beadLAMP for SARS-CoV-2 detection using QuickExtract crude lysate (for hospital)

Introduction

This protocol describes the sensitive detection of SARS-CoV-2 using beadLAMP, a combination of *Reverse Transcription Loop-mediated Amplification* (RT-LAMP) with an upfront magnetic bead-based RNA enrichment step from crude patient samples inactivated in QuickExtract DNA solution. The protocol was established at the Vienna Bio Center as part of the Vienna Covid19 Detection Initiative (VCDI). Detailed findings covering the method establishment and experimental validation can be found in our pre-print '**A rapid, highly sensitive and open-access SARS-CoV-2 detection assay for laboratory and home testing**' (https://www.biorxiv.org/content/10.1101/2020.06.23.166397v2)

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Several key considerations before performing this protocol:
A) SARS-CoV-2 is a highly contagious virus and requires personal protection with appropriate safety standards. Users must follow Biosafety level guidelines outline by the CDC https://www.cdc.gov/coronavirus/2019-ncov/lab/lab-biosafety-guidelines.html

B) Seperate working areas for sample preperation, reaction set-up and analysis are highly recommended. Cleaning work surfaces and pipettes with 5-10% Bleach solution (Caution)

C) Do not open RT-LAMP reactions in order to prevent carry-over cross contamination leading to false positives. The colorimetric master mix from NEB (M1804L) already contains dUTP and UDG to prevent cross-contamination and lowers the risk of reaction contamination.

D) 96-well plates must be properly sealed. This is absolutely crucial to prevent cross-well contamination. Proper plate seals (e.g. aluminium seals), a proper plate-roller (e.g. BioRad) or even better a PCR plate sealer (e.g. BioRad PX1 PCR plate sealer) can help prevent cross-well contamination.

E) Store reaction components at the appropiate temperatures outline by the manufacturer. When thawing the NEB WarmStart LAMP kit, we advise thawing at room temperature for 10 minutes with vortex mixing for 5 seconds every 5 minutes. This ensures that any Magnesium sulfate precipiate goes into solution, which is essential for proper HNB colorimetric LAMP detection and proper performance. Check Mastermix after thawing to ensure no precipiate is visible.

F) Colorimetric and fluorescent detection can be performed in the same reaction by simple addition of both dyes (the colorimetric kit from NEB already contains PhenolRed for colorimetric read-out). We found that 1-2 µM SYTO-9 fluorescent dye to be comparable to the commercial fluorescent dye sold with NEB's WarmStart LAMP Kit. Alternative fluorescent dyes are SYTO-82. Do not use SYBR I or Eva Green as those were shown to inhibit LAMP reactions.

G) When working with crude samples lysed in QuickExtract, keep samples on ice whenever possible. While we have observed complete inactivation of nucleases after 5 minutes at 95°C, we found considerable RNase activity in biological sample types such as saliva and gargle. Due to the FBS content in Universal Transport Media (UTM), nasopharyngeal swabs in this medium may also contain RNases.

H) RT-LAMP uses 6 primers, two inner loop primers (FIP, BIP), loop primers (LF, LB) and outer bumping primers (F3, B3). A reaction contains all six primers if a single amplicon is tested. It is possible to add primers for 2 amplicons (12 primers) in a single reaction - in this case GuHCl needs to be added. Testing for 2 amplicons increases robustness and also slightly sensitivity of the reaction.

I) Primer choice:
We recommend As1 (ORF1ab) as the 'safest amplicon' particularly for hospital settings since
- it does not overlap a CDC amplicon used in the Roche Cobas assays
- it has performed extremely well in our tests regarding false-positives (no false positives)
- BUT: it is not the most sensitive primer combi we have for LAMP

The most sensitive option for beadLAMP with the colorimetric (PhenolRed) kit and Guanidinium-HCl is the double-combination of As1 and E1 primers.
Disadvantage: The E1 amplicon overlaps the RT-qPCR Cobas E gene assay, thus there is a risk of cross-contamination;

Primer sequences are
As1_F3
CGGTGGACAAATTGTCAC
As1_B3
CTTCTCTGGATTTAACACACTT
As1_LF
TTACAAGCTTAAAGAATGTCTGAACACT
As1_LB
TTGAATTTAGGTGAAACATTTGTCACG
As1_FIP
TCAGCACACAAAGCCAAAAATTTATCTGTGCAAAGGAAATTAAGGAG
As1_BIP
TATTGGTGGAGCTAAACTTAAAGCCCTGTACAATCCCTTTGAGTG

E1-F3
TGAGTACGAACTTATGTACTCAT
E1-B3
TTCAGATTTTTAACACGAGAGT
E1-FIP
ACCACGAAAGCAAGAAAAAGAAGTTCGTTTCGGAAGAGACAG
E1-BIP
TTGCTAGTTACACTAGCCATCCTTAGGTTTTACAAGACTCACGT
E1-LB
GCGCTTCGATTGTGTGCGT
E1-LF
CGCTATTAACTATTAACG

Materials

› Materials

- QuickExtract DNA solution 50ml (Lucigen QE09050), store at -20°C
- AmpureXP RNA beads (A63987), store at 4°C
- Bead dilution buffer (2.5 M NaCl, 10 mM Tris-HCl pH 8.0, 20% (w/v) PEG 8000, 0.05% Tween 20, 5 mM NaN3) **(CAUTION with NaN3 read MDS)**, store at 4°C
- WarmStart® Colorimetric LAMP 2X Master Mix with UDG (NEB M1804L), store at -20°C
- SYTO™ 9 Green Fluorescent Nucleic Acid Stain, 5mM (2500x to 5000x), (Thermofisher S34854), store at -20°C under light protected conditions
- Primer sets E1 (SARS-CoV-2 E-gene), As1 Rabe&Cepko (SARS-CoV-2 Orf1ab-gene), sequences are outlined above, store at -20°C

- HBSS - Hank's Balanced Salt Solution without Phenol (Thermofisher 14025092)
- Nuclease free water (not DEPC-treated) 100ml, (Thermofisher AM9938)
- Guanidine hydrochloride solution, 8M (Sigma, 50937-100ML) (only needed if performing double-amplicon reactions with As1 and E1)
- Magnet for seperation of magnetic beads in PCR plates (96-well) or PCR tubes such as DynaMag (Thermofisher 492025, or NEB NEBNext® Magnetic Separation Rack S1515S)
- 1.5 ml Eppendorf DNA low-bind tubes or equivalent product free from RNases and DNases
- DNase/RNase free PCR-strips or 96-well PCR plates with appropriate seal
- Plate roller (e.g. BioRad MSR0001) or PCR plate sealer (e.g. BioRad PX1 PCR plate sealer 1814000) to ensure proper sealing of the plates
- 2 heat-blocks (e.g. Eppendorf ThermoStat, with heated lid) or 2 PCR machines
- Optional: Twist Synthetic SARS-CoV-2 RNA Controls Isolate MT007544.1 (Twist Bioscience 102019) for positive control testing, store stock (1e5 copies/µl) or working dilutions (1e3 copies/µl) at -80°C. Dilutions can be temperally stored at -20°C. Avoid multiple freeze and thaw cycler.

Procedure

Preperation of reaction components

1. **10x LAMP Primer mixes**

   Thaw 100µM Primer stock and make 100µl of 10x primer mixes. Larger volumes are made by scaling up volumes of individual components

10x LAMP primer mixes

	A	B	C	D
1	**Reagent**	**amount to add for E1 mix**	**amount to add for As1 mix**	**Comments**
2	F3 (100uM stock)	2	2	2µM at 10x
3	B3 (100uM stock)	2	2	2µM at 10x
4	LF (100uM stock)	4	4	4µM at 10x
5	LB (100uM stock)	4	4	4µM at 10x
6	FIP (100uM stock)	16	16	16µM at 10x
7	BIP (100uM stock)	16	16	16µM at 10x
8	Nuclease free water	56	56	
9	Total (µl)	100	100	

2. **Prepare 50x Syto9 solution in DMSO**

   CRITICAL **Store solution under light protected conditions.**

Syto-9 dye stock

	A	B
1	**Reagent**	**amount to add**
2	Syto9 stain 5mM	2
3	DMSO	198
4	Total (µl)	200

3. **Prepare 1:5 diltion of AmpureXP RNA beads**

   CRITICAL Equilibrate AmpureXP RNA beads and Bead dilution buffer at room temperature for 30 minutes prior to use. We further recommend fresh dilution of beads and to avoid using dilution that have been stored for more than 3 days. The mixture below is sufficient for 100 bead-LAMP reactions using 60µl of diluted bead solution per reaction

AmpureBeads Mix

	A	B
1	**Reagent**	**amount to add**
2	AmpureXP RNA beads (µl)	1200
3	Bead dilution buffer (µl)	4800
4	Total (µl)	6000

Sample inactivation and lysis using Lucigen QuickExtract DNA solution

4. Thaw an aliquot of QuickExtract DNA solution on ice and vortex before use. (50µl per patient)

5. On ice, mix 50µl of original sample material (nasopharyngal swab, gargle or saliva) with 50µl of QuickExtract solution in PCR strips or in a 96-well plate; seal the plate (or close the PCR strips with the lids)

   CRITICAL Potentia

6. Heat-inactivate QuickExtract:Sample mix at 95°C for 5 minutes, then cool on ice or store at -80°C

   PAUSE Sample can be stored at -80°C at this point for up to four weeks, potentially longer

Bead-LAMP

7. Prepare the bead-LAMP reaction mix

   If multiple reactions are performed, we strongly recommend to prepare a Mastermix for all reactions including a 10% excess. For example, when intending to run 10 reactions, prepare a Mastermix for 11 total reactions. This minimises pipetting errors and ensures enough reagent mixture to be distributed over individual PCR tubes or PCR wells.

   CRITICAL **If performing only colorimetric based detection, the fluorescent dye can be replaced with the same volume of nuclease-free water. Syto-9 can be added to record real-time fluorescent kinetic profiles and end-point fluorescence. This is especially useful when setting up the techique and optimising reaction condtions such as primers and sample types.**

MasterMix set-up for single (As1) amplicon

	A	B	C	D
1	**Reagent**	**per 20µl bead-LAMP reaction**	**Mastermix for 10 bead-LAMP reactions (+ 10%)**	
2	WarmStartcolorimetric LAMP Kit w UDG and UTP (2x)	10	110	
3	Primer mix As1 (10x)	2	22	
4	Nuclease free water	7.6	83.6	
5	Syto9 (50µM) (optional)	0.4	4.4	
6	**Total**	**20**	**220**	
7				

For performing bead-LAMP using the double-amplicon combi of As1 and E1 and GuHCl, prepare the following mix:

MasterMix set-up for double (As1+E1) amplicon + GuHCl

	A	B	C	D
1	**Reagent**	**per 20µl bead-LAMP reaction**	**Mastermix for 10 bead-LAMP reactions (+ 10%)**	
2	WarmStart colorimetric LAMP Kit w UDG and UTP(2x)	10	110	
3	Primer mix As1 (10x)	2	22	
4	Primer mix E1 (10x)	2	22	
5	GuHCl (8M)	0.1	1.1	
6	Nuclease free water	5.5	60.5	
7	Syto9 (50µM) (optional)	0.4	4.4	
8	**Total**	**20**	**220**	
9				

8. To 100µl of QuickExtract Lysate in PCR tubes, add 60µl of diuted AmpureXP RNA beads (0.6x of lysate volume), mix and incubate for 5 minutes at room temperature

CRITICAL **If real-time fluorescent signal will me measured, transfer lysate to a PCR plate or PCR tubes suitable for real-time measurement. All downstream enrichment and detection steps are performed on this plate/tube.**

CRITICAL **For volumes smaller than 100µl, it is important to bring the volume up to 100µl with HBSS before addition of 60µl diluted beads. We have successfully used initial lysate volumes of 40-100µl.**

9. After 5 minutes, place mixtures containing samples and beads onto a magnetic rack for 5 minutes to allow for complete seperation of beads from the solution

   CRITICAL **It is important to await complete seperation to avoid loss of beads. This is usually complete after 5 minutes and should be visible by accumlation of beads at the magnet-tube interphase.**

10. Without disturbing the beads, carefully remove the solution while keeping the tubes on the magnetic rack.

    CRITICAL **It's important not to disturb the beads as the nucleic acid material is bound to it and would be lost**

11. While keeping the tubes on the magnetic stand, add 200µl of 85% EtOH solution to the beads and wait for 30 seconds, then remove the wash solution

    CRITICAL **It's important not to disturb the beads**
    It's important to remove any residual Ethanol since this can lead to a color-change of the Phenol dye from pink to yellow (without the reaction taking place)

12. Remove tubes from the magnetic rack and allow the beads to air-dry for 3-5 minutes.

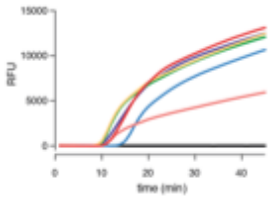
    CRITICAL **Do not exceed 5 minutes to prevent full drying of the beads.**

13. To each well/tube containing beads, add 20µl of LAMP reaction mixture and mix by pipetting to bring the beads into solution.

    CRITICAL Ensure that the beads went into solution. The mix should appear brownish with no transparent phase
    CRITICAL If real-time fluorescence is recorded, transfer reaction to a suitable detection vessel (qPCR plate)

14. Transfer the reaction to a suitable stable heat-source such as a qPCR/PCR thermocycler or heat-block and run reactions at 63°C for 30 minutes.

    For real-time fluorescence data aquisition of beadLAMP in a qPCR thermocycler, we typically perform 30 cycles at 63°C with 1-minute cycle length and reading at the end of each cycle. SYTO-9 or NEB fluorescent Dye require data aquisitons using a standard FAM filter (494nm/518nm absorption/emission).



    After 30 minutes run-time, remove reactions and allow to cool briefly at room temperature. Then proceed to inspect reactions visually.
    Negative reactions are pink (Phenol)
    Positive reactions yellow (Phenol)

15. CRITICAL **Discard reactions. DO NOT open and DO NOT run on a gel**