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LAMP/RT-LAMP COVID positive control

In 1 collection

Anibal Arce Medina¹, Isaac Núñez², Tamara Matute², Fernan Federici²

¹Pontificia Universidad Catolica de Chile; ²SynBioUC

Laboratorio de Tecnologías Libres

Reclone.org (The Reagent Collaboration Network)

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Tamara Matute

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We use this protocol and it's working

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ABSTRACT

This protocol explains how to prepare dsDNA and ssRNA target positive control for LAMP and RT-LAMP reactions. Particularly it describes the pipeline for SARS-CoV-2 Nucleocapsid (N) gene positive control which is included in the ReClone collection. The general procedure involves amplifying the gene of interest with primers which add T7 promoter and T7 terminator to its sequence followed by T7 in vitro transcription to obtain the ssRNA.

Figure 1: General pipeline. It shows how dsDNA is amplified from control plasmid at the time pT7 and tT7 are incorporated to its sequence which is used to perform the ssRNA T7 in vitro transcription.

Sequence elaboration:

Sequence [OrfN_5](#) was designed and synthesized based on IDT SARS-CoV-2 positive control plasmid ([CAT#10006625_2019](#)).

IMAGE ATTRIBUTION

Isaac Núñez. GNU General Public License v3.0.

MATERIALS

Materials and Tools:

- Pipettes p2, p10, p200
- Pipette tips
- PCR tubes (0.2 uL)
- rNTPS

Kits and enzymes:

- [Promega Wizard® SV Gel and PCR Clean-Up System](#)
- [Qiagen RNeasy Kit](#)
- HiScribe™ ([NEB E2040S](#))
- PPI ([NEB M0361S](#))
- RNasin® ([Promega N2111](#))
- DNase I ([NEB M0303S](#))

Equipment:

- Thermocycler
- Centrifuge
- spin
- Nano-Spectrophotometer

N gene Amplification

- 1 Amplify SARS-Cov-2 Nucleocapsid (N) gene fragment dsDNA from ReClone or IDT positive control plasmid with the *ultramers* indicated in Table 1.

Name	Sequence
NT7_Fw	CGA AAT TAA TAC GAC TCA CTA TAG GGG CAA CGC GAT GAC GAT GGA TAG
T7_Nter_Rv	ACT GAT CAA AAA ACC CCT CAA GAC CCG TTT AGA GGC CCC AAG GGG TTA TGC TAG TTA GGC CTG AGT TGA GTC AG

Table 1: Primers/Ultramers used to amplify N-gene positive control

The amplification can be carried out using any standard PCR reaction formulation (e.g. Phusion DNA polymerase with HF buffer), adding 0.01 ng to 1 ng of the plasmid template and 1 µL of each primer at 10 µM concentration and following this thermocycler program:

Initial denaturation at 98 °C for 00:00:30

Followed by **35 cycles** of:

98 °C per 00:00:05

57 °C per 00:00:15

extension at 72 °C per 00:01:00

End by a final extension at 72 °C per 00:05:00

dsDNA positive control

- 2 **dsDNA purification**
Perform PCR reaction purification with any commercially available kit.

We use [Promega Wizard® SV Gel and PCR Clean-Up System](#)

Expected result

Typical yields are around 25 µL at 30 - 100 ng/?L.

- 3 Store sample under -20 °C

Note

In case you are not making RT-LAMP positive control **go to step 10**.

ssRNA positive control

4 In vitro transcription preparation

In vitro transcription has to be done from previously prepared dsDNA. We do it using HiScribe™ ([NEB E2040S](#)) system plus PPI ([NEB M0361S](#)) and RNasin® ([Promega N2111](#)):

Compound	Volume (µL)
rATP	2,0
rUTP	2,0
rGTP	2,0
rCTP	2,0
Buffer 10X	2,0
T7 pol	2,0
RNasin	0,5
PPI (NEB)	0,5
DNA (from PCR)	7,0
Total	20,0

Table 2: *In vitro* transcription formulation.

- 5 Incubate reaction at 37 °C Overnight

- 6 Treat the reaction with DNase I ([NEB M0303S](#)) by diluting the reaction with 70 µL H₂O (molecular grade) and 10 µL 10X buffer DNase I.

7 Incubated at 37 °C per 00:15:00 .

Note

Heat inactivation of DNase is not recommended because it can harm RNA.

8 Perform RNA purification with any available kit. We use [Qiagen RNeasy Kit](#).

Expected result

Typical yields are around 50 µL at 1000ng/?L.

9 Perform aliquots and store the samples at -80 °C

Copy number computation and dilutions

10 In order to compute the sample **copy number concentration**, it's necessary to measure its **mass concentration** in a nano-spectrophotometer, plate reader, or estimate it by gel electrophoresis imaging.

Equipment

NanoDrop™ 3300 Fluorospectrometer	NAME
Fluorospectrometer	TYPE
NanoDrop™ 3300	BRAND
nd-3300	SKU
https://www.thermofisher.com/order/catalog/product/ND-3300#/ND-3300	LINK

Note

Repeat steps 8 to 10 for ssRNA each time you perform experimentation because samples and aliquots concentration is not stable even though keeping them frozen.

11 Compute the sample copy number concentration [copies/?L] by replacing the length [bp] (**Table 3**) and mass concentration [ng/?L] (C_(mass)) in the next mathematical expressions :

- (1.a) $\frac{\text{copies}}{\text{mol}} \text{ d s D N A} = \frac{\text{length} \text{ d s D N A [bp]} * 617.96 \frac{\text{mol}}{\text{bp}} + 36.04 \frac{\text{mol}}{\text{bp}}}{C_{(mass)}}$
- (1.b) $\frac{\text{copies}}{\text{mol}} \text{ ssR N A} = \frac{\text{length} \text{ s s R N A [bp]} * 321.47 \frac{\text{mol}}{\text{bp}} + 18.02 \frac{\text{mol}}{\text{bp}}}{C_{(mass)}}$

(2)
$$\frac{Y}{\text{copies}} \{n\} g = \left(\frac{Z}{\text{mol}} \{g\} \{ \text{ssRNA} \} * \frac{10^9}{n\} g \right)^{-1} * 6,02210^{23} \frac{\text{copies}}{\text{mol}} \{ \text{dsDNA} \}$$

(3)
$$\frac{X}{\text{copies}} \{ \mu L \} = C_{\text{mass}} \frac{n\} g \{ \mu L \} * \frac{Y}{\text{copies}} \{n\} g$$

	Length [bp]
dsDNA	1427
ssRNA	1374

Table 3: dsDNA and ssRNA fragment sizes.

Note

use eq. 1.a for dsDNA and eq. 1.b for ssRNA.

Expected result

Typical amounts are:

dsDNA: $10^8 - 10^9$ copies/ng

ssRNA: $10^9 - 10^{10}$ copies/ng

12 Make serial dilutions with molecular grade H₂O to reach the target $\frac{\text{copies}}{\mu L}$ concentration.

As positive control we typically use 1 μL of sample at $10.000 \frac{\text{copies}}{\mu L}$

Note

You can store aliquots of dsDNA samples at target concentrations at -20 °C or -80 °C .
Freezing and thawing cycles may affect the concentration then use each aliquot just one time.