Rapid, specific and sensitive visual detection of SARS-CoV-2 (COVID-19) virus RNA using colorimetric duplex loop-mediated isothermal amplification (LAMP)

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The provided protocol corresponds to the version valid on June, 2020 and may be subject to changes without notice.

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Rapid, specific and sensitive visual detection of SARS-CoV-2 (COVID-19) virus RNA using colorimetric duplex loop-mediated isothermal amplification (LAMP)

Keywords : SARS-CoV-2, COVID-19, diagnostic, RNA, LAMP, RT-qPCR, gene N

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LAMP is an isothermal nucleic acid amplification technique that allows to detect DNA or RNA molecules of specific sequence such as viral genomes. The reaction is carried at a constant temperature, and does not require expensive thermocyclers used in (q)PCR. It is thus a useful method to perform infectious disease diagnosis at point of care in low- and middle-income countries.

The target sequence is amplified using 2 or more sets of 4 or 6 oligonucleotidic primers each and a polymerase with high strand displacement activity.

The LAMP principle is described here:

https://www.youtube.com/watch?v=L5zi2P4lggw

https://www.youtube.com/watch?v=V4PyySdk3Jo

LAMP produce amounts of DNA considerably higher than PCR. The amplification product can be detected via different method. The protocol described here is based on a commercial assay (New England Biolabs) and allows visual detection of amplification using pH-sensitive indicator dyes to detect pH change resulting from DNA polymerization.

The protocol was tested on 182 clinical samples which were previously analyzed by conventional RT-qPCR. The duplex LAMP assay detected 100% of the positive samples with CT≤ 35 (N=110) in RT-qPCR ; 72 % of samples with 35<CT<37 (N= 18) and a minority of samples with 37<CT<40. The sensitivity was 100% (no false positive among the 27 negative tested samples).

1. Equipment

- Vortex apparatus (only for RNA extraction)
- High speed 1,5ml tube centrifuge (only for RNA extraction)
- Thermostatic oven (preferred) or water bath or dry bath

2. Vessels

- 1.5 tubes for RNA extraction
- 96 well sealable microwell plates and sealing tape
  (alternatively: 1.5ml or 0.5 ml or 0.2 ml tubes) for LAMP
  NB : microwell plates are preferred to tubes because :
  - easy management of a large number of samples.
  - easy record of the visual inspection readout (wells are numbered and a single picture of the plate)
  - possibly more robust results (probably because of reduced volume resulting in less evaporation)

3. Reagents for RNA extraction

- High speed 1,5ml tube centrifuge
- TRI-Reagent from Sigma-Aldrich or TRIzol from Invitrogen or QIAzol from QIAGEN
- Chloroform (Sigma-Aldrich)
- GlycoBlue (Thermofisher Scientifc) (or Glycogen or yeast tRNA or any other RNase free ballast for ethanol co-precipitation to increases visibility of the RNA pellet)
- Isopropanol (Sigma-Aldrich)
- Ethanol 75% (Sigma-Aldrich). In a falcon 50ml tube, add 12,5 ml of RNAse free water to 37,5ml of ethanol 96-100% (Sigma-Aldrich).
- RNAse free water

4. Reagents for Colorimetric LAMP

- WarmStart® Colorimetric LAMP 2X Master Mix New England Biolabs cat # M1800
- Two mix of 6 primers (synthetic oligonucleotides) each (10 x)

Primers should be HPLC purified (at least FIP and BIP primers)

"GeneN_B" set of primers	
GeneN-B-F3	5' ACCGAAGAGCTACCAGACG
GeneN-B-B3	5' TGCAGCATTGTTAGCAGGAT
GeneN-B-FIP	5' TCTGGCCCAGTTCCTAGGTAGTTCGTGGTGGTGACGGTAA
GeneN-B-BIP	5' AGACGGCATCATATGGGTTGCACGGGTGCCAATGTGATCT
GeneN-B-LF	5' CCATCTTGGACTGAGATCTTTCATT
GeneN-B-LB	5' ACTGAGGGAGCCTTGAATACA

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"GeneN_ID21" set of primers	
F3_ID21	5' GCCAAAAGGCTTCTACGCA
B3_ID21	5' TTGCTCTCAAGCTGGTTCAA
FIP_ID21	5' TCCCCTACTGCTGCCTGGAGGCAGTCAAGCCTCTTCTCG
BIP_ID21	5' TCTCCTGCTAGAATGGCTGGCATCTGTCAAGCAGCAGCAAAG
LF_ID21	5' TGTTGCGACTACGTGATGAGGA
LB _ID21	5' ATGGCGGTGATGCTGCTCT

Prepare the two 10X LAMP Primer Mixtures (one with primers « GeneN-B », the other with primers « ID21 »). Alternatively, a single mixture of all 12 primers can be used.

10X LAMP Primer Mix
FIP 16 µM (1x final concentration in LAMP reaction = 1.6 µM)
BIP 16 µM (1x final concentration in LAMP reaction = 1.6 µM)
F3 2 µM (1x final concentration in LAMP reaction = 0.2 µM)
B3 2 µM (1x final concentration in LAMP reaction = 0.2 µM)
LF 4 µM (1x final concentration in LAMP reaction = 0.4 µM)
LB 4 µM (1x final concentration in LAMP reaction = 0.4 µM)

5. Inactivation of viral infectivity and RNA extraction protocol

Inactivation of viral infectivity and RNA extraction according to Coupeau et al. (https://www.narilis.be/sana/2020_unamur_sars-cov-2_rtqpcr).
Other RNA extraction protocols can be used.

This step and the preparation of LAMP amplification should be performed in a "preLAMP" area to avoid contamination.

RNA is extracted from 100 µl of swab transport medium using TRI Reagent from Sigma-Aldrich or TRIzol from Invitrogen or QIAzol from QIAGEN and dissolved in 30 µl of RNase free water. Store at -20°C (or -80°C if available).

Clinical specimen processing will be performed in a class II biological safety cabinet by experienced researcher wearing appropriate protective equipment (gowns and gloves).

From now, the whole process is carried out using filtered tips.

- Spin the collection tube 200 g, 1min, to make sure the sample is at the bottom of the tube. Use a centrifuge with sealed buckets.
- Clean the centrifuge
- Transfer 100 µl fraction of the clinical sample in a 1,5 ml tube containing 1.0 ml of TRI Reagent.
- Mix gently by inverting the tube. This procedure inactivates sample upon contact with TRI Reagent.
- Incubate 5 min at room temperature. If necessary, inactivated samples can be stored at -20 °C (or -80 °C) for further processing.

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- Add 200 µl of chloroform and vortex for 10 sec
- Incubate for 5 min at room temperature
- Centrifuge at 12,000 g for 10 min (preferably at 4 °C)
- Transfer 500 µl of the colorless upper aqueous phase (containing the RNA) in 1.5 ml tube containing 3 µl of Glycoblue (co-precipitant to increases visibility of the RNA pellet)
- Importantly avoid any contact with the ring or the lower organic phase (pink)
- Important note: the organic and aqueous phase can be inverted, i.e. the organic pink phase can be above the clear aqueous phase. In this case, add 100ul of RNAse free water to the sample, vortex and centrifuge at 12,000 g for 10 min preferably at 4 °C)
- Add 500 µl of isopropanol and vortex for 3 sec
- Incubate for 5 min at room temperature
- Centrifuge at 12,000 to 14,000 g for 10 min preferably at 4 °C)
- The RNA pellet forms a blue pellet on the bottom of the tube
- Discard the supernatant
- Add 900 µl of 75 % ethanol
- Gently mix by inverting the tubes (avoid to detach the pellet)
- Centrifuge for 5 min at 12,000 to 14,000 g preferably at 4 °C)
- Aspirate slowly the supernatant with a 1 ml pipette by avoiding contact with the blue pellet.
- Centrifuge briefly again.
- Use a narrow tip to remove residual ethanol
- To dry the pellet, leave the tube open under the chemical hood until complete ethanol evaporation. This step lasts about 5 min. Do not over dry the RNA by letting the sample dry more than 10 min.
- Dissolve the pellet in 30 µl of RNAse free water
- Incubate at room temperature until complete dissolution of the blue pellet
- If necessary, RNA can be stored at -80°C for further processing

6. Colorimetric LAMP protocol

Prepare on ice (if available) a mixture (scale up) for the desired number of samples plus positive (positive RNA or synthetic DNA or RNA) and negative controls (negative sample and H_2O).

Per sample:

Colorimetric LAMP 2X Master Mix (must be pink when thawed)	12.5 µl
10 X Primer Mix GeneN_B	2.5 µl
10 X Primer Mix GeneN_ID21	2.5 µl
RNAse-free H_2O	3.5 µl

- Dispense 21 µl in wells (or tubes)
- Put on ice (if available)
- Add 4 µl of RNA sample, mix carefully by up and down pipetting
- Seal the plate with plastic film
- Incubate at 65°C for 35 minutes.

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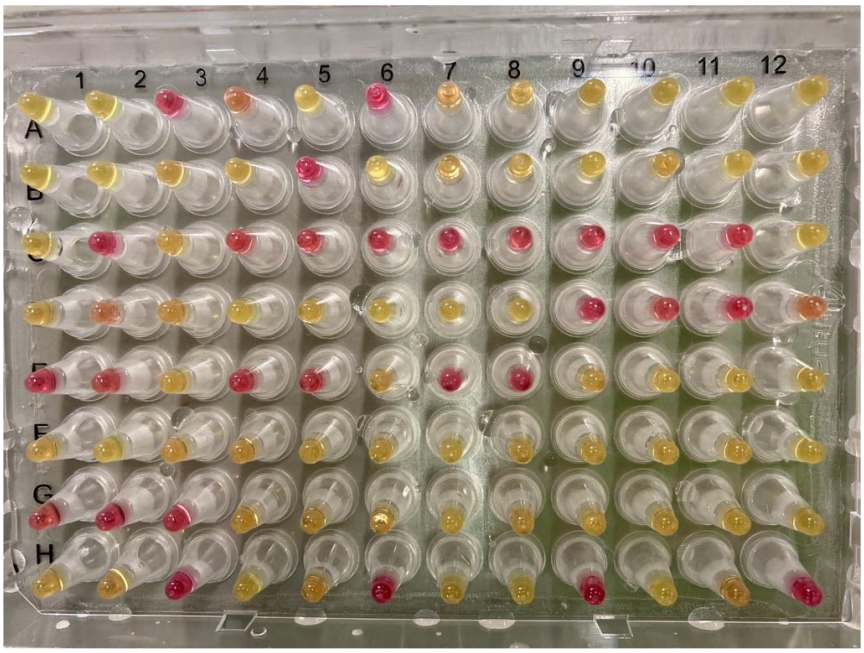
We incubate the plate in a home-made small sandbox placed in an air incubator. Check that the sand is at 65°C.

Sandbox in air incubator.

- Inspect visually the plate.
  Positive controls should be yellow, negative controls should be pink. Orange is also to be considered as positive.
- Take a picture of the plate upside down.
  See below for an example.
  The plate can be frozen and snapped later.
  <u>Do not open the plate to avoid contamination</u>.



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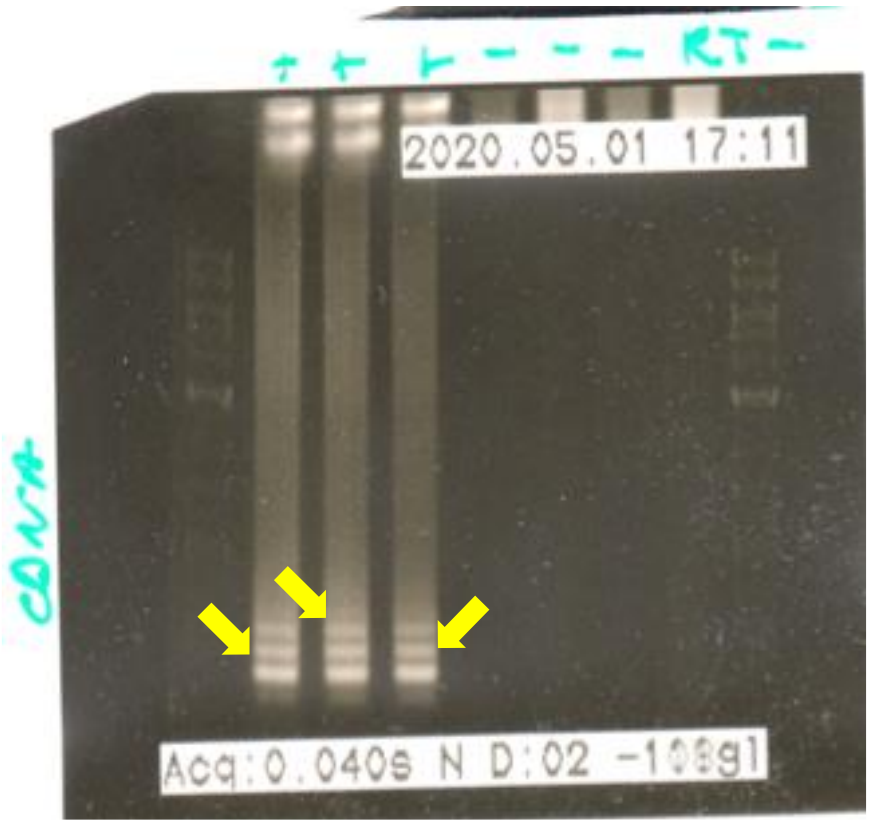
Electrophoresis readout (optional)

Run 15 µl of the reaction mixture on a 0.7% agarose gel containing ethidium bromide. Positive sample should display amplification products including discrete bands corresponding to the “amplicon” and concatemers of the amplicon (see arrows on the picture).



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Gene N sequence showing the location of F3 (sense, left) and B3 (antisense, right) primers as follows:

Gene N-B
ID21

ATGTCTGATAATGGACCCCAAAATCAGCGAAATGCACCCCGCATTACGTTTGGTGGACCCTCAGATTC
AACTGGCAGTAACCAGAATGGAGAACGCAGTGGGGCGCGATCAAAACAACGTCGGCCCCAAGGTTT
ACCCAATAATACTGCGTCTTGGTTCACCGCTCTCACTCAACATGGCAAGGAAGACCTTAAATTCCCTC
GAGGACAAGGCGTTCCAATTAACACCAATAGCAGTCCAGATGACCAAATTGGCTACT**ACCGAAGAG**
CTACCAGACGAATTCGTGGTGGTGACGGTAAAATGAAAGATCTCAGTCCAAGATGGTATTTCTACTA
CCTAGGAACTGGGCCAGAAGCTGGACTTCCCTATGGTGCTAACAAAGACGGCATCATATGGGTTGCA
ACTGAGGGAGCCTTGAATACACCAAAAGATCACATTGGCACCCGCA**ATCCTGCTAACAATGCTGCA**A
TCGTGCTACAACTTCCTCAAGGAACAACATT**GCCAAAAGGCTTCTACGCA**GAAGGGAGCAGAGGCG
GCAGTCAAGCCTCTTCTCGTTCCTCATCACGTAGTCGCAACAGTTCAAGAAATTCAACTCCAGGCAGC
AGTAGGGGAACTTCTCCTGCTAGAATGGCTGGCAATGGCGGTGATGCTGCTCTTGCTTTGCTGCTGC
TTGACAGA**TTGAACCAGCTTGAGAGCAA**AATGTCTGGTAAAGGCCAACAACAACAAGGCCAAACTG
TCACTAAGAAATCTGCTGCTGAGGCTTCTAAGAAGCCTCGGCAAAAACGTACTGCCACTAAAGCATA
CAATGTAACACAAGCTTTCGGCAGACGTGGTCCAGAACAAACCCAAGGAAATTTTGGGGACCAGGA
ACTAATCAGACAAGGAACTGATTACAAACATTGGCCGCAAATTGCACAATTTGCCCCCAGCGCTTCA
GCGTTCTTCGGAATGTCGCGCATTGGCATGGAAGTCACACCTTCGGGAACGTGGTTGACCTACACAG
GTGCCATCAAATTGGATGACAAAGATCCAAATTTCAAAGATCAAGTCATTTTGCTGAATAAGCATATT
GACGCATACAAAACATTCCCACCAACAGAGCCTAAAAAGGACAAAAAGAAGAAGGCTGATGAAACT
CAAGCCTTACCGCAGAGACAGAAGAAACAGCAAACTGTGACTCTTCTTCCTGCTGCAGATTTGGATG
ATTTCTCCAAACAATTGCAACAATCCATGAGCAGTGCTGACTCAACTCAGGCCTAA

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