Electronic Supplementary Information

Rapid and Quantitative Loop-mediated Isothermal Amplification (LAMP) Assays for Discriminatory Detection of *Vibrio cholerae*

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Contents

Digital PCR (dPCR) assay

In this study, the PCR primers and probes targeting *ompW*, *O1rfb*, *O139rfb*, *tcpA* were designed, and their sequences are provided in the table below**.** FAM, HEX, and Cy5 were used in the probes for each target. The as-designed primers and probes were purchased from Microsynth AG (Balgach, Switzerland). Standard desalting was used for all primers and HPLC purification steps were included for probes. The primers were shipped as solids and suspended in nuclease-free water prior to use. The suspended primers were stored at -20 ˚C.

Table. Sequences for PCR primers and probes for *ompW*, *O1rfb*, *O139rfb*, and *tcpA*

Target gene		Sequences (5'-3')
ompW	Forward	CACCAAGAAGGTGACTTTATTGTG
	Reverse	GGAAAGTCGAATTAGCTTCACCAA
	Probe	FAM/ACATAAGATTTCTACCTCTGGTGGT/BHQ-1
O1rfb	Forward	GTTTCACTGAACAGATGGG
	Reverse	GGTCATCTGTAAGTACAAC
	Probe	HEX/CATGCCTATTCTGACGTAAT/BHQ-1
O139rfb	Forward	TGGGATGCCAGTCATGCTGT
	Reverse	GTCAAACCCGATCGTAAAGG
	Probe	Cy5/CACTGTGGTGGGTATTTTAC/BHQ-2
tcpA	Forward	TTTCCACGAAACTCTGCA
	Reverse	ACTTAATTACGCCAGCGC
	Probe	FAM/TGCTTGGGTCAAGCCACC/BHQ1

The 25-µL of PCR reaction mixture contained 2.5 and 1.0 µL of Buffer A and B from the naica® multiplex PCR MIX (Stilla Technologies, France; R10104), 0.1 µM of fluorescein sodium salt (VWR, Cat. No. 0681-100G), 500 nM forward and reverse primers, 200 nM probe, 5 µL of DNA template. Each PCR reaction component was stored at -20 ˚C and thawed on ice immediately prior to assay preparation. The components were mixed in a PCR box except the DNA template was added in a separate area to avoid possible cross-contamination. After adding the DNA template, the reaction mixture was gently vortexed and spun down. The mixture was loaded into Sapphire Chip (Stilla Technologies, Villejuif, France), and the Chip was transferred to Geode (automated droplet generator and thermocycler; Stilla Technologies, France). The reaction condition consisted of 12 minutes at 40 °C of partitioning mastermix into droplets under a 1.3 psi pressure, followed by 45 cycles of denaturation step at 95 ˚C for 10 s and the annealing/extension step at 59 ˚C for 1 minute, and a release step that lowers the pressure and temperature**.** After the reaction, the Chips were transferred into the Naica Prism3 (three-color fluorescence imager; blue, green, red channels) and scanned using Crystal Reader (Stilla Technologies, France).

The scanned images in the Chips were analyzed using Crystal Miner software (Stilla Technologies, France). The total number of generated droplets was counted and as a quality control, the sample was considered failed when the number was < 15,000. The number of positive and negative droplets were counted based on their fluorescence signals for three-color channels (blue, green, and red). The Crystal Miner software automatically calculateed the absolute concentration in the unit of gc/µL in the reaction using Poisson distribution analysis to the number of positive droplets out of the total.

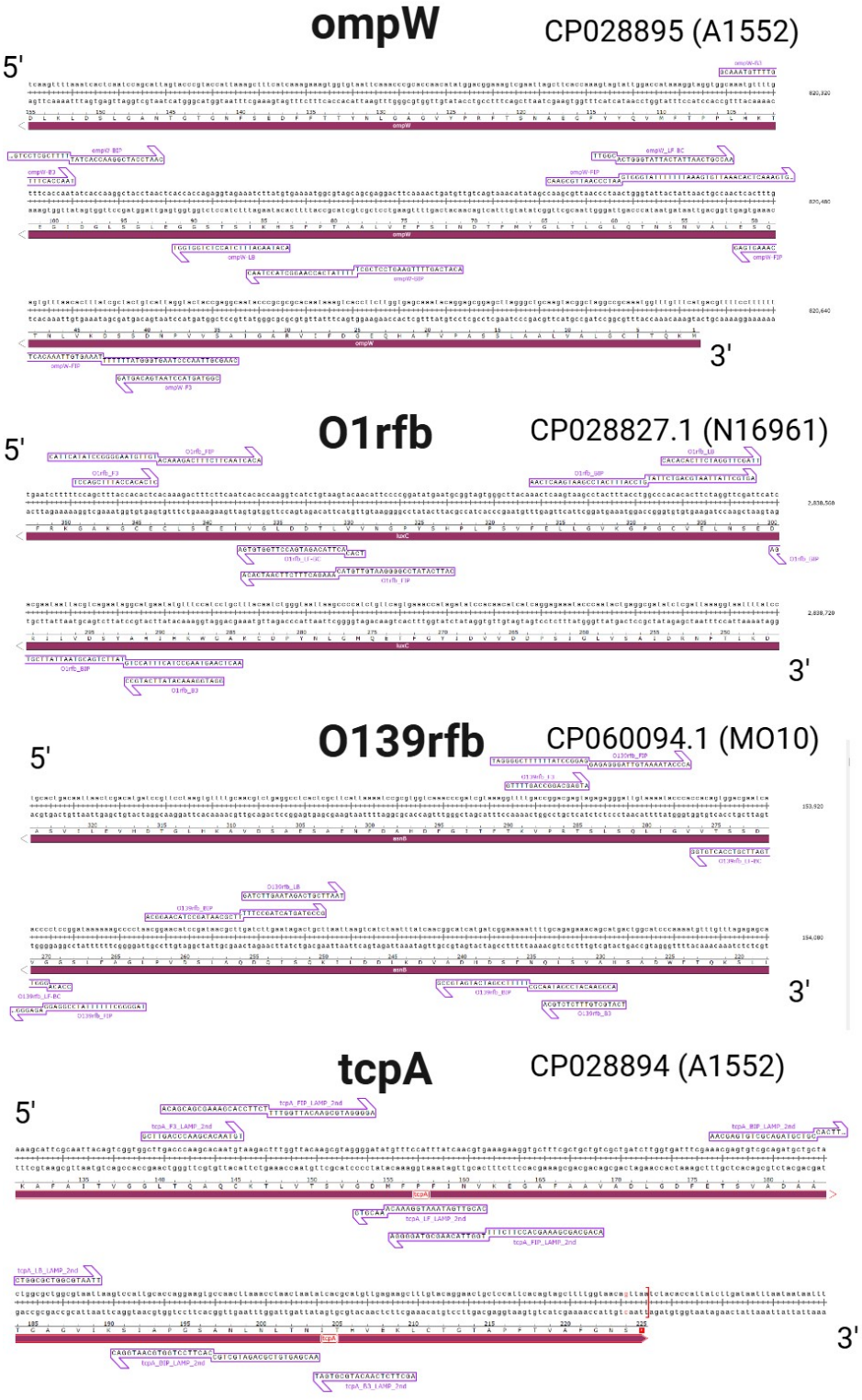

Figure S1 Alignment of loop-mediated isothermal amplification (LAMP) primers for four molecular indicators of *Vibrio cholerae* (*V. cholerae*) against reference genomes, *ompW*, *O1rfb*, *O139rfb*, and *tcpA* using the SnapGene software (v. 7.0.1, GSL Biotech LLC, Boston, MA).

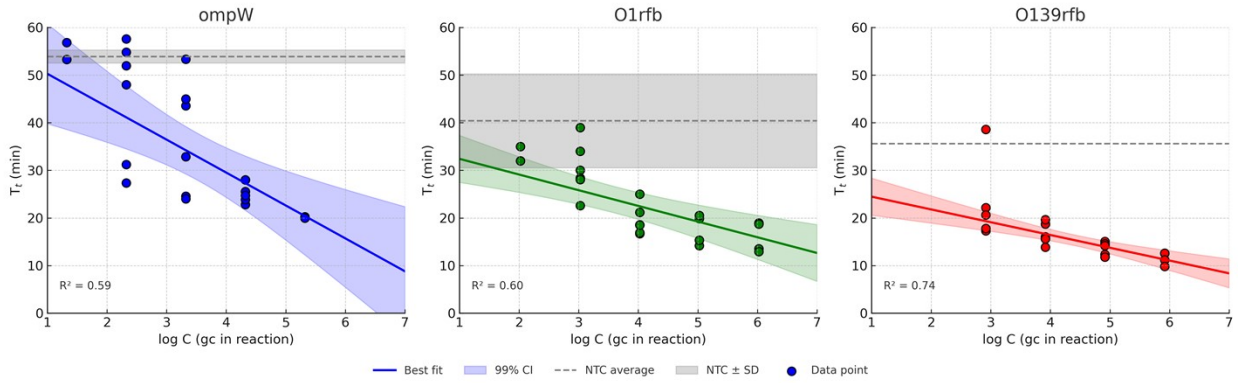

Figure S2 Three loop-mediated isothermal amplification (LAMP) assays for detection of *Vibrio cholerae* (*V. cholerae*) O1 and O139 serogroups. Three molecular markers, *ompW* for *V. cholerae*, *O1rfb* and *O139rfb* for respective serogroups, were targeted by each assay. The plots between the logarithmic concentration and the time to reach fluorescence threshold, T_t values, for each assay were established. Data points indicate different LAMP reactions. A solid line and shaded area indicate a best-fit line with R^2 value and 95% confidence interval (CI) with respective colors. Dashed horizontal lines and the shaded region indicate the T_t mean for NTCs and its standard deviation.

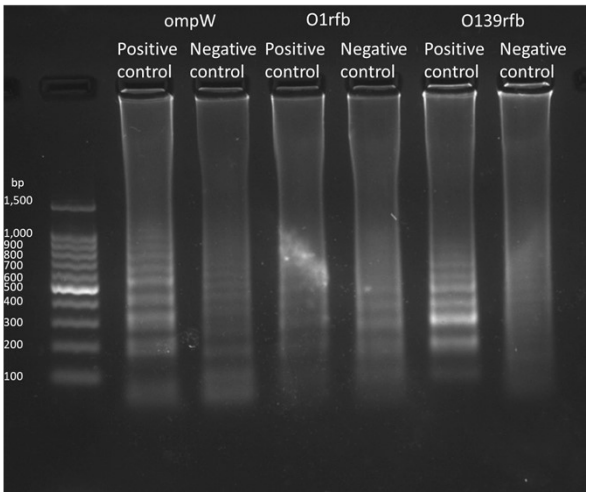

Figure S3 Gel electrophoresis of LAMP products from positive controls (~10^4 gc/µL) and no-template control (NTC) for three singplex LAMP assays: ompW, O1rfb, and O139rfb.

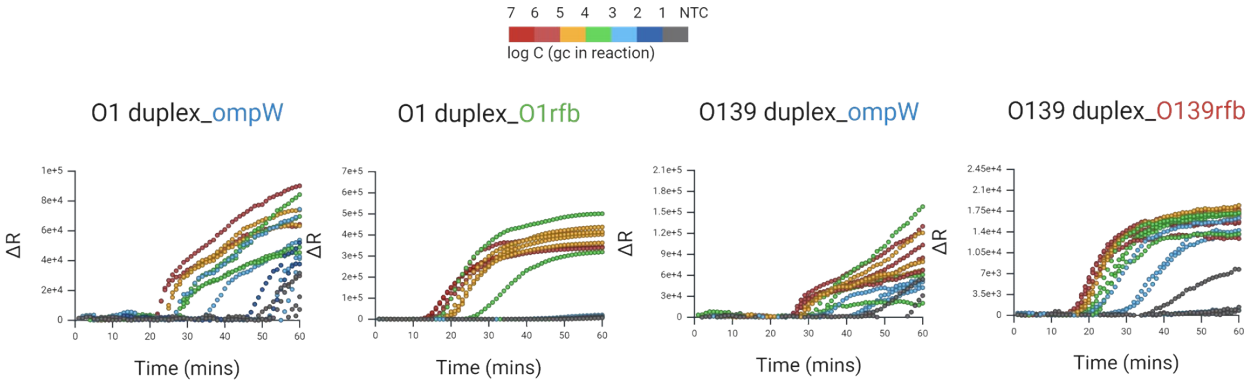

Figure S4 Two duplex loop-mediated isothermal amplification (LAMP) assays for the detection of *Vibrio cholerae* (*V. cholerae*) O1 and O139 serogroups: the *ompW-O1rfb* and *ompW-O139rfb* assay. Real-time monitoring of relative fluorescence signal, ΔR, over 60 minutes, measured every minute with serially diluted synthetic segments of targets and no-template controls (NTCs). Double synthetic gene segments for each duplex assay were prepared.

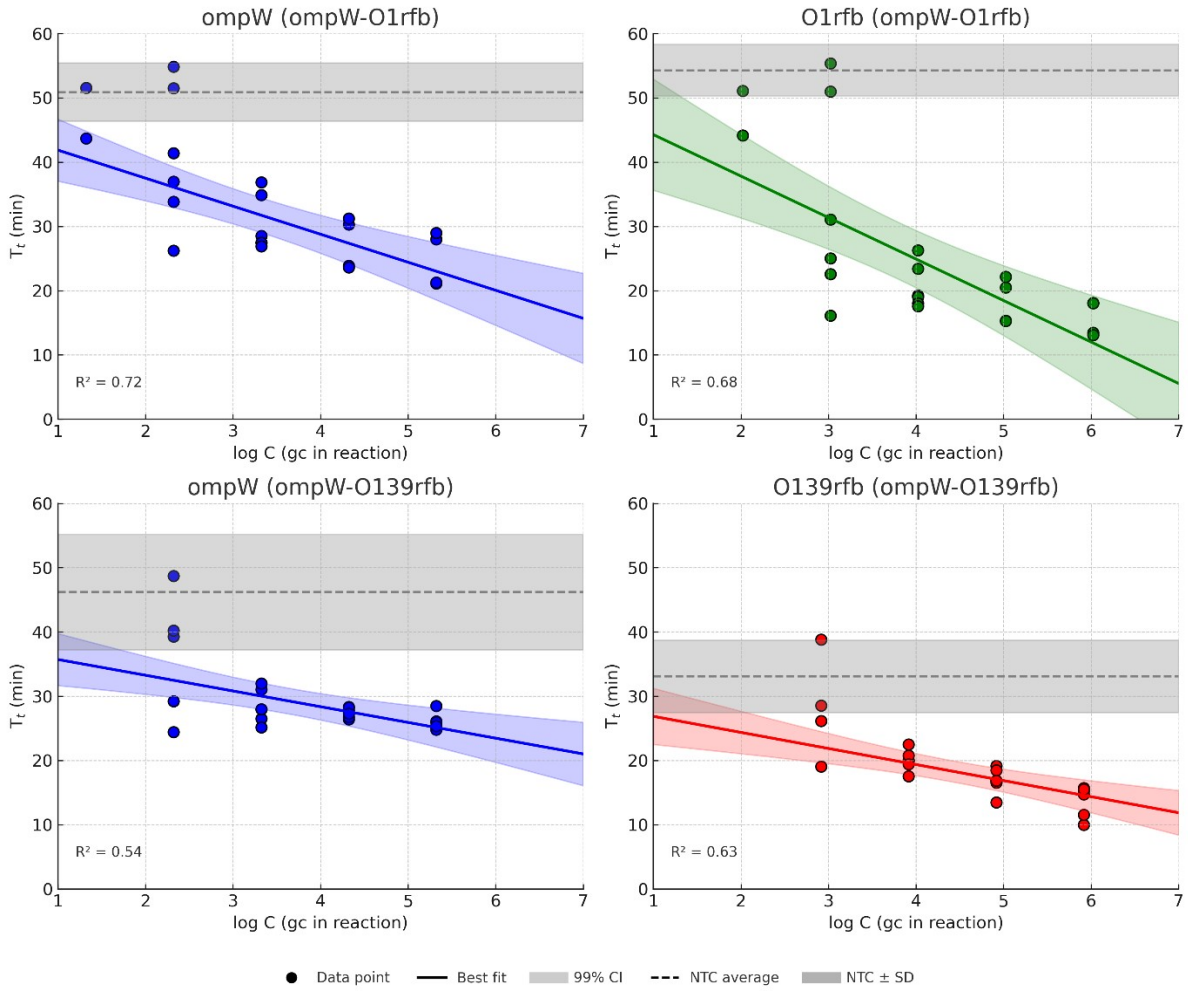

Figure S5 Two duplex loop-mediated isothermal amplification (LAMP) assays for detection of *Vibrio cholerae* (*V. cholerae*) O1 and O139 serogroups. Three molecular markers, *ompW* for *V. cholerae*, *O1rfb* and *O139rfb* for respective serogroups, were targeted by each assay. The upper two images contain the data of the *ompW-O1rfb* duplex and the lower two graphs the results of the *ompW-O139rfb* duplex LAMP assay. The plots between the logarithmic concentration of the synthetic segments and the time to reach threshold, T_t values, for each assay were established. Data points indicate different LAMP reactions. The solid line and surrounding shaded area represent the best-fit line with R^2 value and 95% confidence interval (CI) with respective colors. Dashed horizontal lines and the shaded region indicate the T_t mean for NTCs and its standard deviation.

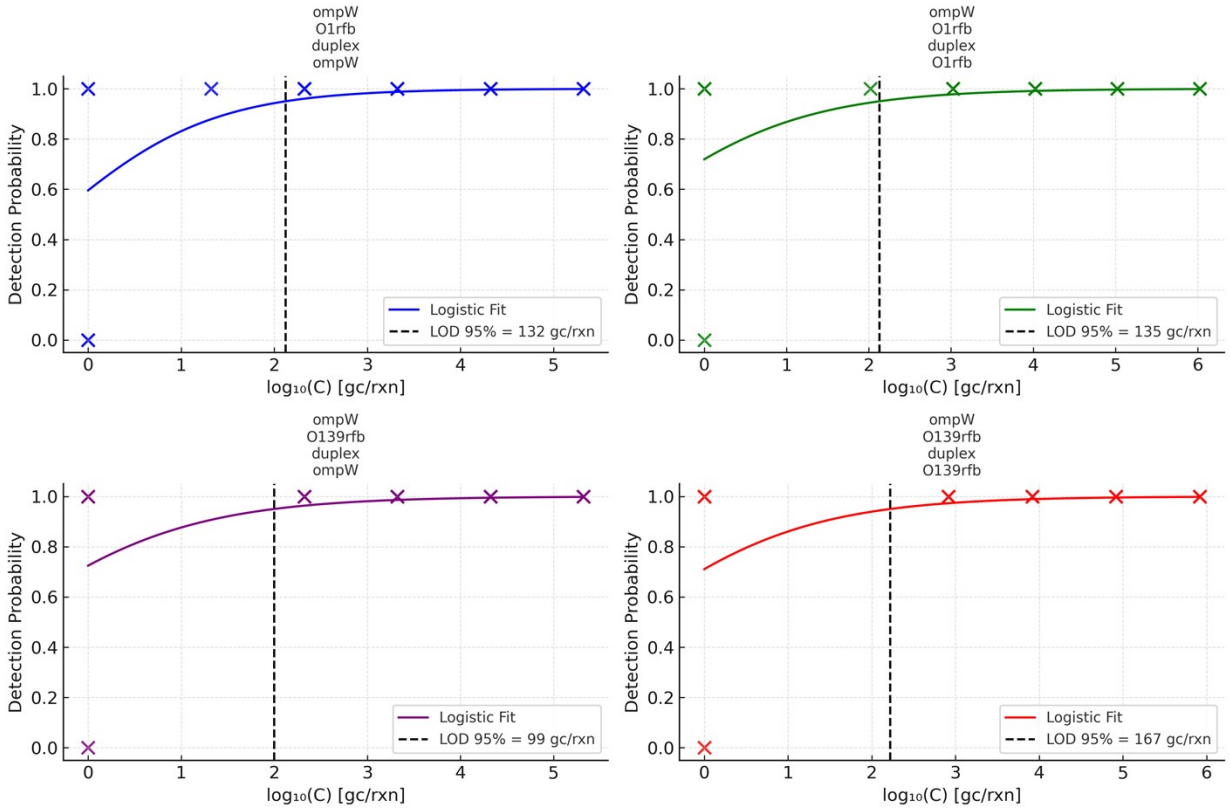

Figure S6 Calculation of limit of detection (LOD) for two duplex loop-mediated isothermal amplification (LAMP) assays, *ompW-O1rfb* (top) and *ompW-O139rfb* (bottom), using a logistic regression-based method. Detection probability of duplex assay. The LODs were defined as the target concentration where the detection probability was 0.95, indicated by a vertical dashed line.

Table S1 Hetero-dimer analysis across loop-mediated isothermal amplification (LAMP) forward, inner, and loop (F3, B3, FIP, BIP, LF, and LB) primers targeting *ompW*, *O1rfb*, and *O139rfb*. Risk level was classified by ΔG (Gibbs free energy, kcal/mol) from low (ΔG > -9), moderate (-12 < ΔG <-9), and high (ΔG <-12)

Primer 1	Primer 2	ΔG (kcal/mol)	Primer interaction	Risk level
$F3_{ompW}$	$B3_{ompW}$	-4.41	ompW <-> ompW	Low
$F3_{ompW}$	FIP_{ompW}	-5.37		Low
$F3_{ompW}$	BIP_{ompW}	-6.97		Low
$F3_{ompW}$	LF_{ompW}	-5.37		Low
$F3_{ompW}$	LB_{ompW}	-6.97		Low
$B3_{ompW}$	FIP_{ompW}	-7.18		Low
$B3_{ompW}$	BIP_{ompW}	-5.84		Low
$B3_{ompW}$	LF_{ompW}	-6.97		Low
$B3_{ompW}$	LB_{ompW}	-8.32		Low
FIP_{ompW}	BIP_{ompW}	-10.29		Moderate
FIP_{ompW}	LF_{ompW}	-7.48		Low
FIP_{ompW}	LB_{ompW}	-5.37		Low
BIP_{ompW}	LF_{ompW}	-6.97		Low
BIP_{ompW}	LB_{ompW}	-8.32		Low
LF_{ompW}	LB_{ompW}	-5.37		Low
$F3_{O1rfb}$	$B3_{O1rfb}$	-6.6	O1rfb <-> O1rfb	Low
$F3_{O1rfb}$	FIP_{O1rfb}	-5.49		Low
$F3_{O1rfb}$	BIP_{O1rfb}	-6.68		Low
$F3_{O1rfb}$	LF_{O1rfb}	-6.59		Low
$F3_{O1rfb}$	LB_{O1rfb}	-4.47		Low
$B3_{O1rfb}$	FIP_{O1rfb}	-6.71		Low
$B3_{O1rfb}$	BIP_{O1rfb}	-3.89		Low
$B3_{O1rfb}$	LF_{O1rfb}	-5		Low
$B3_{O1rfb}$	LB_{O1rfb}	-3.52		Low
FIP_{O1rfb}	BIP_{O1rfb}	-5.49		Low
FIP_{O1rfb}	LF_{O1rfb}	-6.82		Low
FIP_{O1rfb}	LB_{O1rfb}	-4.99		Low
BIP_{O1rfb}	LF_{O1rfb}	-5.86		Low
BIP_{O1rfb}	LB_{O1rfb}	-6.01		Low
LF_{O1rfb}	LB_{O1rfb}	-6.59		Low
$F3_{O139rfb}$	$B3_{O139rfb}$	-3.53	O139rfb <-> O139rfb	Low
$F3_{O139rfb}$	$FIP_{O139rfb}$	-11.32		Moderate
$F3_{O139rfb}$	$BIP_{O139rfb}$	-8.26		Low
$F3_{O139rfb}$	$LF_{O139rfb}$	-11.17		Moderate
$F3_{O139rfb}$	$LB_{O139rfb}$	-2.94		Low
$B3_{O139rfb}$	$FIP_{O139rfb}$	-4.77		Low
$B3_{O139rfb}$	$BIP_{O139rfb}$	-6.96		Low
$B3_{O139rfb}$	$LF_{O139rfb}$	-4.89		Low
$B3_{O139rfb}$	$LB_{O139rfb}$	-5.09		Low
$FIP_{O139rfb}$	$BIP_{O139rfb}$	-8.26		Low

$FIP_{O139rfb}$	$LF_{O139rfb}$	-7.48		Low
$FIP_{O139rfb}$	$LB_{O139rfb}$	-3.42		Low
$BIP_{O139rfb}$	$LF_{O139rfb}$	-5.19		Low
BIP_{O1rfb}	LB_{O1rfb}	-4.62		Low
LF_{O1rfb}	LB_{O1rfb}	-4.99		Low
$F3_{ompW}$	$F3_{O1rfb}$	-5.37	ompW <-> O1rfb	Low
$F3_{ompW}$	$B3_{O1rfb}$	-5		Low
$F3_{ompW}$	FIP_{O1rfb}	-6.68		Low
$F3_{ompW}$	BIP_{O1rfb}	-5.5		Low
$F3_{ompW}$	LF_{O1rfb}	-4.41		Low
$F3_{ompW}$	LB_{O1rfb}	-6.97		Low
$B3_{ompW}$	$F3_{O1rfb}$	-3.89		Low
$B3_{ompW}$	$B3_{O1rfb}$	-8.76		Low
$B3_{ompW}$	FIP_{O1rfb}	-7.18		Low
$B3_{ompW}$	BIP_{O1rfb}	-4.87		Low
$B3_{ompW}$	LF_{O1rfb}	-10.26		Moderate
$B3_{ompW}$	LB_{O1rfb}	-4.41		Low
FIP_{ompW}	$F3_{O1rfb}$	-6.68		Low
FIP_{ompW}	$B3_{O1rfb}$	-5.24		Low
FIP_{ompW}	FIP_{O1rfb}	-6.83		Low
FIP_{ompW}	BIP_{O1rfb}	-7.79		Low
FIP_{ompW}	LF_{O1rfb}	-8.18		Low
FIP_{ompW}	LB_{O1rfb}	-8.18		Low
BIP_{ompW}	$F3_{O1rfb}$	-4.74		Low
BIP_{ompW}	$B3_{O1rfb}$	-6.21		Low
BIP_{ompW}	FIP_{O1rfb}	-9.02		Moderate
BIP_{ompW}	BIP_{O1rfb}	-9.41		Moderate
BIP_{ompW}	LF_{O1rfb}	-14.93		High
BIP_{ompW}	LB_{O1rfb}	-6.97		Low
LF_{ompW}	$F3_{O1rfb}$	-6.62		Low
LF_{ompW}	$B3_{O1rfb}$	-8.16		Low
LF_{ompW}	FIP_{O1rfb}	-3.9		Low
LF_{ompW}	BIP_{O1rfb}	-6.21		Low
LF_{ompW}	LF_{O1rfb}	-6.97		Low
LF_{ompW}	LB_{O1rfb}	-3.29		Low
LB_{ompW}	$F3_{O1rfb}$	-7.71		Low
LB_{ompW}	$B3_{O1rfb}$	-5.46		Low
LB_{ompW}	FIP_{O1rfb}	-5.12		Low
LB_{ompW}	BIP_{O1rfb}	-4.41		Low
LB_{ompW}	LF_{O1rfb}	-5.13		Low
LB_{ompW}	LB_{O1rfb}	-6.01		Low
$F3_{ompW}$	$F3_{O139rfb}$	-8.02	ompW <-> O139rfb	Low
$F3_{ompW}$	$B3_{O139rfb}$	-5		Low
$F3_{ompW}$	$FIP_{O139rfb}$	-6.68		Low
$F3_{ompW}$	$BIP_{O139rfb}$	-6.68		Low

$F3_{ompW}$	$LF_{O139rfb}$	-6.24		Low
$F3_{ompW}$	$LB_{O139rfb}$	-4.89		Low
$B3_{ompW}$	$F3_{O139rfb}$	-5.84		Low
$B3_{ompW}$	$B3_{O139rfb}$	-5.09		Low
$B3_{ompW}$	$FIP_{O139rfb}$	-5.89		Low
$B3_{ompW}$	$BIP_{O139rfb}$	-6.71		Low
$B3_{ompW}$	$LF_{O139rfb}$	-5.02		Low
$B3_{ompW}$	$LB_{O139rfb}$	-5.47		Low
FIP_{ompW}	$F3_{O139rfb}$	-7.42		Low
FIP_{ompW}	$B3_{O139rfb}$	-7.18		Low
FIP_{ompW}	$FIP_{O139rfb}$	-17.7		High
FIP_{ompW}	$BIP_{O139rfb}$	-14.54		High
FIP_{ompW}	$LF_{O139rfb}$	-11.02		Moderate
FIP_{ompW}	$LB_{O139rfb}$	-6.68		Low
BIP_{ompW}	$F3_{O139rfb}$	-6.78		Low
BIP_{ompW}	$B3_{O139rfb}$	-3.55		Low
BIP_{ompW}	$FIP_{O139rfb}$	-7.82		Low
BIP_{ompW}	$BIP_{O139rfb}$	-6.96		Low
BIP_{ompW}	$LF_{O139rfb}$	-5.02		Low
BIP_{ompW}	$LB_{O139rfb}$	-5.5		Low
LF_{ompW}	$F3_{O139rfb}$	-4.41		Low
LF_{ompW}	$B3_{O139rfb}$	-5.09		Low
LF_{ompW}	$FIP_{O139rfb}$	-13.81		High
LF_{ompW}	$BIP_{O139rfb}$	-8.16		Low
LF_{ompW}	$LF_{O139rfb}$	-5.02		Low
LF_{ompW}	$LB_{O139rfb}$	-6.32		Low
LB_{ompW}	$F3_{O139rfb}$	-4.41		Low
LB_{ompW}	$B3_{O139rfb}$	-3.43		Low
LB_{ompW}	$FIP_{O139rfb}$	-7.84		Low
LB_{ompW}	$BIP_{O139rfb}$	-3.54		Low
LB_{ompW}	$LF_{O139rfb}$	-6.37		Low
LB_{ompW}	$LB_{O139rfb}$	-6.59		Low

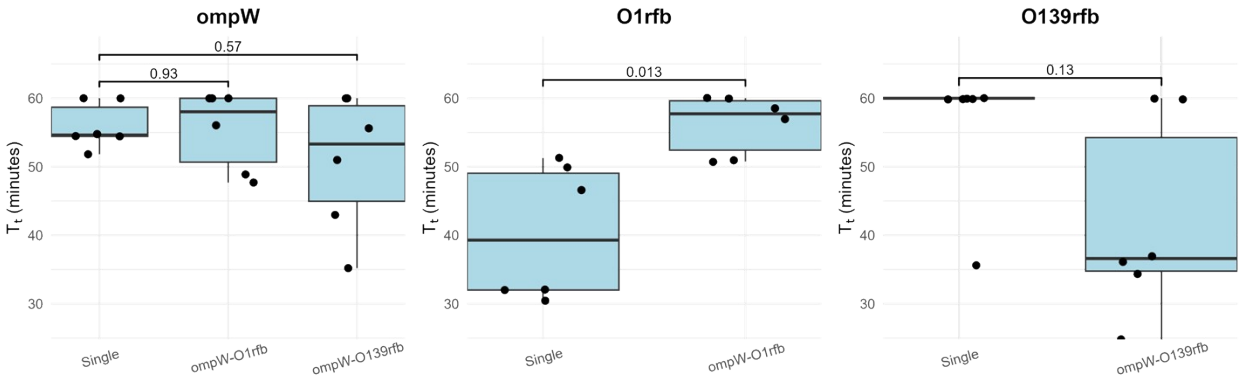

Figure S7 Pair-wise comparison of mean of time to reach threshold, T_t values, for no-template controls (NTCs) for single and duplex LAMP assays targeting *ompW*, *O1rfb* and *O139rfb*. The boxplot indicates the distribution of data points for NTCs. T_t values for the reaction to have no amplification within reaction period were set to 60 minutes.

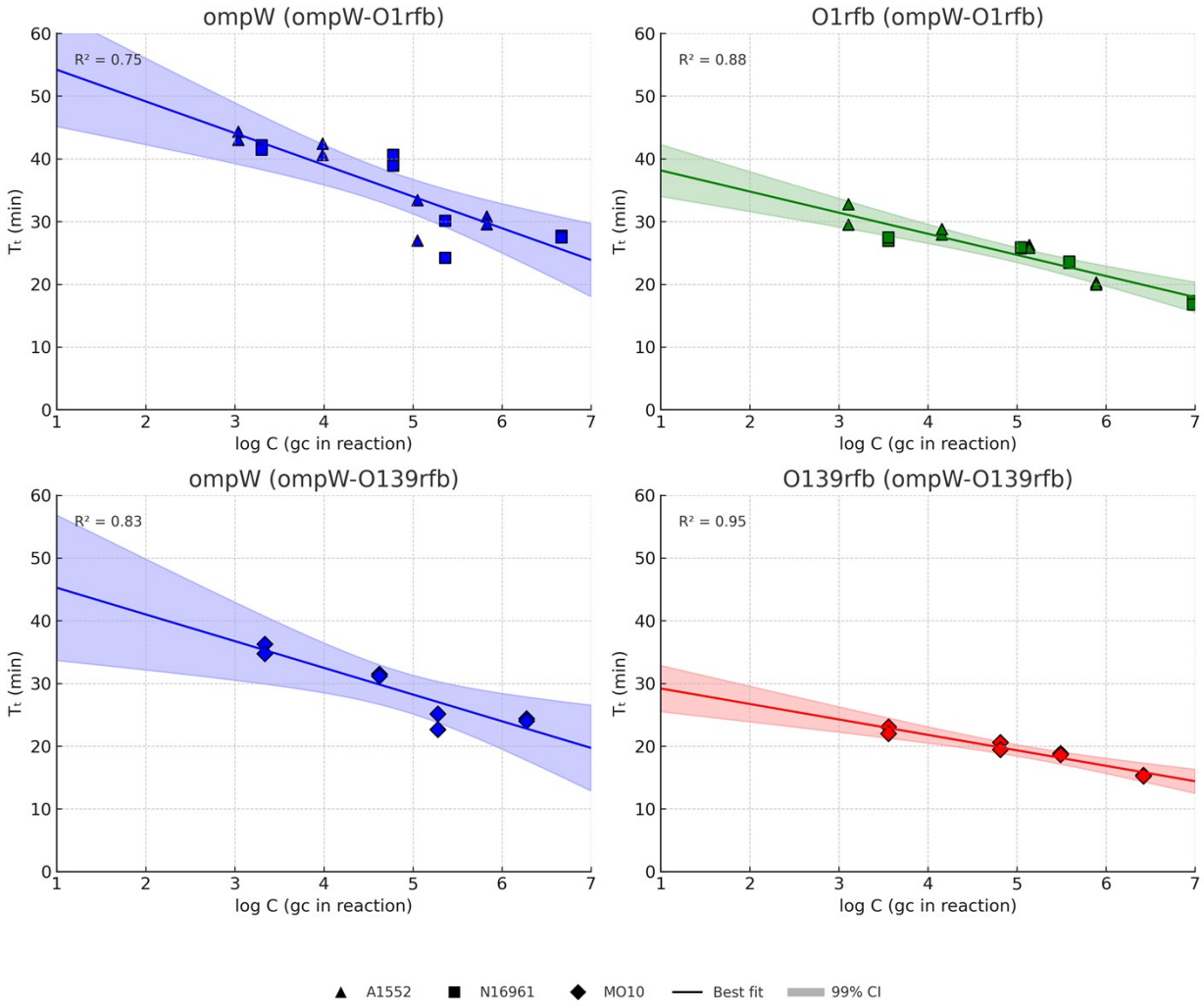

Figure S8 Two duplex loop-mediated isothermal amplification (LAMP) assays for three for *V. cholerae* molecular markers, *ompW*, *O1rfb* and *O139rfb*: (top) *ompW-O1rfb* duplex and (bottom) *ompW-O139rfb* duplex. (Left) The plots between the logarithmic concentration of the synthetic segments and the time to reach threshold, T_t, values for each assay were established. (Right) The plots between the logarithmic concentration of the DNA extracts from three strains and the time to reach the threshold. Data points indicate different LAMP reactions. A solid line and shaded area represents the best-fit line with R^2 value and 95% confidence interval (CI) with respective colors. Dashed horizontal lines and the shaded region correspond to the T_t mean for NTCs and its standard deviation.

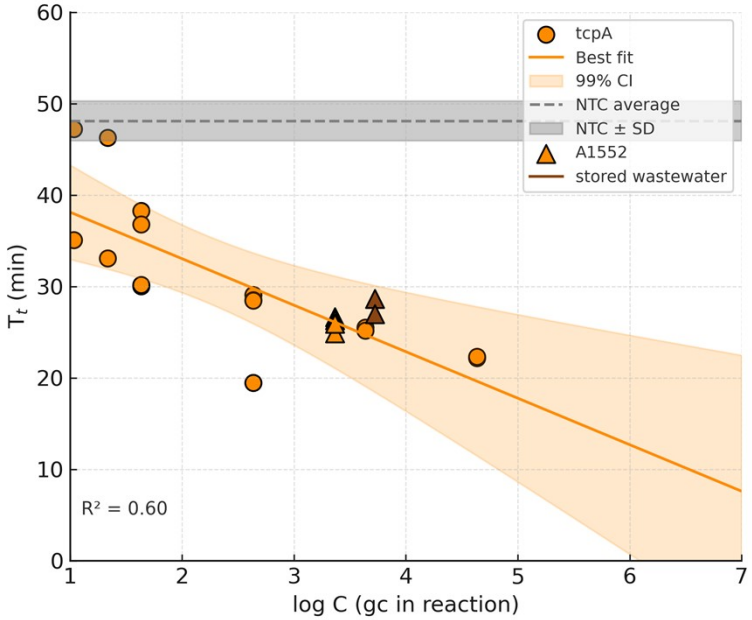

Figure S9 The plot between the logarithmic concentration of the synthetic segment and the time to reach threshold, T_t, values for the assay was established. Data points represent different LAMP reactions for synthetic segments (orange circle), DNA extracts from the *V. cholerae* A1552 strain in phosphate-buffered saline (PBS) (orange triangle), and in stored wastewater (dark brown triangle). A solid line and shaded area correspond to the best-fit line with its R^2 value, and 95% confidence interval (CI) retrieved from data points for synthetic segments. Dashed horizontal lines and the shaded region indicate the T_t mean for NTCs and its standard deviation.

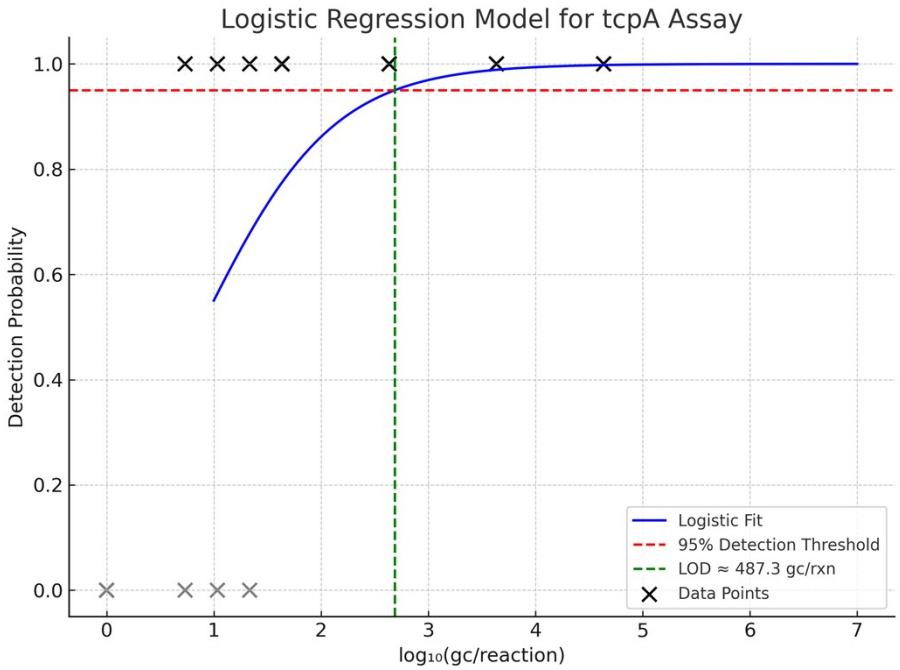

Figure S10 Calculation of limit of detection (LOD) for loop-mediated isothermal amplification (LAMP) assay for *tcpA* using a logistic regression-based method. Detection probability of the assay. The LODs were determined where the detection probability is 0.95, indicated by a vertical dashed line.