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3 RT qLAMP--Direct Detection of SARS-CoV-2 in Raw Sewage
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28 **Introduction**. The purpose of this report is to describe the ability of loop-mediated isothermal
29 amplification, LAMP, in the form of RT qLAMP to detect and quantify SARS-CoV-2 in raw
30 sewage, directly, i.e., without sample processing for virus concentration or RNA extraction.
31 We provide information on the routine application, equipment, and facilities used to illustrate
32 the feasibility of RT qLAMP application for detailed monitoring of SARS-CoV-2 for wastewater
33 based epidemiology (WBE). The most important and novel aspect of this report is
34 demonstration that even at low reported case rates e.g. 1-10/100,000, in a community,
35 SARS-CoV-2 virus is present in raw sewage at concentrations > 1-5/μL, sufficient for LAMP-
36 based detection directly avoiding the qPCR need for cumbersome time-consuming
37 concentration and RNA extraction. Incorporation of this analytical approach will facilitate
38 development of data supporting wastewater-based epidemiology as an important component
39 of policy advice directed to COVID-19 control.

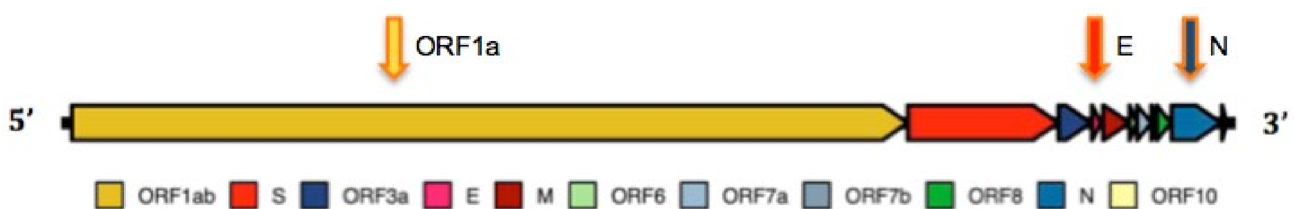
40 Loop-mediated isothermal amplification, LAMP, is not novel and it is not new. It is a
41 thoroughly demonstrated and well-understood nucleic acid amplification procedure, first
42 described 20 years ago, Notomi, 2000, since developed for largely clinical applications but
43 equally demonstrated for detection of DNA or RNA in a wide variety of viral, microbial, and
44 protozoan pathogens as well as identifying gene-specific targets in plants and animals, e.g.,
45 Salar et al, 2013; Becherer et al, 2020. The principal characteristics of LAMP include the use
46 of four to six primers annealing to initially four then six target sites selected to meet well
47 established criteria, e.g., Eiken, 2018. The amplification mechanism is strand extension with
48 loop formation producing what has been described as a cauliflower-like product, producing a
49 characteristic ladder band appearance on confirming gels. The multiple primer-target
50 combination gives the process a very high degree of specificity, enabling target detection in
51 crude preparations containing extraneous nucleic acids. The specificity also permits effective
52 multiplex applications. The process uses a polymerase having strand extension activity,
53 typically Bst, acting at constant temperature in the 60-70°C range. Operation at constant

54 temperature permits amplification with simple means of maintaining constant temperature
55 such as a water bath facilitating application in areas of limited laboratory facilities. The
56 process has been found insensitive to interferences common to conventional PCR processes
57 applied to analysis of environmental samples. Furthermore, the process is fully as sensitive
58 as conventional PCR and amplification times, e.g., cycle threshold (Ct), are typically short,
59 i.e., 20-40 min, Becherer et al, 2020.

60 The relatively slow adoption of LAMP procedure for environmental monitoring specifically to
61 water and wastewater is partly characteristic of processes having few detailed published
62 reports to stimulate the interest of other investigators. Two features of LAMP may be
63 described as disadvantages: 1) the rather intricate process of primer design, testing, and
64 optimization needed to permit routine application; and 2) the very high sensitivity would permit
65 cross contamination if not recognized and precluded by proper laboratory procedure and
66 careful technique.

67 The world-wide spread of SARS-CoV-2 infection and COVID-19 disease throughout the
68 human population has stimulated massive effort to develop and improve ability to detect and
69 to monitor the virus e.g., Color, 2020. The standard method being applied at the beginning of
70 the 1st quarter of 2021 both to clinical detection and environmental monitoring, sewage, is
71 PCR, typically RT qPCR (CDC, 2020; WHO, 2020). However, building on previous clinical
72 applications many variations of LAMP-based procedure have been reported, Becherer et al,
73 2020. Not requiring a thermocycler, the LAMP process lends itself to both scale up and
74 miniaturization and can be combined with increasingly sophisticated technology and
75 downstream refinements including sequencing. Numerous reports of LAMP-based assays for
76 SARS-CoV-2 detection in clinical samples have been developed, Dong et al, 2020;
77 Thompson et al, 2020; Chaouch, 2020) providing information and encouragement for
78 development of a LAMP-based assay for SARS-CoV-2 in raw sewage.

79 The potential for successful application of LAMP to SARS-CoV-2 monitoring in raw sewage
80 is illustrated by previous experience applying a multiplex LAMP to detection of
81 *Cryptosporidium* and *Giardia* in surface water samples, Gallas-Lindeman et al, 2012; Ongerth
82 and Saaed, 2020. That work showed: 1) target organisms were detectable in the complex
83 untreated water and wastewater matrix; 2) that LAMP could be multiplexed for detection of
84 both simultaneously; 3) organisms are detectable at low concentration, ca. 1-5/10L; 4)
85 detection was not affected by extraneous components in a complex sample concentrate; and
86 5) quantification using a qPCR instrument (Roche Light Cycler 480) was possible. From early
87 reports on monitoring SARS-CoV-2 in raw sewage, Ahmed et al, 2020: Wurtzer et al, 2020;
88 Wu et al 2020, calculation of likely virus concentrations at a sewage treatment plant serving
89 a population having COVID-19 daily reported cases in the range 5-10/100,000, suggested
90 that the virus would be detectable without concentration and that LAMP would not be affected
91 by extraneous sewage components. To test this potential, taking advantage of many well-
92 described LAMP primer sets reported for clinical application since February 2020, we
93 selected primers for three potential targets, Figure 1, ORF-1a (Lamb et al, 2020) E-gene and
94 N-gene (Broughton et al, 2020), Table 1, assembled essential materials, and arranged to
95 obtain raw sewage samples with the local wastewater agency, East Bay Municipal Utility
96 District Special District1 (EBMUD SD1).



97 Figure 1. SARS-CoV-2 genome and subunit arrangement with primer locations

98 **Methods**. Development, selection, and optimization of amplification conditions for each set
99 of primers, ORF-1a, E-gene, and N-gene, was described in the original references, Lamb et
100 al 2020, and Broughton et al, 2020. Primers were applied here using all of the concentration
101 and amplification conditions established in the original references, (ORF1a, Lamb et al 2020;

102 E-gene and N-gene, Broughton et al, 2020). To facilitate testing the approach we used off-

103 the shelf materials where possible. Materials used included:

104 - Primers prepared with standard desalting, IDT, Coralville, IA
105 - Master mix: WarmStart LAMP Kit E1700, E2019, New England BioLabs, Ipswich, MA
106 - Control: Synthetic SARS-CoV-2 RNA, Control 6 (MT118835), Twist Bioscience, S. San
107 Francisco, CA.
108 - Raw sewage: East Bay Municipal Utility District SD1 (EBMUD), Oakland, CA

109 Table 1. Primer sequences used for direct raw sewage RT qLAMP

Gene	Primer	Sequence	No. of Bases
		(Ref. Lamb et al. 2020, 63 °C; Color, 2020, 65 °C)	
ORF 1a	F3	TCCAGATGAGGATGAAGAAGA	21
ORF 1a	B3	AGTCTGAACAACTGGTGTAAG	21
ORF 1a	FIP	AGAGCAGCAGAAGTGGCACAGGTGATTGTGAAGAAGAAGA	41
ORF 1a	BIP	TCAACCTGAAGAAGAGCAAGAACTGATTGTCCTCACTGCC	40
ORF 1a	LF	CTCATATTGAGTTGATGGCTCA	22
ORF 1a	LB	ACAAACTGTTGGTCAACAAGAC	22
		(Ref. Broughton et al, 2020, 62 °C; Color, 2020, 65 °C)	
N	F3	AACACAAGCTTTCGGCAG	18
N	B3	GAAATTTGGATCTTTGTCATCC	22
N	FIP	TGCGGCCAATGTTTGTAATCAGCCAAGGAAATTTTGGGGAC	41
N	BIP	CGCATTGGCATGGAAGTCACTTTGATGGCACCTGTGTAG	39
N	LF	TTCCTTGTCTGATTAGTTC	19
N	LB	ACCTTCGGGAACGTGGTT	18
		(Ref. Broughton et al, 2020, 62 °C; Color, 2020, 65 °C)	
E	F3	CCGACGACGACTACTAGC	18
E	B3	AGAGTAAACGTAAAAAGAAGGTT	23
E	FIP	ACCTGTCTCTTCCGAAACGAATTTGTAAGCACAAGCTGAT	41
E	BIP	CTAGCCATCCTTACTGCGCTACTCACGTTAACAATATTGCA	41
E	LF	TCGATTGTGTGCGTACTGC	19
E	LB	TGAGTACATAAGTTCGTAC	19

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111 Reactions for RT-qLAMP were 25 µL total volume, according to proportions listed in Table 2.

112 Table 2: Direct raw sewage RT qLAMP reaction mix components

Reaction Mix Component	Volume
WarmStart master mix (1700 or 2019)	12.5 µL, includes dNTPs at 1.4 mM; RTx, 8 mM $MgSO_4$
10X Primers	2.5 µL (1.6 µM FIP/BIP, 0.2 µM F3/B3, 0.4 µM Loop F/B)
NEB green fluorescent dye w/ E1700 kit	0.5 µL (@ 0.5 µM)
Nuclease free H_2O	4.5 - 0 µL (adjust w/ template to 25 µL total rxn volume)
Template: Control or Raw sewage	5 - 9.5 µL
Total Reaction Mix	25 µL

113 Upon receiving fresh sewage samples, representative aliquots were distributed along with
114 standards and no template controls, as appropriate, to reaction components distributed into
115 0.2 mL PCR reaction tubes on ice during preparation. All reactions were conducted in
116 triplicate. Reactions were prepared in a PCR hood using routine lab technique designed to

preclude potential cross contamination. Amplifications were conducted using a Rotor-Gene Q programmed according to Qiagen protocol for constant temperature, 65°C (63°C after initial runs), for 30-40 minutes followed by high resolution melting. Reaction tubes were not opened after run completion and were frozen to permit future analysis.

Raw sewage was obtained from the EBMUD SD1 serving a population of ca. 650,000 largely in Alameda County. Separate regions of the service area contribute flows to the three interceptor sewers, North (N), Adeline (ADA), and South (S) terminating at the single treatment plant, Figure 2a. Samples of 1L total volume were collected from each interceptor individually at a point just before the treatment plant. Samples were 24-hour composites representative of the period 9:00 am to 9:00 am, Figure 2c. Samples were transported on ice to the laboratory for processing and analysis, kept refrigerated w/o preservative until analysis. Sampling dates were July 29, Sept. 9, Sept. 22, and Oct 1, 2020.

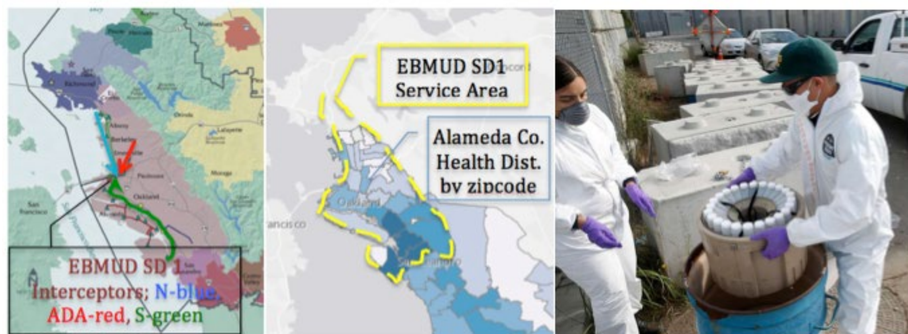
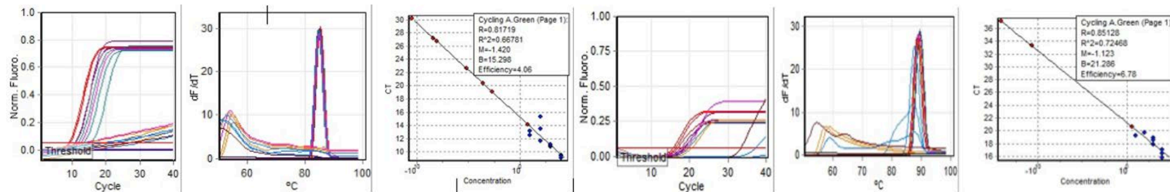


Figure 2. Left: EBMUD SD1 service area (mauve) with North (blue), Adaline (Red), and South (green) interceptors; Center: Alameda County Health District COVID-19 monitoring by zip code; Right: EBMUD SD1 staff retrieving 24-hour samples for compositing.

<u>Results</u>

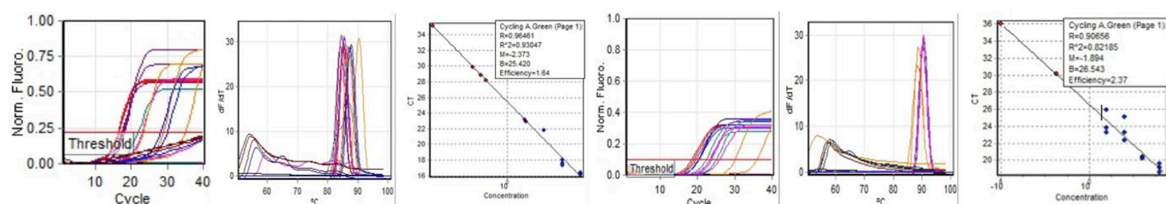
Initial testing of RT LAMP to determine basic performance characteristics was begun using primers to the ORF1a gene. Testing consisted of 5-logs reference standard dilution from 10^4-10^0, including raw sewage. No acceptable standard curve could be obtained although raw sewage produced consistent amplification with Ct ca. 20-35, details below. Performance of RT qLAMP was then compared for E-gene and N-gene primers, Figure 3a-f. Standard curve quality was improved but remained low and significant differences in synthetic control

141 amplification were apparent between E-gene and N-gene amplifications. We continued to
142 include raw sewage with continued apparent amplification. Using melt curves as indicator,
143 raw sewage SARS-CoV-2 RNA amplification appears specific. Not all replicates of either
144 standards or sewage produced product, generally, standards at < 10^2 copies gave
145 inconsistent reproducibility.



146 Figures 3a-f (left to right): E-gene amplification, melt, and standard curve; N-gene
147 amplification, melt, and standard curve.

148 Further comparison of E-gene and N-gene performance was made examining the effect of
149 reaction mix components, continuing to include raw sewage in amplification runs. Direct
150 comparison between the previous run, September 9, without alteration of conditions was
151 made on September 23. Standard curve quality for both E and N primers was improved.
152 Amplification of synthetic RNA standards was less consistent although more consistent
153 amplification from sewage was observed, Figures 4a-f.



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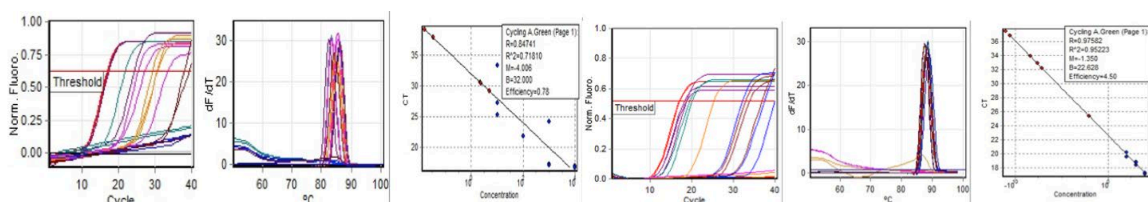
155 Figure 4a-f (left to right): E-gene amplification, melt, and standard curve; N-gene
156 amplification, melt, and standard curve.

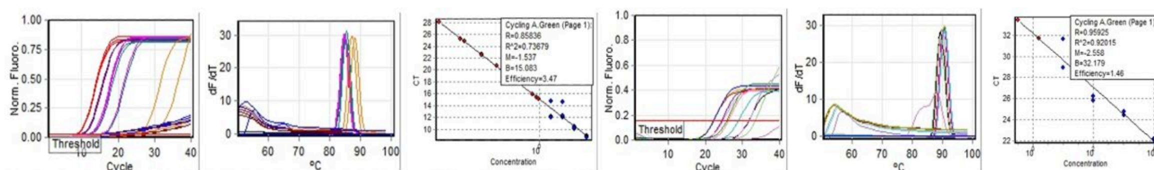
157 In companion runs on the same day, different mix components were used; NEB reaction mix
158 2019 was used instead of NEB reaction mix 1700.



160 Figure 5a-f (left to right): E-gene amplification, melt, and standard curve; N-gene
161 amplification, melt, and standard curve.

162 Raw sewage was collected again, October 1, 2020. Samples, 1 L each from interceptors N,
163 ADA, and S were composited from 24-hour discrete samplers. The 1 L samples were iced
164 for transport to Cel Analytical, San Francisco, and refrigerated for analysis, October 2, 2020.
165 Analysis consisted of preparing 25 µL reactions in triplicate in four separate runs, each
166 consisting of triplicate standards, Twist, M 118835 at dilutions 10^4-10^1, plus triplicate 7 µL
167 raw sewage from each of the three interceptors. Each of the four separate runs were
168 conducted using different primer sets: Run 13: N-gene primers, Broughton et al, Feb. 2020;
169 Run 14: E-gene primers, Broughton et al, Feb. 2020; Run 15, Figures 6a-f, and ORF1a
170 primers, Lamb et al., Feb. 2020; and Run 16: New England BioLabs E2019 Kit N2-gene +
171 E1-gene, Zhang et al, July 2020, Figures 7a-f.

172 The amplification efficiency for the Twist standards ranged by more than a factor of 2, with
173 the N-gene (Broughton et al, 2020) Figure 6d-f, least efficient, the E-gene (Broughton et al,
174 2020) most efficient, Figure 6a-c. Note that the offset (intercept, B) also ranged by a factor of
175 about 2, with N-gene highest and the E-gene lowest. A significant difference in the time to
176 initiation of amplification was observed with the E-gene amplifying in as little as 10 minutes,
177 N-gene slowest not amplifying until after 20 minutes. with the ORF1a and NEB N2+E1 in
178 between.

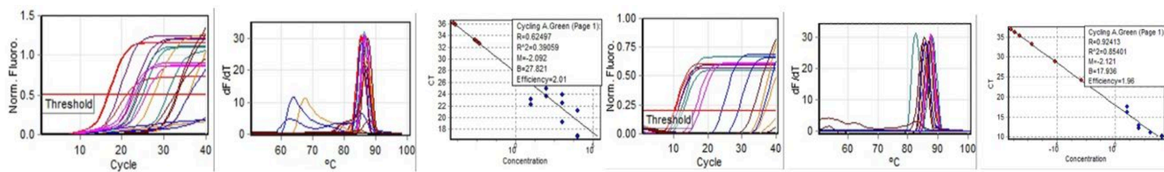
179 

180 Figure 6a-c (left to right). Run 14, E-gene amplification, melt curve, and standard
181 curve, and 6d-f Run 13, N-gene amplification, melt curve, and standard curve

182 Amplifications using the ORF1a primers were least consistent in both amplification and
183 melting temperature with no acceptable standard curve. Amplification using the NEB
184 combined N1+E1 primers was observed to initiate at times between that of the E-gene
185 and the Broughton N-gene primers, Figure 7d. Somewhat overlapping (slight peak

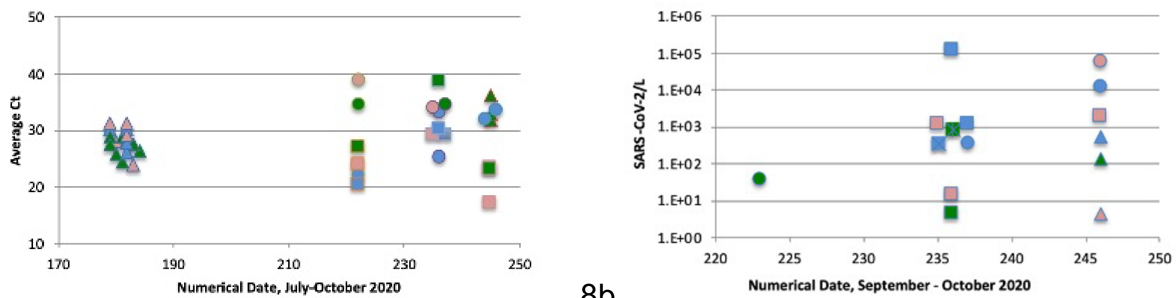
186 separation) in melt curves of the NEB primers suggested comparable amplification by

187 both N1 and E1 primers, Figure 7e.

188 

189 Figure 7a-c (left to right). Run 15, ORF1a-gene amplification, melt curve, and standard
190 curve, and 7d-f Run 16, NEB N1+E1 amplification, melt curve, and standard curve

191 As noted above, detection of SARS-CoV-2 in raw sewage was apparent throughout testing
192 of RT LAMP and RT qLAMP performance. Analysis includes four sets of raw sewage samples
193 from each of the three EBMUD SD1 interceptor sewers. Samples from July 29 produced only
194 qualitative results due to inability to produce a standard curve using the ORF1a primers.
195 Accordingly, all raw sewage amplification results are summarized in terms of Average Ct for
196 each of the four sampling dates (7.29, 9.9, 9.22, 10.1), Figure 8a. Based on more acceptable
197 performance resulting from amplifications summarized above, Figures 3, 4, 5, 6, and 7,
198 quantitative product was determined based on 7µL raw sewage component of the 25 µL
199 reaction mix, expressed as SARS-CoV-2/L, Figure 8b.



200 8 a 8b

201 Figure 8a. Raw sewage amplification product, Average Ct, samples Jul 29, Sep 9, Sep 22,
202 and Oct 1. Figure 8b. Representative calculated concentration, copies/L, samples Sep 9, Sep
203 22, Oct 1. Symbol Key: **Site**: N=Blue; ADA=Red; S=Green; **Gene**: ORF1a=triangle, N=circle;
204 E =square; **Mix**: 1700=filled; 2019=open. Note: some data points are plotted offset from
205 actual dates due to overlap.

206 **Discussion**

207 Finding that RNA from SARS-CoV-2 in raw sewage can be amplified directly without
208 pretreatment of concentration using RT LAMP may seem surprising. However, several
209 carefully considered factors support the strength of the findings. First, the RT LAMP process

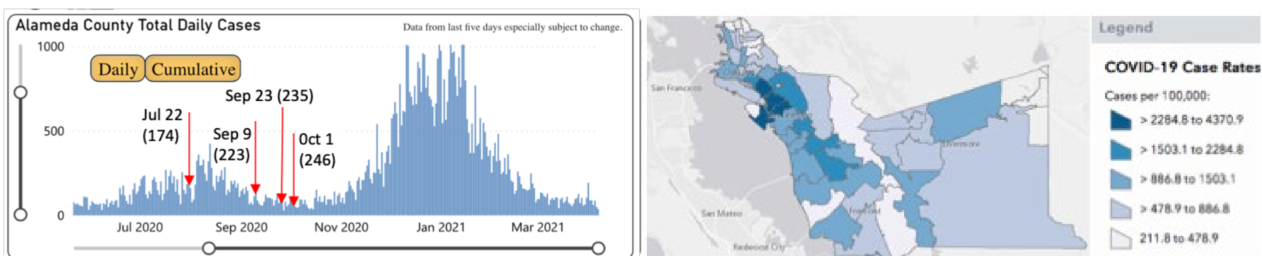
210 has been widely and successfully applied to detection of SARS-CoV-2 in clinical samples
211 beginning early this year, 2020, in response to COVID-19 monitoring needs, Thompson et al,
212 2020. The specific primers used in the work described here were selected from recent and
213 thorough work to develop effective diagnostic tools. Second, accumulating information from
214 both clinical assays and wastewater monitoring, e.g., Wu et al, July 2020, indicate that early
215 COVID-19 infection, likely preceding onset of symptoms, results in fecal shedding bursts,
216 estimated in the 10^{12}/day range. Based on this level, shedding by a single individual in
217 100,000 would contribute to a SARS-CoV-2 concentration at the sewage treatment plant of
218 ca. 2.5 x 10^{5}/L or ca. 1.5 in a 5 µL template volume for a 25 µL LAMP reaction. And, third,
219 LAMP as a process has been widely demonstrated to be sufficiently sensitive to amplify
220 template at this level, i.e., 1-10 target copies/µL. Finally, and equally important, due to the
221 multiple primer design and isothermal action polymerase, the LAMP process has selectivity
222 permitting specific amplification in the presence of extraneous nucleic acids and other
223 components of environmental media, sewage specifically that interfere with more common
224 PCR analytical methods.

225 The work described here should be considered preliminary although providing clear evidence
226 of SARS-CoV-2 detection. The poor performance of the ORF1a primers limiting quantification
227 may have been partly due to its timing early in the sequence of testing. Although we made
228 no procedural changes in succeeding runs, the quality of LAMP performance does appear to
229 improve over the course of the six-week testing period. Continuing work is in progress to
230 retest ORF1a performance.

231 Amplification characteristics observed differed between E-gene and N-gene primers, Figures
232 4, 5, and 6. Initiation of amplification appeared somewhat earlier with E-gene primers and
233 appreciably more product resulted. Variation in melt curve peaks was observed with E-gene
234 amplifications less apparent in N-gene results. The peak melting temperature for N-gene
235 product was 2-3° higher than for E-gene product.

236 The reactions in Figure 5 were conducted using the NEB E2019 reaction mix. It differs from
237 the E1700 mix used in Figure 4 reactions by inclusion of dTTP, dUTP, and a thermolabile
238 uracil DNA glycosylase (UDG). With consistent use in a sequence of amplification runs and
239 incorporation of dU into amplification products, the presence of UDG will prevent potential
240 carryover from previous reactions but will not affect amplification of the subsequent run due
241 to complete inactivation at 65°C. Our reactions had no predecessors using the E2019 mix so
242 that effects observed would have been due to action of the additional mix components on
243 amplification of both control synthetic RNA and components in the raw sewage. Whatever
244 the mechanism, use of the mix increased product formation from both E and N gene primers,
245 and appreciably improved the efficiency of N-gene reactions. It is important to note that
246 although the NEB E2019 kit is supplied with E-gene and N-gene primers (both sets different
247 from the E and N-gene primers used in our work (Table 1)), except for Run 16 (Oct 1, Figure
248 7d-f) we did not use the NEB primers. All other reactions used only the Table 1 primers.

249 Ability to detect SARS-CoV-2 in sewage is fundamentally dependent on the extent of COVID-
250 19 infection in the community. A reasonably detailed record of infection history in the EBMUD
251 SD1 service area is maintained by the Alameda County Health Department (ACHD), ACHD,
252 2020. From the end of July through September corresponding to the period of our sewage
253 analysis, the daily reported cases averaged ca. 150/day or for a population of just over
254 150,000, ca. 10/100,000 per day, Figure 9. The data show highly differentiated rates of
255 infection among areas of the County and sewerage service area, cataloged by zipcode.

256 

257 Figure 9. COVID-19 cases reported daily, Jun 2020-Mar 2021 w/ sewage sampling
258 dates, and cumulative cases/100,000 by postal code, Alameda County, California.

259 The total cases reported, March 15 to September 30 (200 days) was 21,240, an average of
260 100 per day or 6.3/100,000 per day. Cumulative case rates among post code areas range
261 from ca. 300/100,000 to nearly 2000/100,000. Understanding approximate incidence rates is
262 important to the utility of monitoring sewage for understanding COVID-19 dynamics.

263 Considering how monitoring for SARS-CoV-2 in sewage may be of use in control of
264 transmission several factors must be taken into account. These considerations have a direct
265 bearing on the LAMP method described here and on needs for its refinement. A critical factor
266 is the shedding rate in early, perhaps pre-symptomatic infections. Calculations described
267 above indicate that detectable virus would be present in sewage for shedding at 10^{12} per day
268 for a single infection/100,000 population. But, detection at this level is not a challenge to
269 current detection methods. Many reports have shown that ample SARS-CoV-2 can be
270 measured at the sewage treatment plant indicating only that infection is amply distributed in
271 the community. To be useful for infection control the need is to be able to identify and if
272 possible to isolate and trace contacts of the small number of early, high, perhaps super
273 spreading, infections. Accordingly, monitoring focused on population concentrations of 100-
274 1000 such as in institutions, hotels, multistory apartment building, industrial sites, will
275 increase the sensitivity to detect by factors of 100 to 1000 in relation to the original
276 assumption of 1 infection/100,000.

277 Recognizing that the real value of a method is ability to apply it to focused upstream sampling
278 serves as a guide to features of the analytical method needing refinement and optimization.
279 The most challenging problem of an RT qLAMP is refinement at minimal target concentration.
280 While theoretically capable of amplification from a single copy, i.e., a single SARS-CoV-2 in
281 a volume compatible with a 25 μL reaction mix for example, i.e., 5-10 μL, if the concentration
282 in the sewage being sampled is that low, Poisson statistics dictate that a single copy will be
283 present in only a minor proportion of replicated 5-10 μL aliquots analyzed. However, as
284 suggested above, upstream sampling magnifies the concentration from a single shedder in

285 direct proportion to the reduced population in the target sewage source. Thus, refinement of
286 a RT qLAMP procedure for lowest limit of detection would not be important. Demonstration
287 of consistently reproducible amplification at moderate concentrations is essential. Such
288 demonstration should be readily achievable. Additional features require further attention
289 including treatment of samples to make viral RNA available for amplification. Pretreatment of
290 samples to release and denature viral RNA is important. The Mg^{++} concentration is a reaction
291 mix component important to optimize. The primer-polymerase combination is sensitive to the
292 total Mg^{++} concentration, optimized for our primers at 8 mM. Raw sewage includes Mg^{++}; in
293 our raw sewage samples ca. 33 mg/L. We did not adjust or re optimize to take this into
294 account for the work described here. Future work must account for this component, likely to
295 vary widely from among communities. Finally, the samples prepared by EBMUD SD1 staff
296 were 24-hour composite samples. Samples collected at upstream sources are more likely to
297 be grab samples and may be subject to variation over the typical 24-hour cycle of human
298 activities that affect sewage composition around the clock.

299 Features of an RT qLAMP that are attractive for the type of application outlined above are
300 those referenced in virtually all publications describing its advantages: LAMP is faster,
301 cheaper, highly specific, insensitive to interferences, and more flexible than the RT qPCR
302 procedure, currently used almost exclusively for sewage monitoring of SARS-CoV-2. Sewage
303 samples collected in the morning, returned to the lab can be combined with reaction mix
304 components immediately and amplified, using the same thermocycler (or simple water bath)
305 programmed for constant temperature, producing interpretable results in less than an hour.
306 No preprocessing for virus concentration, RNA extraction, and interference mitigation is
307 needed saving time, effort, and materials.

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Figure 1

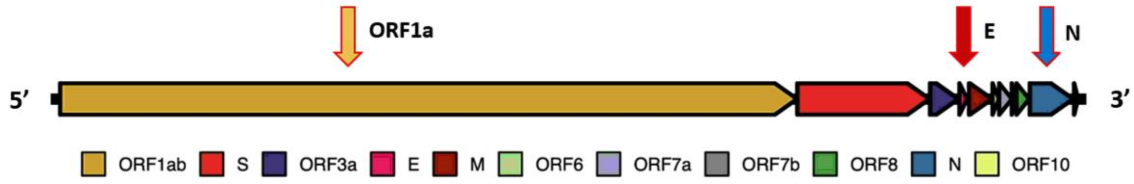
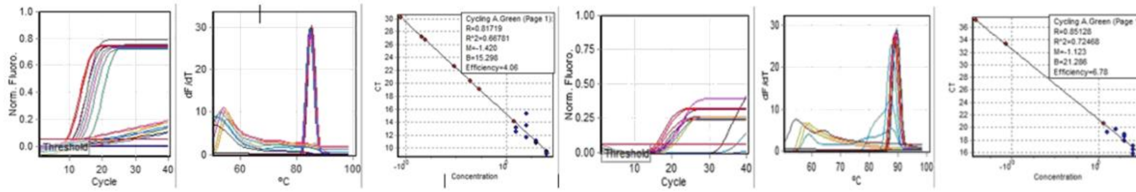


Figure 2

Figure 3



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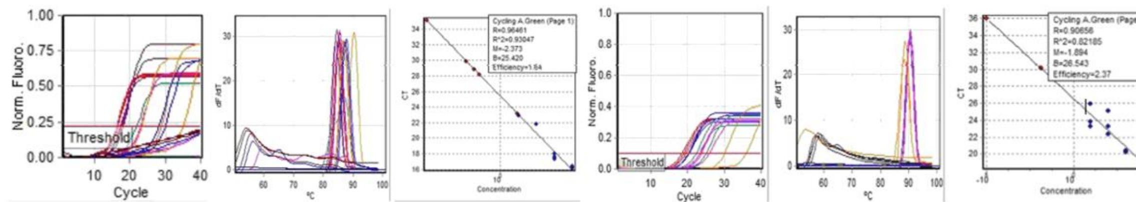

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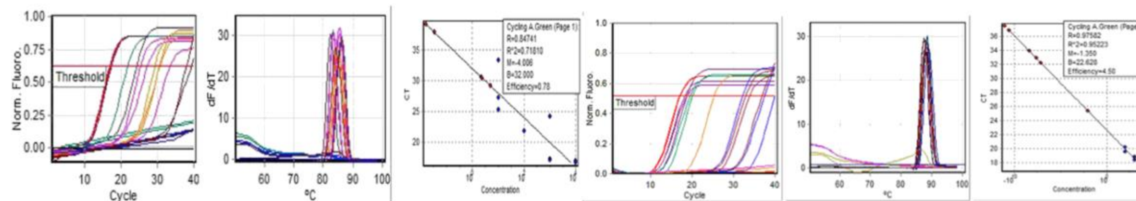

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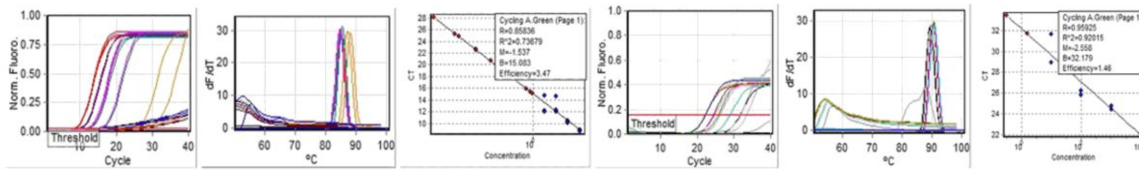

Figure 7

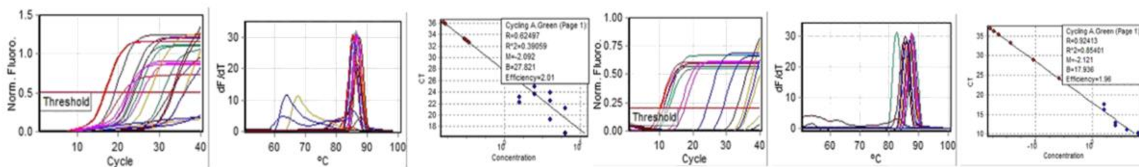

Figure 8

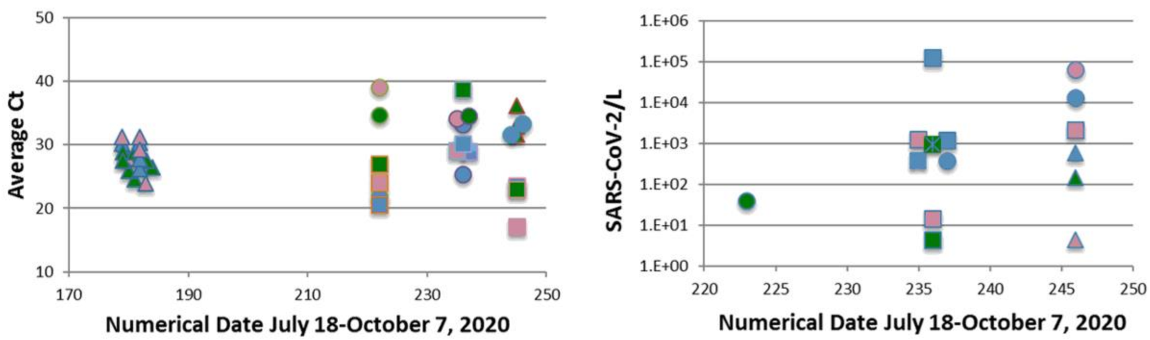

Figure 9