Full title

Reference materials for SARS-CoV-2 molecular diagnostic quality control: validation of encapsulated synthetic RNAs for room temperature storage and shipping

Short title

Room temperature storage and shipping of RNA reference materials for covid-19 detection quality control

Authors

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Abstract

The Coronavirus pandemic unveiled the unprecedented need for diagnostic tests to rapidly detect the presence of pathogens in the population. Real-time RT-PCR and other nucleic acid amplification techniques are accurate and sensitive molecular techniques that necessitate positive controls. To meet this need, Twist Bioscience has developed and released synthetic RNA controls. However, RNA is an inherently unstable molecule needing cold storage, costly shipping, and resource-intensive logistics. Imagene provides a solution to this problem by encapsulating dehydrated RNA inside metallic capsules filled with anhydrous argon, allowing room temperature and eco-friendly storage and shipping. Here, RNA controls produced by Twist were encapsulated (RNAshells) and distributed to several laboratories that used them for COVID-19 detection tests by amplification. One RT-LAMP procedure, four different RT-PCR devices and 6 different PCR kits were used. The amplification targets were genes E, N; RdRp, Sarbeco-E and Orf1a/b. RNA retrieval was satisfactory, and the detection was reproducible. RNA stability was checked by accelerated aging. The results for a 10-year equivalent storage time at 25 °C were not significantly different from those for unaged samples. This room temperature RNA stability allows the preparation and distribution of large strategic batches which can be stored for a long time and used for standardization processes between detection sites. Moreover, it makes it also possible to use these controls for single use and in the field where large differences in

43 temperature can occur. Consequently, this type of encapsulated RNA controls, processed
44 at room temperature, can be used as reference materials for the SARS-Cov-2 virus as well
45 as for other pathogens detection.

46 Introduction

47 The Coronavirus pandemic has led to an unprecedented need for diagnostic tests to rapidly
48 detect the presence of this pathogen in the population. Real-time RT-PCR and RT-LAMP
49 amplification techniques proved to be accurate and sensitive molecular tools for virological
50 diagnosis [1,2,3,4,5].

51 A key aspect of any ongoing pathogen detection effort is using quality control measures for
52 a wide range of applications from diagnostic assay development to day-to-day testing. This
53 includes verifying and validating diagnostic tests of both Next-Generation Sequencing
54 (NGS) and reverse transcription-amplification (RT-PCR or RT-LAMP) assays [6,7,8,9].
55 Controls based on viral nucleic acids extracted from infected patients or live viruses
56 propagated in cell culture have safety and security concerns. Synthetic RNA controls, like
57 those generated through gene synthesis by Twist Bioscience, mitigate the biohazard risk
58 and broaden access across diverse strains [10]. Additionally, as DNA synthesis is a readily
59 available technology, new RNA controls can be rapidly developed in response to the
60 emergence of new strains or pathogens.

61 However, RNA is an inherently unstable molecule. In solution or dry i), RNA rapidly
62 degrades through hydrolysis or oxidation (for a review, see [11]. This instability presents
63 storage and cost challenges for laboratories that require large stocks of control RNA (for a
64 review see [2]). Imagene's technology consists of confining dehydrated RNA, in the

65 presence of proprietary stabilizing solutions, inside metallic capsules filled with anhydrous
66 argon (RNAshells®), keeping away the samples from deleterious factors. Thus, it offers a
67 solution to the stability problem by allowing extensive and eco-friendly storage and
68 shipping a room temperature [2].

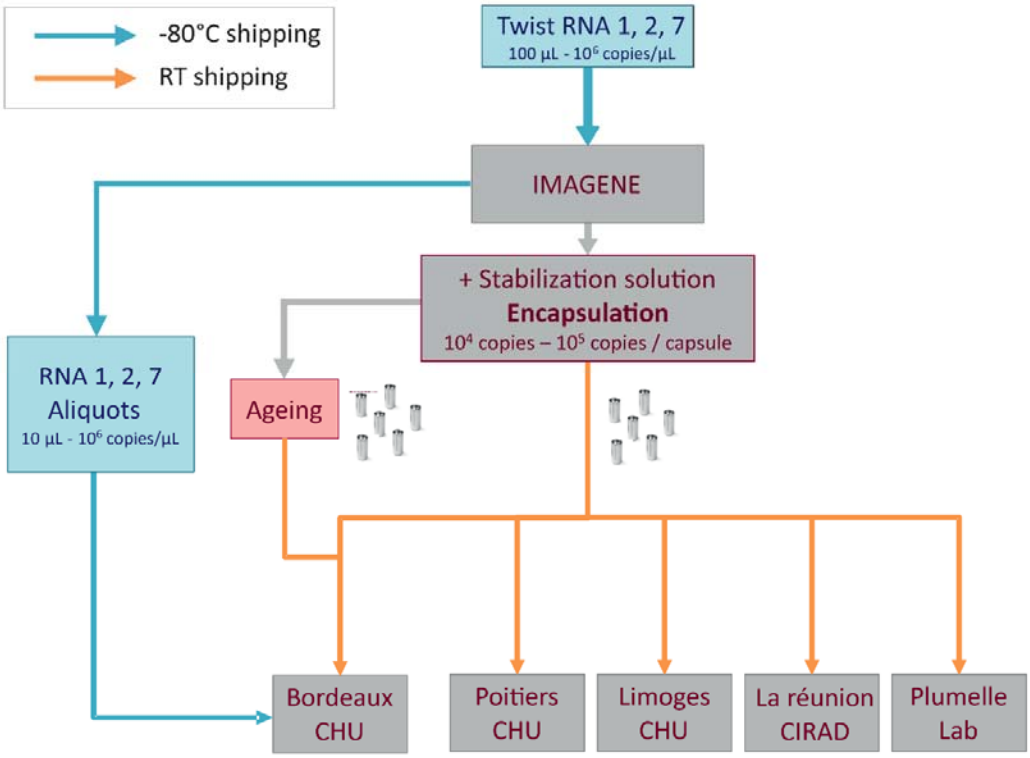
69 Here, Imagene encapsulated synthetic RNA controls (RNAshells) produced by Twist
70 Bioscience. These controls were then distributed to five laboratories for fitness-for-purpose
71 analysis. The objective was to verify, first, that the detection of the virus was reliable and
72 second that the RNA stability was insured for a minimum of a 10-year storage period at
73 room temperature. For that, we used four different RT-PCR devices with six different PCR
74 kits and one LAMP procedure on RNA from three SARS-Cov-2 variants and five gene
75 targets.

Material and methods

Workflow

The RNA samples synthetized by Twist Bioscience were sent on dry ice to Imagene. One part of these samples was aliquoted and sent on dry ice to Bordeaux CHU. The other part was aliquoted and encapsulated. A set of these aliquots was heated to simulate 10 years of room temperature storage and sent to Bordeaux CHU. The other aliquots were sent to Bordeaux, Limoges, Poitiers CHUs, the CIRAD Réunion and a private clinical laboratory (Plumelle, Salon de Provence).

The experimental strategy we followed is summarized in Fig 1.



87 Fig 1. **Experimental strategy for the preparation, distribution and validation of**

88 **RNAshell® encapsulated RNA controls for SARS-CoV-2 virus detection**.

89 RNA Twist RNA controls

90 Synthetic SARS-CoV-2 RNA control molecules for three SARS-CoV-2 variants were graciously

91 supplied by Twist Bioscience. Details are given Table 1.

92 **Table 1. Synthetic SARS-CoV-2 RNA controls used in this study**

Control	GenBank ID	GISAID name	Name used
Control 1	MT007544.1	Australia/VIC01/2020	RNA 1
Control 2	MN908947.3	Wuhan-Hu-1	RNA 2
Control 7	EPI_ISL_418227	France/HF2393/2020	RNA 7

93 The controls are ssRNA *in vitro* transcribed from synthetic DNA. Each one consists of

94 six non-overlapping 5 kb fragments, providing greater than 99% coverage of the viral

95 genome.

96 RNA encapsulation and accelerated ageing

97 RNA controls were sent to Imagene over dry ice as 100 µL solutions at a concentration of

98 approximately 10^6 copies per microliter [10]. They were distributed in 10 µL aliquots and

99 stored at -80°C. Aliquots were thawed, diluted with an Imagene proprietary stabilization

100 solution and distributed into glass inserts fitted in capsules to obtain either 10^4 copies (low)

101 or 10^5 copies (high) per capsule.

102 RNA samples were vacuum-dried and encapsulated under anhydrous and anoxic
103 argon/helium according to the process developed by Imagene [12,13] to give RNAshells®. A
104 set of 3 capsules of each variant RNA and each copy number (a total of 18 capsules) were
105 heated at 90 °C for 16 h to simulate 10 years of storage at 25 °C according to our previously
106 published Arrhenius equation for encapsulated RNA [11]. RNAshells® were then shipped at
107 room temperature to the participating laboratories.

108 RNAshell® opening and RNA rehydration

109 The RNAshells® were opened with a portable ShellOpener™ supplied by Imagene following
110 the given instructions. After opening, a given volume of RNAse-free ultrapure water was
111 added in the capsule and the RNA was left 10-20 minutes on the bench to rehydrate. At
112 that point, the RNA was ready for analysis (RT-qPCR or RT-LAMP). The different volumes
113 used to rehydrate and analyze the RNA by RT-qPCR in the different laboratories are given in
114 Table 2.

115 **Table 2. Summary of the material and methods for the different RT-qPCR analysis.**

Site	**Bordeaux CHU**	**Bordeaux CHU**	**Limoges CHU**	**Poitiers CHU**	**Poitiers CHU**	**Private clinical Laboratory**
Instrument	Light Cycler 480 (Roche)	Light Cycler 480 (Roche)	CFX96 (BioRad)	CFX96 (BioRad)	CFX96 (BioRad)	SLAN®-96P Real-Time PCR System (Sansure)
Kit	GeneFinder Covid-19 Plus, Osang Healthcare	SARS CoV-2 R Gene, Argene Biomérieux [14,15]	SuperScript™ III One-Step RT-PCR (Thermofisher)	TaqPath COVID-19 Multiplex Diagnostic	Allplex™ 2019-nCoV Assay (Seegene)	Kit Novel coronavirus 2019-ncov (Sansure)

	(distributed by Elitech)[2] [14]			Solution (Thermofisher)		
Nb cycles	45	45	45	40	45	45
Cycles	20 min @ 50°C 5 min @ 95°C 45 cycles : - 15 s @ 95°C - 50 s @ 58°C	5 min @ 50°C 15 min @ 95°C 45 cycles : - 10 s @ 95°C - 40 s @ 60°C - 25 s @ 72°C	ND	2 min @ 25 °C 10 min @ 53°C 2 min @ 95°C 40 cycles : - 3 s @ 95°C - 30 s @ 60°C	20 min @ 50 °C 15 min @ 95°C 45 cycles : - 15 s @ 94°C - 30 s @ 58°C	30 min @ 50°C 1 min @ 95°C 45 cycles : - 15 s @ 95°C - 30 s @ 60°C
Positivity	Ct < 43	ND	fluo signal > 6 000 for IP2 > 13 000 for IP4	Ct < 37	Ct < 40	ND
Target genes	E, N, RdRp	E, N, RdRp	IP2-RdRp, IP4-RdRp Sarbeco- E	S, orf1, N	E, N, RdRp	ORF1ab, N
RNA Controls	RNAshells #1, 2, 7 10^4 copies 10^5 copies 18 aged RNAshells (3 of each) Solution #1, 2, 7 10 µL @ 10^6	RNAshells #1, 2, 7 10^4 copies 10^5 copies 18 aged RNAshells (3 of each) Solution #1, 2, 7 10 µL @ 10^6	RNAshells #1, 2, 7 10^4 copies 10^5 copies	RNAshells #1, 2, 7 10^4 copies 10^5 copies	RNAshells #1, 2, 7 10^4 copies 10^5 copies	RNAshells #2 6x 10^4 copies 6x 10^5 copies

	copies / µL	copies / µL				
Rehydration volume	10 µL	10 µL	20 µL	25 µL	25 µL	ND
RNA volume in reaction	5 µL	5 µL	5 µL	10 µL	8 µL	ND
Theoretical number of copies / reaction	5,000 or 50,000	5,000 or 50,000	2,500 or 25,000	4,000 or 40,000	3,200 or 32,000	ND

ND: non disclosed

Analysis of RNA samples in solution

The non-diluted and non-encapsulated aliquots of RNA 1, 2 and 7, at approximately 10^6 copies per µL, were prepared and sent by Imagene on dry ice to Bordeaux CHU. The samples were thawed and diluted to match the concentrations of the rehydrated capsules (here, 10^3 and 10^4 copies per µL). They were analyzed in parallel by RT-qPCR (see Table 2).

RT-qPCR analysis

124 The different available RT-qPCR materials and methods used in the study are summed up in
125 Table 2.

126 RT-LAMP analysis

127 Here, the RT-LAMP system was first tested on a dilution series of control RNA 2 Low or
128 High, rehydrated with 50 µL of RNAse-free pure water. For Control 2 Low: a single analysis
129 was done for 1000 copies/reaction, 2 replicates were done for 500 and 250 copies/reaction
130 and 3 replicates for 100, 50 and 25 copies/reaction. For Control 2 High: 3 replicates were
131 done for 1000 copies/reaction and 4 replicates for 200, 40 and 8 copies/reaction.

132 To test the encapsulated RNA 2 as a positive control in the field, 60 independent RT-LAMP
133 runs were performed for the presence of SARS-CoV-2. Each run included 14 samples, a
134 negative control and a positive control. The positive control consisted of RNA 2 rehydrated
135 with water and further diluted in *Amies* medium to have a final concentration of 1000
136 copies/reaction.

137 RT-LAMP was performed according to the protocol RunCov developed by the CIRAD-UMR
138 PVBMT (3P) and validated by the National Reference Center [16]. The reactions were
139 performed in a 25 µL total reaction volume containing 15 µL of ISO-DR004-RT300
140 Isothermal Mastermix (OptiGene Ltd, Horsham, United Kingdom), 2.475 µL of each primer
141 pair (N and S), 0.05 µL (0.5 units) of AMV transcriptase (Promega, Charbonnières-les-Bains,
142 France) and 5 µL of template RNA. LAMP reactions were run on a Genie II instrument
143 (OptiGene) at 65°C for 25 min followed by an annealing step during which the temperature
144 was decreased from 92°C to 78°C at a speed of 0.05°C/s to determine the annealing
145 temperature (Ta) of the produced amplicon.

146 ## Statistical analysis

147 All statistical analyses including ANOVA, Fisher Test and previous verification of data
148 normality (histogram and normal: Q-Q plot), were performed using the R statistical
149 software, version 4.1.1 (2021-08-10; R Development Core Team, Vienna, Austria) with the
150 different packages: stats, multcomp, emmeans and ggplot2).

151 # Results and discussion

152 ## Objectives and strategy

153 The aim of this work was to validate whether encapsulated RNA controls stored and
154 shipped at room temperature were fit for purpose as reference materials for internal
155 quality control of SARS-CoV-2 detection.

156 Bordeaux CHU received non-diluted, non-encapsulated, frozen samples in solution, to
157 compare the controls before and after encapsulation (i.e. to check the potential effect of
158 the process and stabilization solution). They also received the aged RNAshells® to validate
159 that 10 years of storage at room temperature did not alter the performance of the
160 encapsulated controls. Two other laboratories (Poitiers CHU and Limoges CHU) received
161 RNAshells® to verify that the controls gave the same performances when run with other
162 RT-qPCR techniques. The CIRAD-UMR PVBMT (3P) laboratory tested the controls in
163 multiple experiments while developing a RT-LAMP test for field use. Finally, RNAshells®
164 were sent to a private clinical laboratory to check that the capsules can be routinely used
165 by any operator.

Controls in solution

First, we checked the performance of the controls on non-encapsulated samples. The results of these experiments are presented Fig 2.

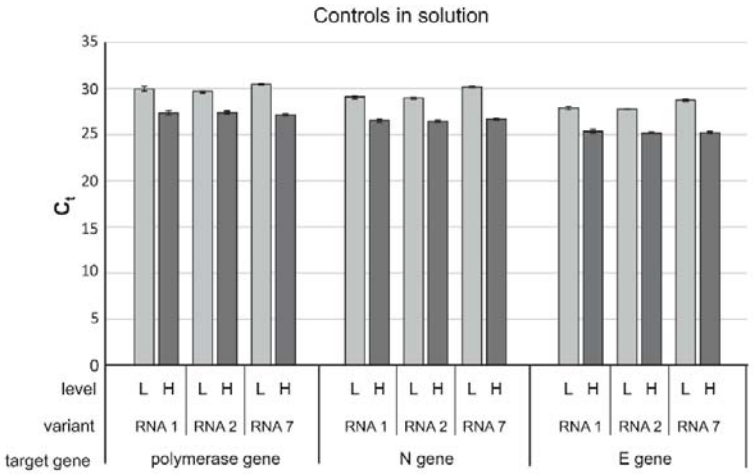


Fig 2. RT-qPCR for controls in solution. The controls were thawed and diluted at reception. Measures were done at Bordeaux CHU on 3 variants RNA (RNA 1, 2, 7), 2 copy numbers: 5,000 (low, L) and 50,000 (high, H) copies/reaction, 3 target genes (polymerase gene, N gene and E gene).

All the controls gave positive results. For a given low or high concentration and a given target gene, the Ct were comparable between the different variants ($p = 0.647$). The highest Ct differences came from the gene targets ($p=7.79 \times 10^{-4}$), probably due to differences in PCR efficiency.

The chosen copy numbers gave Ct values in a suitable range (around 30 and 27 respectively for the low and high controls), clearly different from the negative control (above 40-45). The Ct were also not too low. This is advantageous because a positive control with too

181 many more copies than in patient samples could result in carry-over contamination in the

182 nearby wells of the PCR plate leading to false positive samples.

183 ## RNA controls encapsulation and accelerated aging

184 Then, we needed to verify, that encapsulation does not induce alterations in the controls.
185 Another important point is to test whether RNA controls could be stored at room
186 temperature for an extended period of time in RNAshell®, so a set of capsules were
187 submitted to an accelerated aging by heating to simulate a storage period of 10 years at
188 25°C according to our previously published Arrhenius study [11]. The results are shown Fig
189 3.

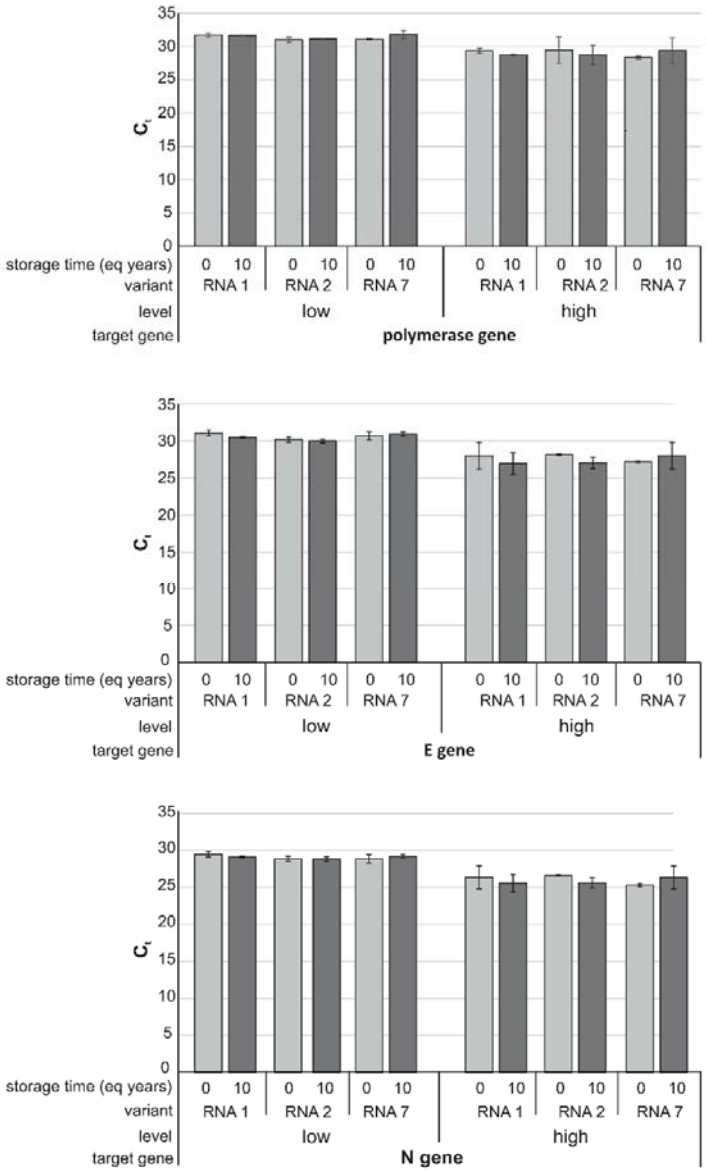
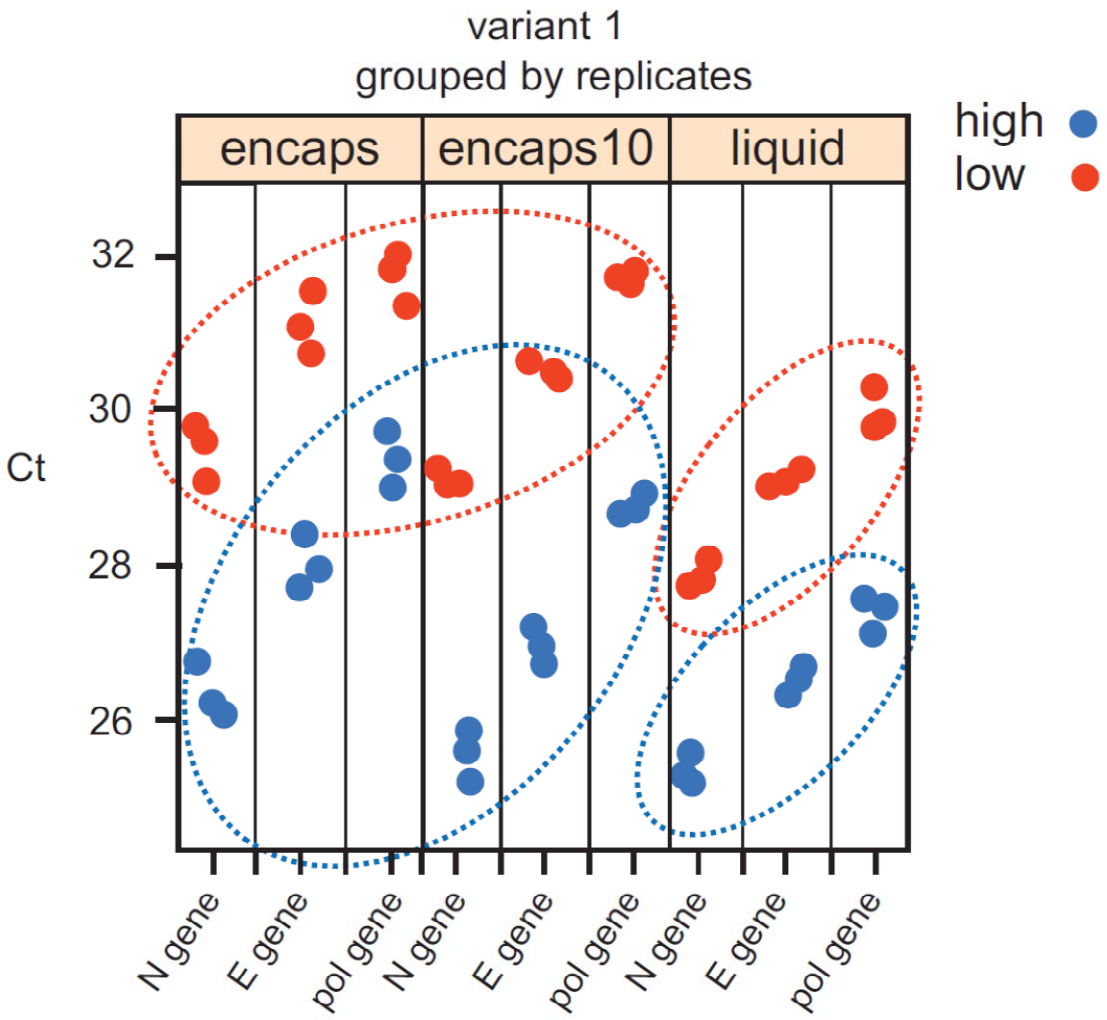


Fig 3. RT-qPCR on RNAshell®-encapsulated and aged controls. RT-qPCR measures were done by Bordeaux CHU on 3 variants RNA (RNA 1, 2, 7), 2 copy numbers: 5,000 (low, L) and 50,000 (high, H) copies/reaction, 3 target genes (polymerase gene, N gene and E gene) and after 2 storage times: time 0 post-encapsulation and after heating for a period equivalent to 10 years at room temperature (0 and 10).

197 All analyzed RNA, whatever the variant, target gene or storage time were successfully
198 amplified.

199 To analyze the extent and sources of variations on these experiments we used variant 1.
200 The results are shown Fig 4.



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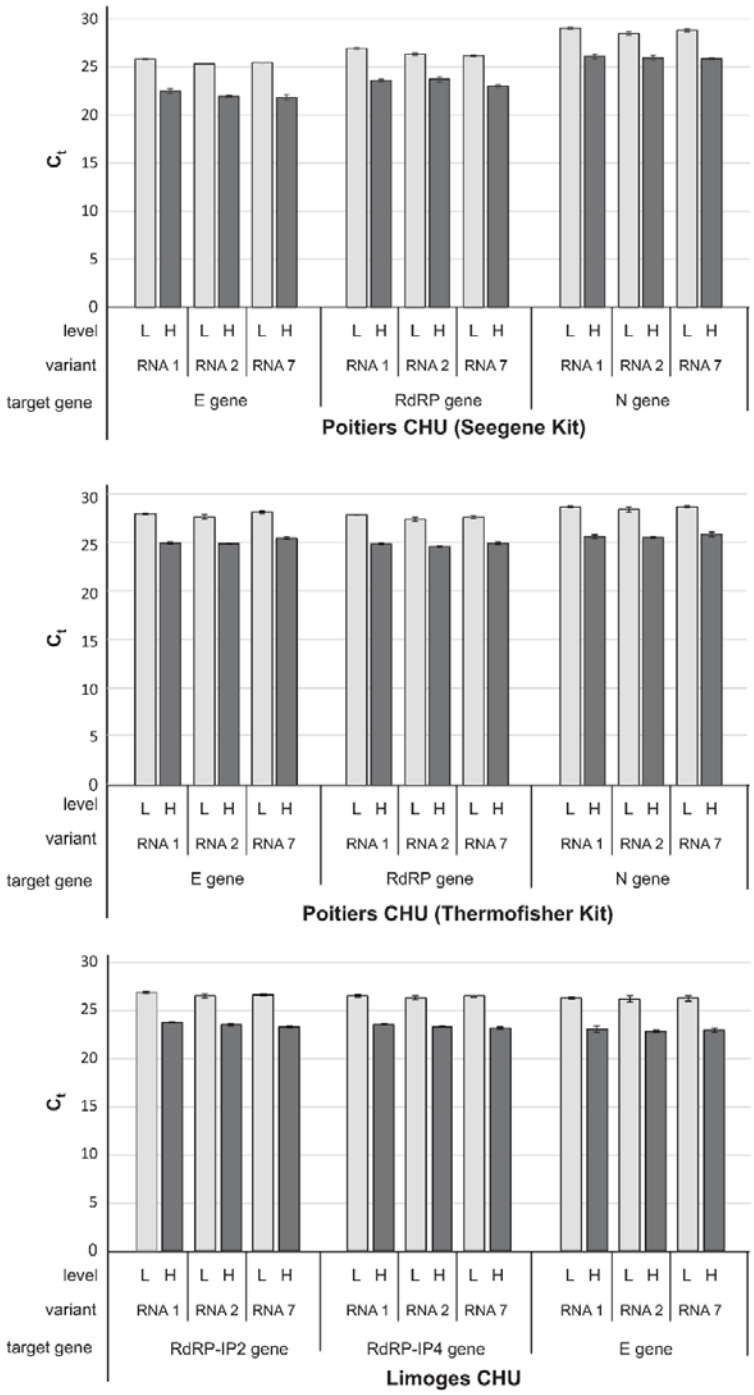
202 **Fig 4. Group analysis for Ct on variant 1 controls.** Ct (3 replicates) are shown
203 according to: a) the state: encapsulated (encaps), encapsulated and aged (encaps
204 10) or in solution (liquid) and b) the number of copies (5,000 (low) and 50,000
205 (high) of each target gene. Groups of Ct values from the same state (dry vs liquid)
206 and copy number are circled.

207 The encapsulated controls and the controls in solution show significant Ct differences
208 (p=0.001999), due partly to the encapsulation step, including the addition of a stabilization
209 solution, but more probably to the fact that the controls in solution did not come from the
210 same diluted samples. However the Ct values of encapsulated RNA are maintained in the
211 same suitable range, meaning that the encapsulation process did not lead to RNA loss,
212 degradation or PCR inhibition to an extent that would make RNAshell®-encapsulated RNA
213 unfit for purpose.

214 On the other side, no significant difference was observed between 0- and 10-years
215 RNAshells® (Fig 3, p=0.7606) meaning that the simulated long-term storage of RNA did not
216 lead to significant degradation. One can therefore consider that the encapsulated RNA
217 controls are stable over at least 10 years at room temperature and are suitable reference
218 materials.

219 Compatibility with other RT-qPCR kits and instruments

220 To broaden the validation, capsules were distributed to laboratories that performed tests
221 with three other RT-amplification techniques, including the method used by the National
222 Reference Center for SARS-CoV 2 (on RdRp IP2 and IP4 regions). The results are shown
223 Fig 5.



224

225 **Fig5. RT-qPCR results from Limoges and Poitiers CHU.** For Limoges CHU, the RT-
226 qPCR measures were done on 3 variants RNA (RNA 1, 2 and 7), 2 copy numbers:
227 2,500 (Low, L) and 25,000 (High, H) copies/reaction), and 3 target genes (RdRp-IP2,
228 RdRp-IP4 and E genes). For Poitiers CHU, RNAshells® were rehydrated with 25 µL of

229 water. Eight µL were used for the Seegene kit and 10 µL for the Thermofisher kit. Ct
230 values were obtained by RT-qPCR measures done on 3 variants RNA (RNA 1, 2 and
231 7), 3 target genes (E, N and RdRp genes), with 2 copy numbers: 10,000 (Low, L) and
232 100,000 copies/capsule (high, H) giving 3,200 and 32,000 copies per reaction for the
233 Seegene kit and 4,000 and 40,000 copies per reaction for the Thermofisher kit. For
234 each technique, the control capsules were run in 3 distinct experiments (Poitiers
235 CHU) and the mean and SE of the 3 Ct were calculated.

236

237 Although the experiments were run over 3 days (at Poitiers CHU), the sampling from a
238 given capsule gave constant results. In all the experiments, techniques and targets, the
239 results obtained in the different laboratories were reproducible among all the capsules,
240 showing that the encapsulated control RNA are suitable for their use as internal quality
241 controls. The Ct for variants were strictly equivalent. The highest variability came from
242 the analysis kits and target genes as demonstrated above with the controls in solution.

243 Compatibility with a RT-LAMP procedure

244 SARS-Cov-2 genome was successfully amplified using the RT-LAMP assay from both RNA 2
245 low and RNA 2 high capsules with a high sensitivity (100% positive samples at 25 copies per
246 reaction for RNA2 low, 40 copies for RNA2 high and 50% positive samples at 8 copies for
247 RNA 2 high). Annealing temperature peaks were obtained for both S and N regions for 90%
248 of the positive samples with annealing temperatures ranging between 88.4-88.7 °C and
249 83.4-83.8 °C, for N and S regions, respectively. For 10% positive samples only one annealing
250 temperature peak (S or N) was obtained. This last result was principally obtained for low
251 copy numbers. This was already observed in other RT-LAMP experiments involving other

252 types of samples and is probably due to differences in primers binding probabilities to their

253 respective targets.

254 In the field, 1000 copies per reaction were taken so that the signal came out early,

255 validating the run before or at the same time as positive samples came out.

256 The results are given Table 3.

257 **Table 3. RT-LAMP results from CIRAD.**

			Annealing temperature	
RNAshell RNA 2	Copies / reaction	Amplification time (min)	N gene	S gene
Low	1000	10.00	88.5	83.50
Low	500	11.75	88.5	83.7
Low	500	11.00	88.5	83.7
Low	250	12.75	88.5	83.6
Low	250	13.50	88.6	83.7
Low	200	14.50	88.6	83.8
Low	200	14.50	88.4	83.8
Low	200	16.75	88.7	83.7
Low	100	13.00	88.6	83.8
Low	100	12.50	88.8	83.7
Low	100	13.00	88.5	83.7
Low	50	13.50	88.7	83.8
Low	50	17.00	88.7	83.8
Low	50	14.00	88.7	83.7
Low	25	16.75	88.3	83.8
Low	25	14.00	88.6	83.8
Low	25	13.50	88.4	83.6
High	1000	10.30	88.7	83.7
High	1000	10.15	88.7	83.7
High	1000	10.15	88.6	83.7
High	200	12.00	88.6	83.7
High	200	11.30	88.6	83.6
High	200	11.30	88.6	83.6
High	200	10.45	88.7	83.7
High	40	12.30	88.7	83.7
High	40	13.45	-	83.7
High	40	13.15	-	83.7
High	40	13.45	88.6	88.5
High	8	19.45	-	83.4

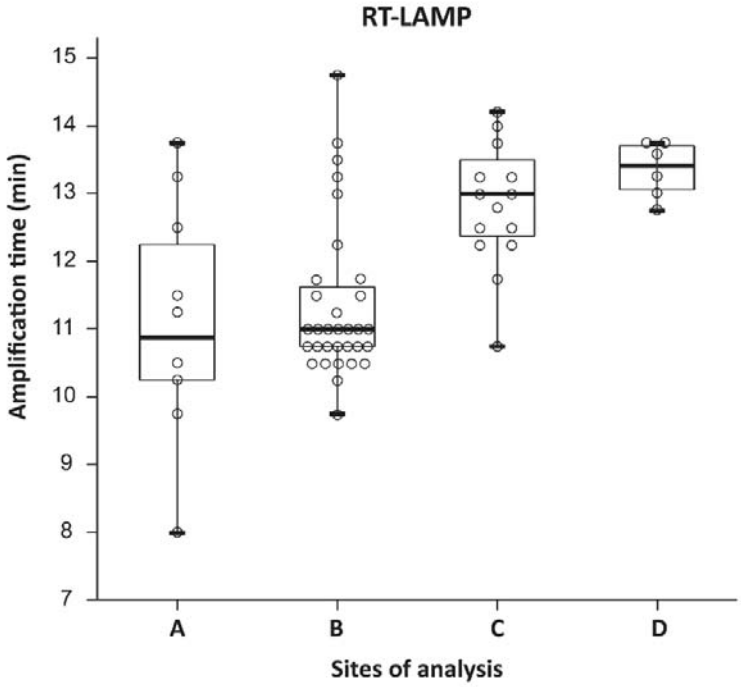
High	8	23.30	88.7	-
High	8	-	-	-
High	8	-	-	-

258 RNA 2 samples High and Low were rehydrated with 50 µL of water and diluted to
259 have between 1000 and 8 copies per reaction and amplified using the Runcov RT-
260 LAMP protocol of the CIRAD La Réunion. The amplification time and annealing
261 temperatures are given.

262

263 ” Real life” use of the capsules as internal quality controls

264 For the monitoring of SARS-CoV 2 detection in the field by RT-LAMP, the RNA 2 variant
265 (1000 copies) was used as a positive control for 60 RT-LAMP tests run on samples taken
266 and analyzed in 4 different sites (A, B, C, D). This control was successfully amplified in each
267 run (Fig 6).



268

269 **Fig 6. LAMP tests** The RNA 2 variant (1000 copies) was used as a positive control
270 for 60 (real life) tests run on samples analyzed at 4 different sites (A, B, C, D). For
271 each test, both N and S regions were amplified.

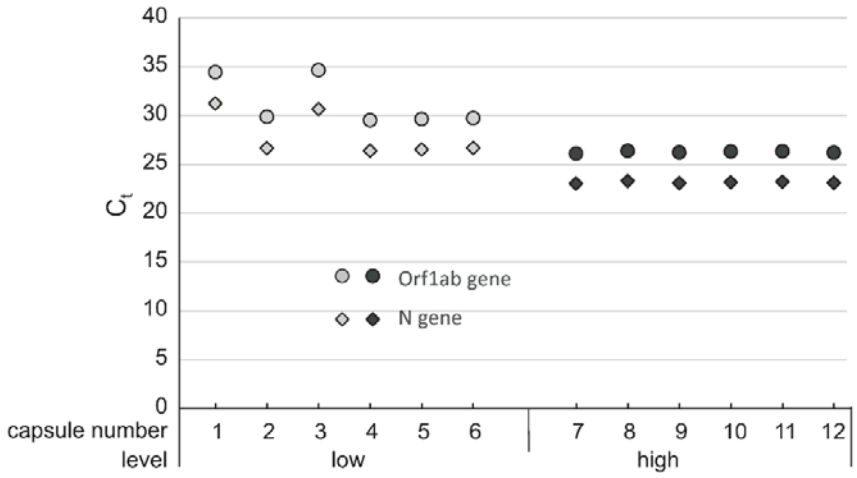
272 For each test, both N and S regions were amplified, generating annealing peaks within the
273 expected annealing temperature ranges (not shown).

274 This field study shows that the capsules can be used as positive controls at the point of care
275 or on site with simplified logistics due to their stability at room temperature.

276

277 Validation of RNA controls for private clinical laboratories

278 To test the robustness of the controls, a series of experiments we conducted in a private
279 clinical laboratory. The laboratory received the capsules and the instructions for use and
280 included the rehydrated RNA in their routine diagnostic test. The results are shown Fig 7.



281

282 **Fig 7. Private clinical laboratory-.** Results on 2 target genes and 2 levels on 6 capsules
283 for each.

284 The results showed a good reproducibility among the capsules except for 2 capsules with a
285 low RNA copy number where the operator probably had rehydrated the RNA with a higher
286 volume of water, decreasing the number of copies in the test.

287 So, the capsules can easily be used as reference materials for internal quality control of the
288 monitoring of the diagnostics procedures, without the need for extensive training of the
289 operators.

290

291 Conclusion

292 This work shows that e RNAs synthetized by Twist Bioscience and encapsulated in
293 RNAshells® by Imagene have been validated as positive controls for SARS-CoV-2 detection.
294 Both 10,000 and 100,000 copy numbers per capsule are suitable for their use in RT-qPCR
295 and RT-LAMP analysis. The signal is reproducible among the variants, the targets and the
296 analysis methods. Moreover, the RNAshell®-encapsulated RNA showed an excellent
297 stability, with an unprecedented shelf life of more than 10 years at room temperature. This
298 stability is a great advantage because, first, it facilitates the use of these controls in the
299 field, in particular when large variations of temperature can be expected, and second,
300 importantly, it allows the preparation and the distribution of large strategic batches which
301 can be kept for a long time and used for standardization between processes or detection
302 sites.

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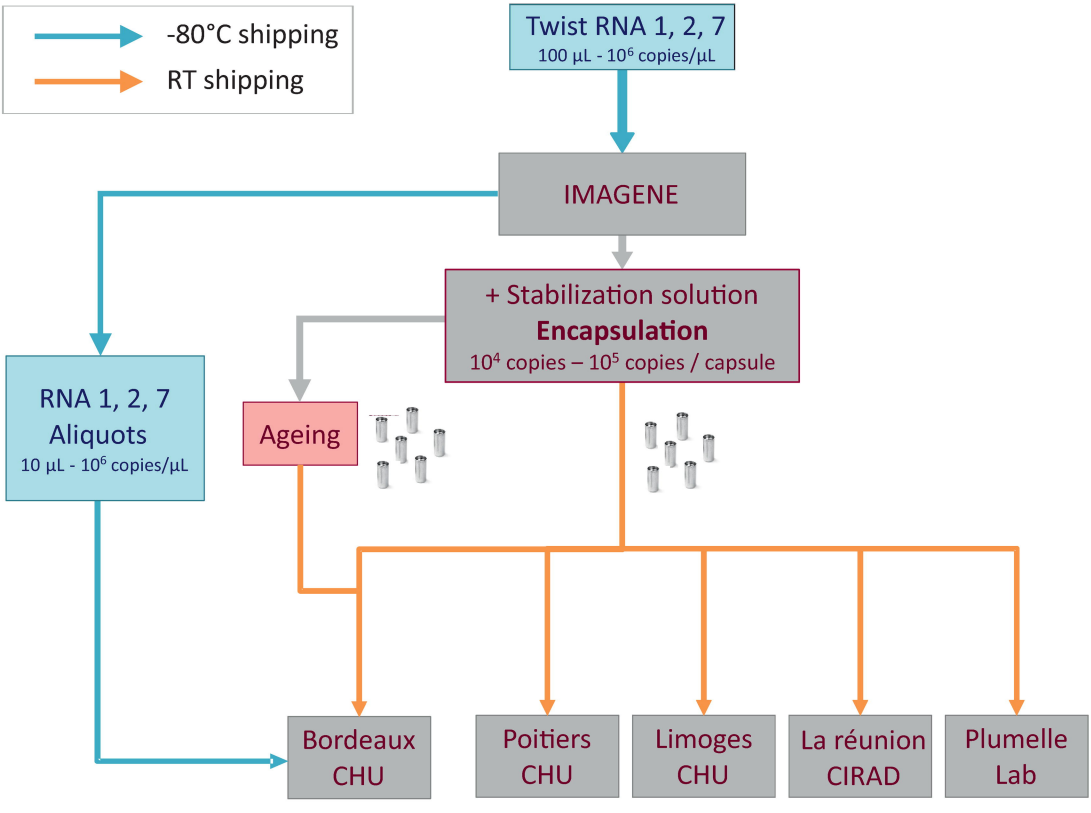
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IMAGENE
+ Stabilization solution
Encapsulation
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RNA 1, 2, 7
Aliquots
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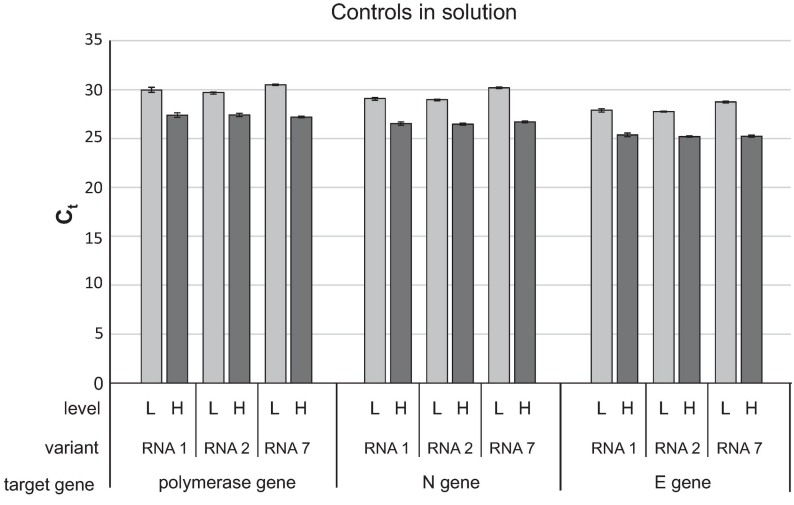
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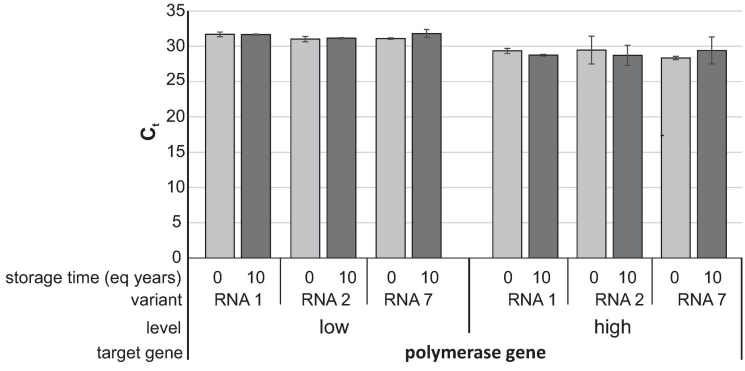
N gene
E gene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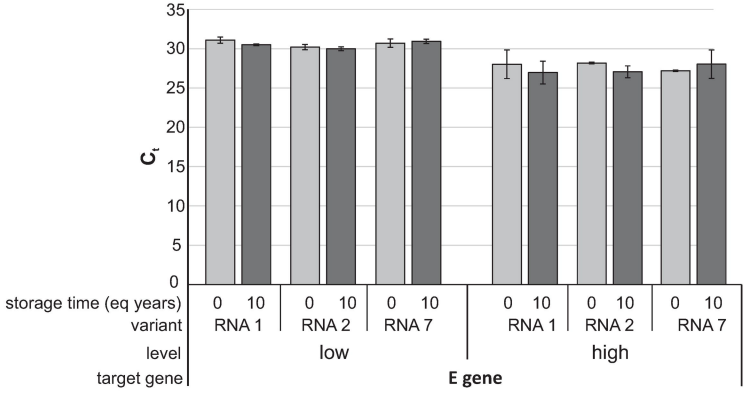
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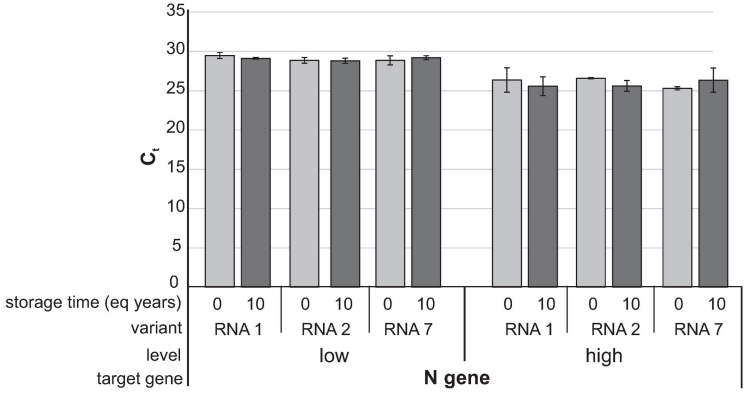
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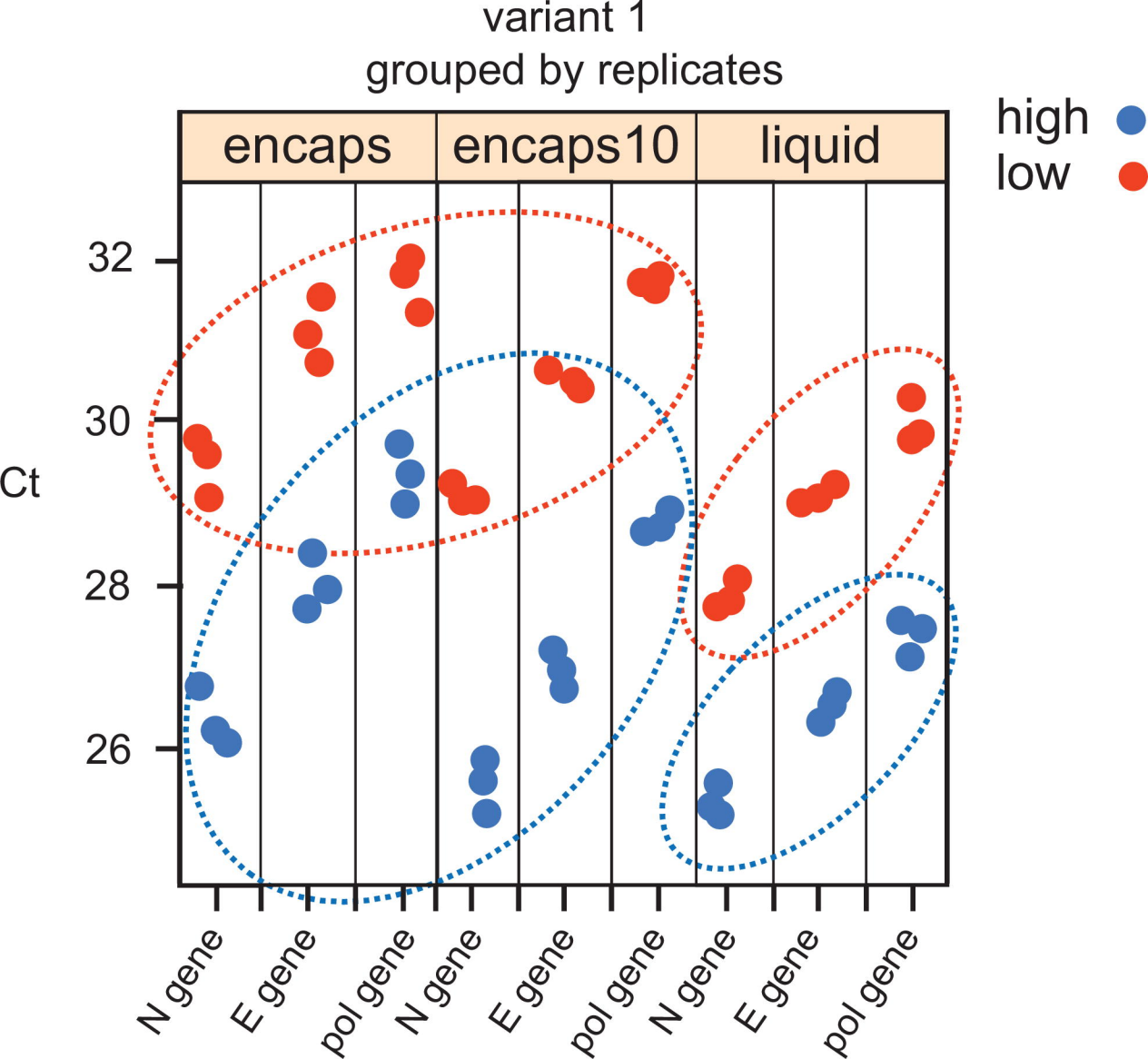
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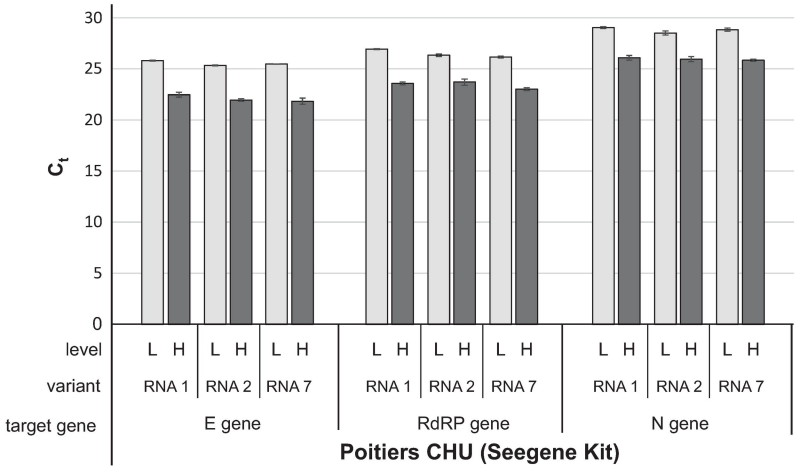
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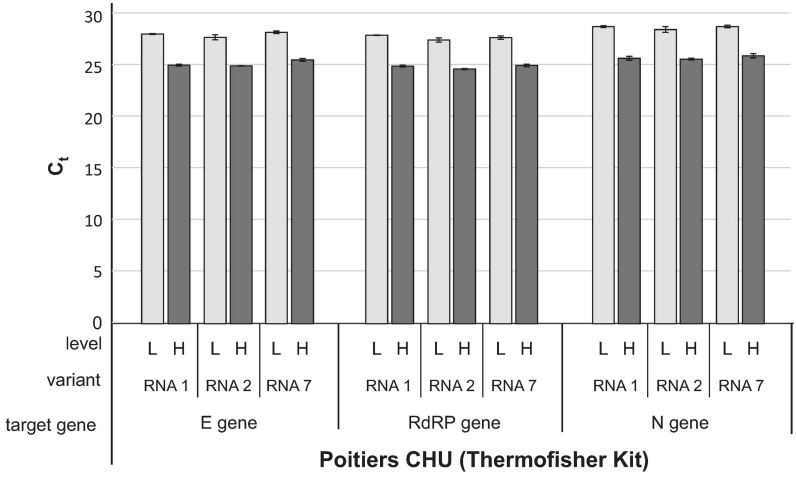
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RdRP gene
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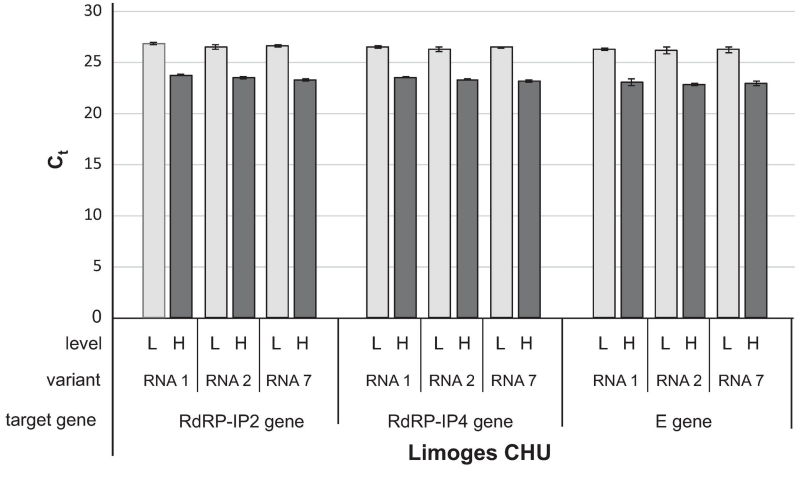
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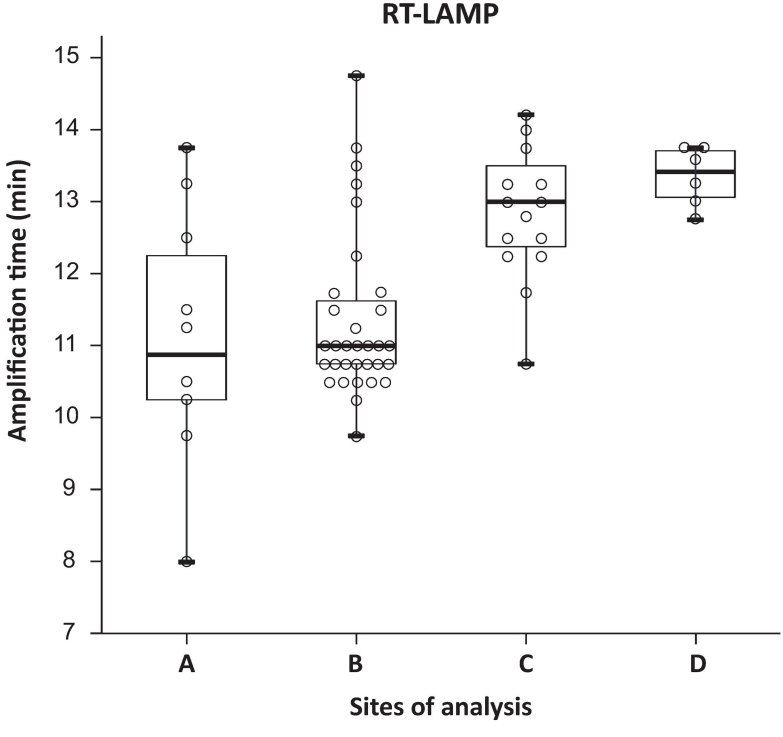
RdRP-IP2 gene
RdRP-IP4 gene
E gene
Limoges CHU

fig 6. CIRAD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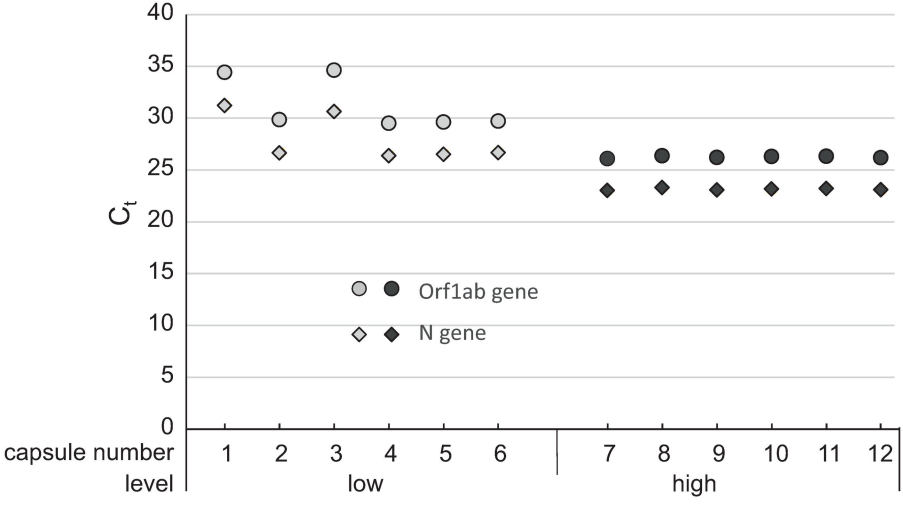
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