Standard Operation Procedure (SOP) for RNA isolation from saliva samples for RT-LAMP detection of SARS-CoV-2 RNA

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This procedure describes the heat inactivation of saliva or gargling fluid samples (to kill the virus for safe handling of the samples) and the isolation of RNA for nucleic acid detection in saliva or gargling fluid samples

Heat inactivation of test samples

Equipment	Company	Material #
Stirrer	various	-
2 l plastic beaker	various	-
Magnetic stirring bar	various	-
Kettle	Severin	WK 3485
Timer	various	-
Thermometer	various	-
Rack for 96 tubes (8x12 layout)	various	-
Table top centrifuge	various	-
96 position racks for 2 ml tubes http://www.kryobox.de/shpSR.php?A=10596&sf=	National Lab	LCRKR96

- place a 2 l beaker containing a magnetic stirring bar onto a stirrer (no heating!)
- Fill the kettle with 1.7 l deionized water
- wear gloves
- place all sample tubes from collection box into the 2 l beaker (check that all tubes are closed). Incubate not more than 94 samples at once.
- Always change gloves after touching samples that are not heat-inactivated before touching anything else
- Boil the water
- Add the boiling water immediately into the beaker
- Stir the samples for 5 min. Make sure that all tubes get immersed.
- Measure the temperature after 5 min (should be >80 °C)
- Spin samples 5 min at 13200 rpm in a table top centrifuge
- place samples into a 96 well rack
- work with samples under safety hood

RNA extraction using magnetic beads

based on: SARS-CoV-2 RNA extraction using magnetic beads for rapid large-scale testing by RT-qPCR and RT-LAMP

Reagent	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 #
Masterblock, 96 well, PP, 0.5 ml, V-bottom (3 plates needed for 96 samples)	Greiner	786261
Masterblock, 96 well, PP, 1 ml, U-bottom (1 plate needed for 96 samples)	Greiner	780261
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 #
Magnet Plate for 96-well deep well plates	Alpaqua	A000400
(Alternative Magnet Plate for 96-well deep well plates->less strong, but works as well)	Magtivio	MDMG0013
Liquidator 96 Manual Pipetting System for 200ul pipet tips)	Mettler Toledo	17010335

1 ml Pipette	various	-
200 µl Pipette	various	-
Multi-stepper pipette	Gilson	
Multi-stepper pipette Multipette® M4	Eppendorf	4982000012
VOYAGER electronic pipette, 6-chanel, 50 - 1250µl,	Integra	4764
Plate shaker	Heidolph	?
Table top centrifuge	Various	-
Heat block	various	-

Prepare stock solutions (prepared in advance)

- 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

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

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

Preparation of working solutions

- Clean surfaces and equipment with RNaseZAP and DNA Away
- Complete GITC lysis buffers for 1x 96-well plate
  - Just before RNA extraction, prepare complete GITC lysis buffer (4.8 M GITC final conc.)
  - Mix the following components
    - 14.40 ml GITC lysis buffer stock
    - 60 µl Glycogen (20 µg/ml final conc.)
    - 600 µl DTT (1M stock) (40 mM final conc.)

- SiMAG-N-DNA beads dilution for 1x 96-well plate
  - Just before RNA extraction, prepare magnetic bead dilution

- 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 (three times)
- 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.
    - 19 ml Ethanol abs.
    - 1 ml SiMAG-N-DNA (5 µg/µl final conc.)
    - Vortex dilution well before use

Preparation of plates

- Prefill 1 Deep Well plate with 70% Ethanol
    - Mix 63 ml of Ethanol abs. and 27 ml of DEPC-treated water
    - Multi-stepper pipette: Fill 900 µl into each well of a 1 ml-well 96 deep well plate
    - Cover plate with lid

- Prefill 1 Deep well plate with water
    - Water, RNase free, non DECP treated (30 ml)
    - Multi-stepper pipette: Fill 300 µl into each well of a 0.5 ml-well 96 deep well plate
    - Cover plate with lid

- Prefill 1 Deep well plate with lysis solution
    - Multi-stepper pipette: Prefill lysis plate with GITC lysis buffer: Add 140 µl freshly prepared complete GITC lysis buffer to each well of a 0.5 ml-well 96 deep well plate
    - Cover plate with lid

RNA extraction procedure

Sample transfer to 96 well plates and lysis of saliva

- Take plate containing lysis solution and saliva samples to a safety hood and continue to work in the safety hood
- Using Integra 6-channel Pipet: Transfer 140 µl heat inactivated sample from the 2 ml tubes with heat inactivated saliva contained in the 96-position rack into each well at the corresponding position of the 96 deep well plate
- Mix by pipetting up and down 8 times
- If a sample is too viscous and cannot be pipetted (usually 1-2 per 96 well plate): remove these tubes and finish the transfer of the other samples into the deep well plate.

        - Samples with highly viscous saliva: add 40 mM DTT (40 µl of 1 M stock per 1 ml sample) to the sample; incubate 5 min at 95 °C in a heat block, spin 5 min at 13 200 rpm in a table top centrifuge and then add 140 µl of the liquified saliva into the corresponding position.
    - Disinfect the safety hood after use
    - Freeze the rack with heat inactivated saliva samples in 96 well rack at -20°.
    - Incubate deep well plate with saliva sample in lysis solution for 10 min at room temperature

RNA binding to magnetic beads

- Vortex again the 20 ml freshly prepared magnetic bead solution
- Multi-stepper pipette: Add 200 µl of magnetic beads into each well of the new 0.5 ml-well deep well plate (make sure that the beads do not settle in multi-stepper pipette during pipetting)
- Using the Liquidator (**fresh tips**): Add the lysed samples (280 µl per well) to the plate containing the beads and resuspend settled beads using Liquidator (15x)
- Seal deep well plate with sticky aluminum foil
- Incubate deep well plate on a shaker for 8 min at ~650 rpm (makes sure that the liquid does not touch the foil
- Open the deep well plate carefully
- Using the Liquidator (same tips): Resuspend settled beads (20x)
- Seal the 96 well plate with sticky aluminum foil
- Incubate for an additional 7 min on a shaker at ~650 rpm (make sure that the liquid does not touch the foil
- Place the deep-well plate on a magnet plate until the supernatant is clear but at least for 5 min
- Clearly visible ring pellets should be formed in each well

Ethanol washes (3x)

- Carefully remove aluminum foil
- Using the Liquidator (same tips): Remove supernatant. Discard tips.
- Remove the deep-well plate from the magnet plate
- Using the Liquidator (**fresh tips**): Add 200 µl 70% Ethanol to each well and resuspend (20x) settled beads
- Check that a brownish suspension is formed
- Place deep-well on magnet plate
- Incubate until ring pellets are formed (ring pellets will form quickly at this step, ca. 1 min)
- Perform this washing step three times
- Using Liquidator (same tips): Remove supernatant from last EtOH was step. Discard tips.
- Do not let plate dry! And proceed immediately!

One quick water wash step and RNA elution

- Using Liquidator (**fresh tips**): Fill up pipet tips with 100 ul of RNAse free water and move Tips close to the bottom of the wells in the center of the ring pellets and pipet in and out the water to rinse the ring pellets while keeping the deep-well plate on the magnet plate. Do not incubate – just a slow rinsing. Discard the water
- Using Liquidator (same tips): add 60 µl of new RNase-free water to each well using the Liquidator
- Remove the plate from the magnet plate
- Using Liquidator (same tips): Resuspend settled beads using the Liquidator (20x)
- Inspect the plate: all pellets should be resuspended, if not use a 200 µl pipet to resuspend the corresponding pellets individually
- Seal deep-well plate with sticky aluminum foil
- Place the deep-well plate on a shaker 15 min
- Place deep-well on magnet plate
- Incubate until ring pellets are formed (ring pellets will form quickly at this step, ca. 1 min)
- Using Liquidator (same tips): Transfer 55 µl of the eluate to new 96 well PCR plate
- Place the new 96 well PCR plate on the magnet plate
- Incubate until ring pellets are formed (ring pellets will form quickly at this step, ca. 1 min)
- Using Liquidator (same tips): To remove any residual beads, transfer 50 µl of the eluate to new 96 well PCR plate (Eppendorf) using Liquidator
- Seal plate with sticky aluminum foil
- Place on ice
- Use directly for LAMP or freeze at -80 °C
- Disinfect surfaces and equipment after use