RNA extraction using magnetic beads by Hamilton ML STAR

Standard Operating Procedure 1 (SOP1, version 1, 25.11.20)
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1. Material and equipment

Reagents	Company	Material #
Guanidinium thiocyanate (GITC)	Sigma	G9277
Sodium citrate	Merck	1.06448.1000
Triton X-100	Sigma	T8787
Glycogen (5 µg/µl)	Invitrogen	AM9510
SiMAG-N-DNA magnetic beads (100 µg/µl)	Chemicell	1104-5
Nuclease-free water	Life Technologies	AM9938
Ethanol abs.	Sigma-Aldrich	32205
Diethyl pyrocarbonate (DEPC)	Sigma-Aldrich	D5758
Dithiothreitol (DTT)	Carl Roth	6908
RNaseZAP	Sigma-Aldrich	R2020
DNA away	Thermo	7010

Consumables	Company	Material #
96 well deep well 2 ml (2 plates needed for 96 samples)	Steinbrenner	978-2200SU
96 well skirted PCR plate (2 plates needed for 96 samples)	4titute	4ti-0960/C
Pipette Tips LQR LTS 200 µl F 960/10 (3 racks needed for 96 samples)	Mettler Toledo	17010646
Multi-stepper tips 12.5 ml (1 tip needed for 96 samples)	Brand	702692
Combitips® advanced 10,0 ml biopur, Farbcode orange (3 tips needed for 96 samples)	Eppendorf	0030089677
1250 µl GRIPTIP, Sterile, Filter, WIDE BORE (1 rack needed per 96 samples)	Integra	6645

Equipment	Company	Material #
Hamilton ML Star (customized)	Hamilton	-
Magnet Plate for 96-well deep well plates	Alpaqua	A000400
1 ml Pipette	various	-
200 µl Pipette	various	-
Heat block	various	-

2. Preparation of buffers and solutions

2.1. Prepare stock solutions (prepared in advance)

a) DEPC-treated water (1000 ml):
- Add 1 ml DEPC (Sigma) to 1000 ml MilliQ (Merck) purified water.
- Shake vigorously.
- Autoclave for 15 min at 121°C to deactivate DEPC.
- Store at room temperature.

b) GITC lysis buffer stock (1000 ml, enough for 65 plates):
Dilute in 400 ml DEPC-treated water at room temperature:
- 590.8 g GITC (c_{final} = 5 M)
- 7.35 g Sodium citrate (c_{final} = 25 mM)
- 10 ml Triton X-100 (c_{final} = 1%)
- Adjust pH to 8 using NaOH.
- Fill up to 1000 ml with DEPC-treated water.
- Aliquot GITC stock (14.4 ml; 1 aliquot for 96 samples) and store it at 4°C (crystals may form in the buffer after a prolonged storage (weeks) at 4°C – warm up before use).

c) DTT (1M stock):
- Dissolve 7.71 g DTT in DEPC-treated water and fill up to 50 ml.
- Aliquot (1.25 ml; 1 aliquot for 2x 96 samples) and store at -20°C.

2.2. Preparation of working solutions

Clean surfaces and equipment with RNaseZAP and DNA Away.

a) Prepare GITC lysis buffers for 1-4 plates just before RNA extraction (c_{final} = 4.8 M GITC) by mixing the following components according to the plate number:

Component	1 plate	2 plates	3 plates	4 plates
GITC	36 ml	48 ml	57.6 ml	72 ml
1 M DTT	1.5 ml	2 ml	2.4 ml	3 ml
Glycogen	150 µl	200 µl	240 µl	300 µl
Zika RNA (10^5)	32.1 µl	42.8 µl	51.4 µl	64.2 µl

b) Prepare SiMAG-N-DNA beads dilution for 1x 96-well plate just before RNA extraction:
- Vortex 1 ml tube containing magnetic bead stock.
- Transfer magnetic bead stock to a 1.5 ml Eppendorf tube.
- Wash magnetic beads with RNAse free water (3x).
- Place on a magnetic rack for 2 min.
- Remove the supernatant.
- Add 1 ml RNAse-free water.
- Mix well by vortexing.
- Perform this washing step three times in total.
- Prepare magnetic bead dilution in Ethanol abs. by adding 1 ml SiMAG-N-DNA to 19 ml Ethanol abs. (c_{final} = 5 µg/µl)
- Vortex dilution well before use.

Component	1 plate	2 plates	3 plates	4 plates

Washed SiMAG-N-DNA beads (stock)	2 ml	3 ml	4 ml	5 ml
EtOH abs.	38 ml	57 ml	76 ml	95 ml

c) Prepare 70% Ethanol by dilution of EtOH absolute in nuclease-free/ DEPC-treated water:

Component	**1 plate**	**2 plates**	**3 plates**	**4 plates**
Nuclease-free/ DEPC-treated water	25.7 ml	45 ml	66 ml	84 ml
EtOH abs.	60 ml	105 ml	154 ml	196 ml

d) Required volume of nuclease-free (non-DEPC-treated!) water:

Component	**1 plate**	**2 plates**	**3 plates**	**4 plates**
Nuclease-free water	36 ml	51 ml	67 ml	82 ml

2.3. Preparation of the Hamilton ML STAR robotic platform

- Check the liquid waste containers to make sure they have enough space for a new RNA extraction run.
  - Glass bottle on the bottom left side: liquid from 1-4 plates method.
  - White plastic container: liquid from 1-96 samples method.
- Check the tip wastes and empty them if required.
- Turn on the computer associated with the Hamilton robot (Username: Hamilton; password: hamilton).
- Switch on the Hamilton ML STAR robot via the green toggle switch on the left side of the robot.
- Switch on the heat-shaker controller via the silver metal box on the left side under the robot
- Switch on the Minichiller (black box on the right side under the robot) using the black toggle switch and the turn on button. Temperature is supposed to be set to 10 °C.
- Start the program in the Hamilton software by clicking on the Hamilton icon (“H”) on the desktop. Select one of the methods according to the number of samples (1-96 samples or 1-4 plates).

2.4. Loading the Hamilton ML STAR robot for the RNA extraction protocol (1-4 plates)

- Precisely follow the step-wise instructions given in the dialog windows of the Hamiton software.
  a) Sample number: Enter the number (1-4) of plates in the dialog box. Click “OK” to continue.
  b) Load 1-4 deep well plates (DWP) at positions shown on the deck layout: The correct positions are highlighted in the dialog box. Positions are numbered with 1-5 beginning at the back of the instrument. The robot will pipe the lysis buffer and then the samples into these DWP. Click “Next” to continue.
  c) Load 300 ml troughs with reagents at shown positions:
     - When pouring liquid into the containers, ensure that there is no foam on the surface of the liquid, because foam may cause pipetting problems.

- Fill the troughs with reagents according to the instructions in the dialog box. Do not overfill reagent troughs.
- To avoid liquid spilling during the carrier movement, 70% EtOH should be loaded after the load carrier moved in if more than two plates are running.
- Cover the nuclease-free Water container with a lid.
- Click “Next” to continue.

d) Load tip racks at shown positions:
- Four types of tips are used during the protocol including 1000 µl tips without filter, 1000 µl tips with filter, 300 µl tips with filters and 300 µl tips with filter and wide-border tips (modified). Do not mix tip size and type (e.g. with or without filter, wide-bore, different volumes). Make sure that the barcode of each tip box is always on the right side (in order to be scanned).
- Remember to place a fresh DWP below 1000µl filter tips (on positions 5-8) as a drip shield.
- Click “Next” to continue.
- The first tip carrier, into which the 1000 µl tips without filter are loaded, will not move out automatically (position 1-4). It needs to be taken out and moved in manually after loading of the tips.
- Click “OK” to continue.

e) Upon the message of the software “Please check the ethanol trough!” check whether the required volume of 70% EtOH was added to the trough. If not, fill it up to the required volume.

f) Load output plates with lids (no barcodes): Full skirted PCR plates used for the first elution. Click “Next” to continue. Note: Final elution plates with barcodes will be placed into the instrument during the RNA extraction procedure.

- Load Micronic sample racks with corresponding barcodes. Make sure, sample tubes are decapped. Click “Next” to continue.
- Start the run by pressing “Ok” when ready to start the method.

2.5. During the RNA extraction run by Hamilton ML Star robot

- After samples have been transferred into the DWP plate sample racks can be removed from the unloaded carrier. Important: Leave the carrier unloaded throughout the method. Close sample tubes and store them at 4°C.
- During incubations do not press “Stop Timers”, otherwise the current step will be stopped and the next step in the protocol is initiated.
- Before the elution step the lid needs to be removed from the water trough on the defined position. A dialog window will open to request the lid removal. As long as the dialog window is open, the method is paused. Press “Ok” to continue.
- Remove all the DWP from the magnet positions and press “Ok” to continue.
- Load final (second) elution PCR plates with corresponding barcodes on the defined position on the unloaded carrier. Press “Next” to continue.
- Reload tips (load more 1000 µl Tips onto the deck). Reload 1000µl noFilter Tips in the first column tips carrier (left, position 1-4). Click “Ok” to continue

2.6. Precautions

- Do not empty the tip waste during a run.

- Do not leave tips on the pipetting channels for a long period of time (e.g. overnight). This may cause damage to the CO-RE O-rings. A daily maintenance procedure will eject the tips.
- For samples with highly viscous saliva add 40 mM DTT (40 µl of 1 M stock per 1 ml sample).

Colorimetric RT-LAMP assay using N2 primers

Standard Operating Procedure 2 (SOP2, version 4, 18.12.20)

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References:

https://doi.org/10.1126/scitranslmed.abc7075
https://doi.org/10.3390/v12080863

1. Material and equipment

Reagents	Company	Material #
Guanidine hydrochloride (GuHCl)	Sigma	G3272
Nuclease-free water	Invitrogen	AM9938
WarmStart Colorimetric LAMP 2x Master Mix	NEB	M1800
Primers (desalted, in water, 500 µM)	Sigma	

Consumables	Company	Material #
96-well skirted PCR plate	4titute	4ti-0960/C
Pipette Tips LQR LTS 20µL F 960/10 (4 needed for 96 samples)	Mettler Toledo	17010646
Pipette Tips 125µL	Integra	3428
Microseal® 'B' seal, adhesive	BioRad	SB1001
Foli Heat Sea, aluminium	4titude	4ti-0536
384-well skirted PCR plate		

Equipment	Company	Material #
Liquidator 96-20 Manual Pipetting System	Mettler Toledo	17014207
12 channel pipette VIAFLO 5-125µL	Integra	4632
Tecan M1000	Tecan	
384-well cooling block	Integra Biosciences	
$4s^2$ automated heat sealer	4titude	
Prime Thermal Cycler, 384-well	Techne	5PRIME / 384
neoLabline plate centrifuge	neoLab	D-6081

2. LAMP primer

Primer name	100X stock [µM]	1X final [µM]	Volume of 500 µM primer stock for 100 µl of a 100X LAMP primer mix [µl]
FIP	160	1.6	32
BIP	160	1.6	32
F3	20	0.2	4
B3	20	0.2	4
LOOP F (or LF)	40	0.4	8
LOOP B (or LB)	40	0.4	8
Water			12

Target	Primer name	5'→3' primer sequence
Internal control (IC)	IC-F3 2-5	AATGAGTGACCTGGCTAAGC
	IC-B3 2-5	AAAAGACACGAGGCCAAGG
	IC-FIP 2-5	CAGCGCCAGATGAGCTACATCTTTTTGATGGGTGCCACCTTC
	IC-BIP 2-5	AGCGGCATTCAAAGTCAGACCATTTCACGGGGTGTCCAATT
	IC-LF 2-5	CCTCCAGTGTTCATTTCCGC
	IC-LB 2-5	GCGTTGCTGGTATCTTTCATCTT
SARS-CoV-2 N2	N2-F3	ACCAGGAACTAATCAGACAAG
	N2-B3	GACTTGATCTTTGAAATTTGGATCT
	N2-FIP	TTCCGAAGAACGCTGAAGCGGAACTGATTACAAACATTGGCC
	N2-BIP	CGCATTGGCATGGAAGTCACAATTTGATGGCACCTGTGTA
	N2-LF	GGGGGCAAATTGTGCAATTTG
	N2-LB	CTTCGGGAACGTGGTTGACC

3. Colorimetric RT-LAMP assay for 96 samples (one plate)

LAMP assays are conducted in clear 384-well PCR plates. Each plate receives an individual plate designation in the following format VT-XXXX, where XXXX represents continuous numeration starting at 0001. Each sample is analysed in three replicates for SARS-CoV-2 detection using the N2 primers, and one replicate for the internal control (IC) for RNA extraction.

3.1. Preparation of RT-LAMP reactions

- Turn on the 4S[2] heat sealer, PRIME thermal cycler and Tecan infinite M1000. Turn on the computer next to the Tecan infinite M1000, open the i-control software connected to the Tecan infinite M1000 and load the method 'LAMP_PhenolRed'.
- Prepare the master mixes for a 96-well plate at room temperature no longer than 15 min before usage. For more or less than one plate, adjust the volume accordingly rounding up in steps of 0.5 plates.
- Per 96 samples, the master mix for SARS-CoV-2 detection is prepared 312X (96 samples in three replicates plus some excess). 100X N2 LAMP primer mix is used in this mastermix.
- Per 96 samples, the master mix for detection of the internal RNA extraction control (IC) is prepared 104X (96 samples in one replicate plus some excess). 100X IC LAMP primer mix is used in this mastermix.

Component	**One LAMP reaction (1X) [µl]**	**104X (IC primer) [µl]**	**312X (N2 primer) [µl]**
WarmStart Colorimetric LAMP 2x Master Mix (NEB)	6.25	650	1950
100X LAMP primer mix (N2/ IC)	0.125	13	39
1 M GuHCl (pH 8-9)	0.5	52	156
Water	1.458	151.7	455
total	**8.333**	**866.6**	**2600**

- From a reservoir, distribute 25 µl N2-master mix into every second row and column of the 384-well plate, starting at A1.
- From a second reservoir, distribute 8.33 µl IC-master mix into every second row and column of the 384-well plate, starting at B2. The 384-well plate is now loaded as shown in the following for the first wells of the plate:

Layout	**1**	**2**
A	25µl N2	empty
B	empty	8.33µl IC

- Briefly centrifuge the plates to remove air pockets at the bottom of the wells.
- Using the liquidator with 20 µl tips add 4.17 µl RNA into all wells of the section B2 and mix by pipetting up and down five times. If one or more samples contain residual magnetic beads after RNA extraction (slightly brown-reddish colour), place the RNA plate on a 96-well magnet stand for at least 2 min prior to pipetting.
- Add 12.5 µl RNA to the wells of section A1, mix by pipetting up and down 10 times and transfer 12.5 µl to sections A2 and B1, respectively. Avoid formation of bubbles by fully submerging the tips and not emptying the full volume when mixing. Do not use the second blow out point.
- Attach a barcode label on the skirt of each plate, facing left. Make sure the barcode of the RT-LAMP plates (format VT-XXXX) corresponds to the barcode on the respective RNA plate (format VT-XXXXr).

- Re-seal the RNA plates with an aluminium foil heat seal, using the 4S^2 heat sealer at 170 °C for 2 s, and store them at -20°C. Seal the 384-well RT-LAMP plates with adhesive optical seal.
- Centrifuge the plates in the neoLab plate centrifuge at 450xg for 30 s.

3.2. Measurement of RT-LAMP reactions

- Place the plates in 384-well cooling blocks on top of a 4°C cool pack for 1 min.
- Measure the absorbance of each well at time point 0 min with the Tecan plate reader using the following settings:
  - Absorbance: 434 nm and 560 nm
  - Flashes: 25
  - Settle time: 0 ms
- Incubate the plate in a 384-well PRIME thermal cycler at 65 °C. Remove it from the thermal cycler for absorbance measurements at the following time points: 10, 15, 20, 25, 30 min.
- Place the plates in 384-well cooling blocks on top of a 4°C cool pack for 1 min prior to absorbance measurement. Read the absorbance of each plate at the Tecan infinite M1000 using the settings above.
- Then place the RT-LAMP plates back into a thermal cycler at 65°C until the next time of measurement.
- Absorbance measurements are saved in an Excel sheet with individual tabs for each time point. Enter the respective time point in the field immediately below the barcode.
- After the 30 min measurement place the plate inside a zip-lock bag and freeze at -20°C.
- Save the Excel file in the directory ‘F:\Heibox\Seafile\VT-plates B-FAST\VT-XXXX’ (shortcut is present on the desktop) inside the folder matching to the barcode on the plate (File name: VT-XXXX_LAMP).