zirconium oxide beads (two spoons, Appendix 1) to Clear RINO brand 1.5 ml screw-cap microcentrifuge tubes.

42 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.

43 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).

44 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.

45 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.

8. TICK HOMOGENIZATION

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

47 Label orange RINO RNA lysis tubes on the cap.

Note

Avoid labeling on the side of tubes due to potential damage during beads beating process.

48 Wash tick using ice-cold 1x PBS:

48.1 For adult ticks: 3 times sequentially in Petri dishes to wash away any external pathogens.

48.2 For nymph ticks: in a clean microcentrifuge tube 1.5mL, add 250 µL of PBS and pipette off the supernatant without pipetting the nymph. Repeat sequentially 3 times to wash away any external pathogens.

48.3

Note

***NOTE**: Because larval ticks are light and small, transfer and wash needs to be done in the same tube to avoid loss during multiple transfers.*

For larval ticks:

- Transfer 60 tick from lint roller to an orange RINO tube containing five 3.2 mm beads or use pooled larval ticks stored at -80 °C in orange RINO tubes (see step 1).
- Add 200 µL 1x PBS to wash the ticks. The ticks will be floating at the top of the 1x PBS.
- To remove the PBS, using a pipette with 200 µL tip, directly insert the tip to the bottom of the tube and aspirate the PBS until all PBS is retrieved. Discard the PBS and repeat the wash twice. The minimum 1x PBS residuals retaining on the beads will not affect the result.

49 Tick transfer:

49.1 For adult ticks: Transfer individual ticks into labeled orange RINO tubes and set On ice.

49.2 For nymph ticks: For a good amount of material used in downstream processing, transfer a pool of ticks (approximately 10-15 nymph, according to their size) until reaching the size of one bead (see Appendix 2), into labeled orange RINO tubes and set On ice.

49.3 For larval ticks: See step 51.3. A pool of ticks reaching the size of one 3.2 mm bead will be sufficient (approximately 60 larvae, according to their size, see Appendix 2). Label the orange RINO tubes from step 51.3 and set On ice.

Note

NOTE: *The size of nymphs or larvae from different tick species could vary.*

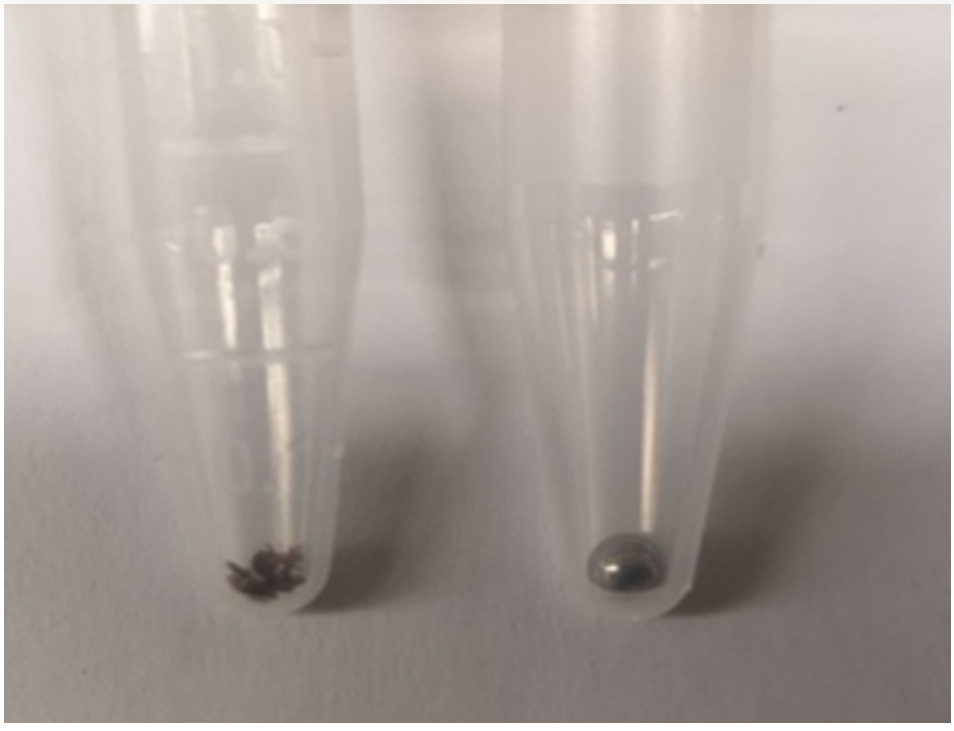


Figure 6.1: Size of 10 Amblyomma americanum nymphs compared to a 3.2 mm diameter bead.

Figure 6.2: Size of 60 Amblyomma americanum larvae compared to a 3.2 mm diameter bead.

50 Ensure the Bullet Blender is fully cooled down. Add more dry ice into the cooling compartment of Bullet Blender, if necessary, then load the RINO tubes with ticks.

51 Set the controls for Speed 10 and Time 3. Press Start.

Note

IMPORTANT: Make sure speed is set at 10, which is sufficient for tick tissue homogenization and at the same time, preserving the intactness of tick-associated microbes.

NOTE: The dry grinding step is used to break the exoskeleton and prevent the sample from floating on the buffer surface.

52 After the run, remove the tubes from the instrument, briefly spin down and set On ice.

53 Add 400 µL cold sterile 1xPBS into each orange RINO tube with ticks.

54 Return RINO tubes to the Bullet Blender.

55 Set the controls for Speed 10 and Time 3. Press Start.

56 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 3 (for larvae, one time beating should be sufficient for homogenization).

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

58 Without disturbing the tubes, carefully transfer the top 320 µL supernatant into 1.5 mL tubes.

9. MICROBE LYSIS

59

Note

***NOTE**: Check section 7 for the buffer preparation and storage details, before setting up microbe lysis and KingFisher instrument.*

Add 80 µL of ATL-DX Lysis Buffer to the 1.5 mL tubes containing 0.1mm beads from step 52.

60 Transfer 320 µL homogenate to each of the pre-labeled bead tubes.

61 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 62. Add 82.5 µL 1x PBS.

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

63 Add more dry ice into the cooling compartment of Bullet Blender, if necessary and then load the all bead tubes (samples and controls).

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

65 Let the samples settle for 00:01:00 and then repeat step 64. 1m

66 Centrifuge the tube at 100 x g, 00:01:00. 1m

67 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 .

10. INSTRUMENT SET UP

68

Note

NOTE: KingFisher Flex only, if using KingFisher Duo Prime, go to section 11

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

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

10.1 SET UP THE PROCESSING PLATES

70 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

	A	B	C	D	E
	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

10.2 EXTRACTION

71 Centrifuge the 1.5 mL tubes with lysate from step 70 for 12000 x g, 00:05:00. 5m

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

73 Transfer 270 µL supernatant of step 71 without any particle carryover to the wells of the Deep-well plate containing proteinase K. This plate becomes the Sample Plate.

74 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.

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

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

10.3. QUANTIFICATION AND STORAGE

77 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.

78 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 3 and Appendix 4)

79 Proceed with sample testing following the REDI-NET SOP T-4 Tick 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 T-3 Tick Storage.

11. INSTRUMENT SET UP

80

> Note
>
> NOTE: KingFisher Duo Prime only, if using KingFisher Flex, go to section 10.

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

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

11.1 SET UP THE SAMPLE PLATE AND ELUTION STRIP

82 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	700 µL
		G	Empty	
		H		

83 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

11.2. EXTRACTION

84 Centrifuge the bead tubes with lysate from step 68 for 12000 x g, 00:05:00. 5m

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

86 Transfer 270 µL supernatant without any particle carryover to the wells of the sample row containing proteinase K.

87 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.

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

89 Start the run, then load the prepared plate/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.

11.3 QUANTIFICATION AND STORAGE

90 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.

91 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 3 and Appendix 4).

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

Note

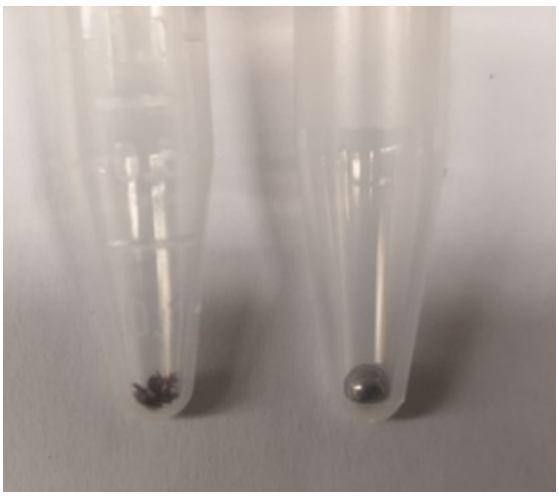
For long-term storage the sample needs to be stored at -80 °C following the REDI-NET SOP T-3 Tick Storage.

APPENDIX 2. SIZING CRITERIA FOR TICK NYMPH AND LARVA PO.

93 **NOTE:** The size of nymphs from different tick species could vary which may influence the number of nymphs needed for pooling.

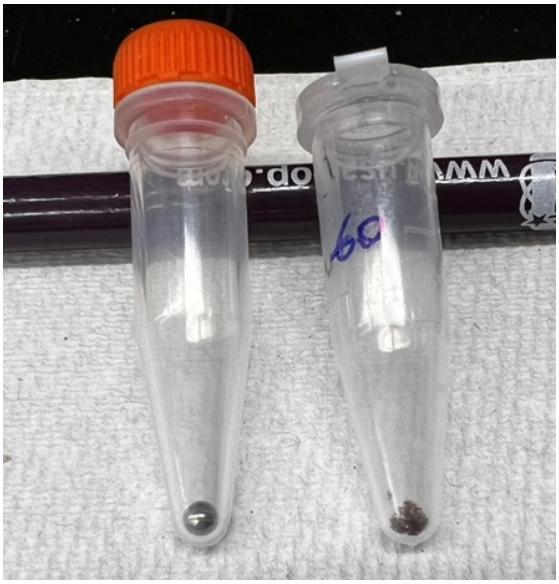
The picture below provides an “actual” size reference for 10 nymphs of Amblyomma americanum pooled in a 1.5 ml microcentrifuge tube compared to a 3.2 mm diameter stainless steel bead in an identical tube (used for tick sample processing).

	A
	Number of nymphs pooled: 10 Amblyomma americanum
	The diameter of the stainless steel bead: 3.2 mm



The picture below provides an “actual” size reference for 60 larvae of Amblyomma americanum pooled in a 1.5 ml microcentrifuge tube compared to a 3.2 mm diameter stainless steel bead in an identical tube (used for tick sample processing).

	A
	Number of larvae pooled: 60 Amblyomma americanum
	The diameter of the stainless steel bead: 3.2 mm



APPENDIX 3. DNA and RNA Measurement Using QUBIT FLUORO..

94 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.

95 RNA Quantification:

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

95.1 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.