RNA extraction from 10µL mouse blood samples (KingFisher Flex 96-well)

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1 *Works for me* dx.doi.org/10.17504/protocols.io.88fhztn

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ABSTRACT

RNA extraction from 5uL mouse blood samples, for subsequent cDNA production and qPCR to detect *Plasmodium* parasites - specifically gametocytes - within the blood. Using KingFisher Flex (96 well) system with MagMAX-96 Total RNA Isolation Kit. Protocol to import into BindIt Software attached.

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Protocol originates from previous method described in: P. Schneider et al, Mol Biochem Parasit 2015, 199: 29-33, DOI: 10.1016/j.molbiopara.2015.03.006. Protocol previously shortly described in P. Birget et al, Proc Roy Soc Lond B, 2017, 284 (1860): 20171229, DOI: 10.1098/rspb.2017.1229; and in P. Schneider et al, PLoS Pathogens 2018, 14(11): e1007371, DOI: 10.1371/journal.ppat.1007371.

GUIDELINES

Please note that RNA was extracted using the semi-automatic Kingfisher Flex Magnetic Particle Processor and the MagMAX-96 Total RNA Isolation Kit (AM1830 Thermo Fisher Scientific) but with adjustments to the manufacturer's protocols (standard protocols AM1830DW) to improve recovery. Note: the adapted protocol requires MORE lysis buffer than is provided in the kit: order extra (AM8500)

MATERIALS

NAME	CATALOG #	VENDOR
RQ1 RNase-Free DNase, 1,000u	M6101	Promega
KingFisher™ Flex™ Systems Consumables, KingFisher Flex Microtiter Deepwell 96 plate, sterile	95040460	Thermo Fisher
KingFisher™ Flex™ Systems Consumables, KingFisher 96 tip comb for DW magnets	97002534	Thermo Fisher
KingFisher™ Flex™ Systems Consumables, KingFisher 96 KF microplate (200µL)	97002540	Thermo Fisher
MagMAX™-96 Total RNA Isolation Kit	AM1830	Thermo Fisher
MagMAX™ Lysis/Binding Solution Concentrate	AM8500	Thermo Fisher

MATERIALS TEXT

In addition you will need: RNAlater, isopropanol, absolute ethanol, and general lab materials (plastics, pipettes, pipette tips)

Sample Collection

1 **Dispense 20 µL of RNAlater** into 0.2 mL strip tubes or 96-well plates, one well per sample. Cover tubes with lids, or plate with a plastic film (*the thick version, not the optical film for qPCRs – that one will NOT come off*). *Note: using more RNAlater ends up inhibiting the downstream process.*

2 **Take a 10 µL blood sample** (we use glass capillaries) and dispense the blood immediately into the RNAlater. Repeat for all samples.

3 **Mix** with pipette and replace lid; or replace lid and mix by carefully tapping the tube.

4 **Freeze samples** at -70°C or immediately extract RNA.

Prepare for RNA extraction

5 **Defrost your samples.**

6 **Get required plastics.** You will need 6 Kingfisher DeepWell (DW; Cat# 95040460) plates, 2 Kingfisher 96 (KF; Cat# 97002540) plates and 1 DW tip comb (Cat# 97002534).

7 **Prepare reagents in kit only if opening a new** MagMAX-96 Total RNA Isolation Kit (Cat#AM1830), Add the appropriate amount of ethanol or isopropanol to the wash buffers (see table below) and mark the bottles clearly by crossing out the word "concentrate", ticking the small checkbox and writing your name/initials and the date.

Reagent	Location	Action
Lysis/Binding Concentrate	Bench	Add 9 mL isopropanol
Wash Solution 1 Concentrate	Bench	Add 6 mL isopropanol
Wash Solution 2 Concentrate	Bench	Add 44 mL 100% ethanol
Elution Buffer	Bench	None
Lysis/Binding Enhancer	-20°C freezer	None
RNA Binding Beads	4°C fridge	None
Turbo DNase	-20°C freezer	None
Turbo DNase Buffer	Bench	None
RNA Rebinding Concentrate	Bench	Add 6 mL isopropanol

New MagMAX-96 Total RNA Isolation Kit - Preparation of reagents (Thermofisher Cat#AM1830)

Note: some of the bench-top reagents crystallise when the ambient temperature is low (below ~18°C). To combat this, set a heat block to 25°C before beginning and place the room temperature reagents on it until ready to use, remembering to mix well!

8 **Prepare bead mix.** Mix 10µL of RNA binding beads (*vortex!)* and 10µL of lysis/binding *enhancer* (total = 20 µL) per sample, in a sterile DNase/RNase-free eppendorf tube. Vortex the mixture after preparation, and again immediately before use. *Note: this stuff sinks to the bottom quickly, vortex regularly if dispensing for many samples.*

9 **Prepare the DNAse mix**. Mix 49 µL Turbo DNase buffer (*mix well before use*) and 1 µL Turbo DNase enzyme per sample, in a sterile DNase/RNase-free eppendorf tube. Mix thoroughly with a pipette, and leave at room temperature until needed.

10 **Mix samples with lysis/binding solution.** Label a Deep Well (DW) plate with the word 'Sample'. Add 175 µL Lysis/Binding solution to the samples; pipette up and down (*multichannel comes in handy*), then move to the Deep Well 'Sample' plate. Add a further 175 µL of Lysis/Binding solution to each - now almost empty - sample tube to collect any remaining blood, mix, and transfer to the corresponding wells of the Deep Well 'Sample' plate.

Note: we add a total of 350µL Lysis/Binding solution to each sample. There's only 20mL per 100 samples available in the kit. Order AM8500 (100mL Lysis/Binding solution to make up the difference)

11 **Fill the plates** as shown in the table:

Plate (type)	Contents /well
Tip plate (KF) + DWTip Comb	N/A
Sample (DW)	filled at step 10: 380 µL sample+lysis/binding solution
Wash 1 (DW)	150 µL wash buffer 1
Wash 2_1 (DW)	150 µL wash buffer 2
DNAse (DW)	50 µL DNAse mix
Wash 2_2 (DW)	150 µL wash buffer 2
Wash 2_3 (DW)	150 µL wash buffer 2
Elution (KF)	75 µL RNA elution buffer

Plate filling instructions.

RNA extraction

12 **Connect the Kingfisher instrument** to the computer using USB cable (*note: this should already be there)*.

13 **Open the BindIt software**. Select 'Your Kingfisher Machine' from the drop-down 'connect' list to connect to your Kingfisher instrument (*note: connection has likely established automatically)*.

14 **Select the protocol** (see step 22), and click on the 'start' (green arrow) button on *the BindIt software*.

15 **Load the plates into the machine.** The machine will turn the turntable and prompt you to load the plates in the correct order. Press the 'start' button on *the instrument* to confirm you have loaded the current plate, this tells the machine to progress to the next plate. Make sure to line up the A1 well of the plate with the marked corner on the turntable position.

16 **Follow dispense step instructions.** The run will begin once all plates are loaded. The instrument will prompt you to add the correct reagents at the dispense steps (grey cells in machine protocol, below). The first dispense step is roughly 2min after start-time. A guide to the approximate length of time until a step ends (so that you can work out how long between dispense steps) is also included in the machine protocol table below. *Note: when adding beads - vortex first!*

17 **Remove plates** when the run has ended (as prompted by machine). Export AND SAVE the run details as a PDF (automatically opened in the software) – it contains details of the times each steps were begun as well as temperatures at frequent intervals, *if something has gone wrong this may give you the answer why. Do not yet discard plates.* Stop and switch off machine.

18 **Check, seal and label RNA extracts.** Check there is liquid in the elution plate (*if not, something has gone wrong!)*. Seal the elution plate with a plastic film (make sure it is freezer-suitable and do not use the optical qPCR films – these do NOT come off) and label it carefully.

19 **Freeze** at -20C (short term) or -70°C (long term) or use immediately for cDNA+qPCR. We recommend 1) Additional DNAse treatment (see step 22-30); 2) diluting at least 1 volume extracted RNA + 4 volumes water before cDNA production to remove the effects of inhibitors 3) if diluted before cDNA production, cDNA can go directly into qPCR without further dilution step.

20 **Discard all plates, except the elution plate**. If you haven't used every well, you can keep them to be reused (be sure to use different, clean wells to the ones you have already used). And, if you do keep them for a while – remove liquids from used wells before storage.

Kingfisher Protocol

21 **Optimized machine Protocol**

File to import into your BindIt software: RNA10uLMouseBlood.bdz

Summary:

Plates (add to BindIt software under the 'Layout' tab):

- Tip plate (KF 96 plate with DW 96 tip comb inserted)
- Sample plate (DW microtiter plate)
- Wash 1 (DW microtiter plate)
- Wash 2_1 (DW microtiter plate)
- Wash 2_2 (DW microtiter plate)
- Wash 2_3 (DW microtiter plate)
- Turbo DNase (DW microtiter plate)
- Elution (KF 96 plate)

Protocol (add to BindIt software under the 'Protocol' tab):

Step name	Description	Plate	Step end (approx. time from start)
Pick up tip		Tip plate	
Mix 1	Heating during mixing 20°C 1 min; medium mix	Sample	2 min 20 s
Add beads	Dispense. Message: "Add 20 µL beads."	Sample	4 min 30s (~2 min)
Bind RNA of lysed sample	Heating during mixing 20°C. 5 min; medium mix. Collect beads count 5, time 5s	Sample	11 min
Release beads 1	Heating during mixing 20°C. Release beads 10s, slow.	Wash 1	11 min 30s
Recollect beads	Heating during mixing 20°C . Collect beads count 5, time 5s.	Sample	13 min 30s
Wash 1	Heating during mixing 20°C. Release beads 10s, slow 45s slow mix Collect beads count 5, time 5s	Wash 1	16 min
Wash 2_1	Heating during mixing 20°C. Release beads 10s, slow 45s slow mix Collect beads count 5, 5s	Wash 2_1	18 min
Dry 1	Dry outside well/tube, 30 s.	Wash 2_1	18 min 30s
DNase 10 min	Heating during mixing 25°C. Release beads 10s, slow 10 min slow mix	Turbo Dnase	29 min 30s
Add rebinding solution	Dispense. Message: "Add 100 µL rebinding solution."	Turbo DNase	32 min 30s (~30 min)
Rebind RNA	Heating during mixing 20°C. 3 min slow mix Collect beads count 5, time 5s.	Turbo DNase	36 min
2nd Wash 2	Heating during mixing 20°C. Release beads 10s slow, 45s slow mix Collect beads count 3, time 5s.	Wash 2_2	38 min
3rd Wash 2	Heating during mixing 20°C. Release beads 10s slow 45s slow mix Collect beads count 3, time 5s	Wash 2_3	40 min
Dry 2	Dry 1 min outside well/tube	Wash 2_3	41 min
Elution	Heating during mixing 65°C. Release beads 10s slow. 7min 30s slow mix Collect beads count 5, time 20s.	Elution	51 min
Release beads into 2_1	Release beads 10s slow	Wash 2_1	52 min
Recollect beads from elu	Collect beads count 5, time 20s.	Elution	53 min
Leave tip		Wash 2_1	53 min

KingFisher Protocol for RNA extraction from 10µL mouse blood samples (BindIt software) with added time frame for extraction

run.

DNAse treatment

22 **We currently use RQ1 RNase-Free DNase**

Defrost. Take RQ1 RNase-Free DNase kit and samples (if frozen) from freezer. Thaw on ice. Vortex RQ1 buffer once defrosted.

23 **Program PCR machine.** You will need 37ºC for 30min and 65ºC for 10min.

24 **Prepare RQ1 reaction mix**. Per sample you will need 2 µL RQ1 10x reaction buffer and 4 µL RQ1 enzyme. Prepare RQ1 reaction mix for all samples and aliquot 6 µL per sample into PCR tubes/plate.

25 **Add RNA extract.** Add 14 µL of RNA extract to each tube/well with RQ1 reaction mix (total volume 20 µL). Mix gently, Seal tubes/plate.

26 **Spin down.** Briefly centrifuge to spin down contents.

27 **37C 30 min.** Place tubes/plate in PCR machine. Run at 37ºC for 30 min.

28 **Add stop solution.** Add 2 µL RQ1 stop solution to each sample.

29 **65C 10 min.** Place tubes/plate in PCR machine. Run at 65ºC for 10 min.

30 **Cool** to 4 °C for 5 mins before using cDNA production, or store samples at -20ºC (short term) or -70ºC (longer term)