Discrimination and surveillance of infectious severe acute respiratory syndrome Coronavirus 2 in wastewater using cell culture and RT-qPCR

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Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA has been extensively detected in raw wastewater in studies exploring wastewater-based epidemiology (WBE) for early warning purposes. Nonetheless, only a few limited studies investigated the presence of SARS-CoV-2 in treated wastewaters to determine the potential health risks across the water cycle. The detection of SARS-CoV-2 has been done mostly by RT-qPCR and ddPCR, which only provides information on the presence of nucleic acids rather than information on potential infectivity. In this study, we set to develop and evaluate the use of viability RT-qPCR for the selective discrimination and surveillance of infectious SARS-CoV-2 in secondary-treated wastewater. Enzymatic (nuclease) and viability dye (Reagent D) pretreatments were applied to infer infectivity through RT-qPCR using porcine epidemic diarrhea virus (PEDV) as a CoV surrogate. Infectivity tests were first performed on PEDV purified RNA, then on infectious and heat-inactivated PEDV, and finally on heat inactivated PEDV spiked in concentrated secondary-treated wastewater. The two viability RT-qPCR methods were then applied to 27 secondary-treated wastewater samples positive for SARS-CoV-2 RNA at the outlet of five large urban wastewater treatment plants in Portugal. Reagent D pretreatment showed similar behavior to cell culture for heat-inactivated PEDV and both viability RT-qPCR methods performed comparably to VERO E6 cell culture for SARS-CoV-2 present in secondary-treated wastewater, eliminating completely the RT-qPCR signal. Our study demonstrated the lack of infectious SARS-CoV-2 viral particles on secondary-treated wastewater through the application of two pretreatment methods for the rapid inference of infectivity through RT-qPCR, showing their potential application in environmental screening. This study

addressed a knowledge gap on the public health risks of SARS-CoV-2 across the water cycle.

Keywords: SARS-CoV-2 infectivity; urban water cycle; Reagent D; nuclease; health risks; viability RT-qPCR

1. Introduction

Severe acute respiratory syndrome Coronavirus 2 (SARS-CoV-2), responsible for the coronavirus disease 2019 (COVID-19), caused until December 9, 2021 more than 266 000 000 cases and almost 5 300 000 deaths worldwide (ECDC, 2021).

Although the most common routes of infection are aerosols and respiratory droplets, SARS-CoV-2 RNA has been commonly found in the feces of infected patients, regardless of the severity or absence of symptoms (Klompas *et al*., 2020; Wang *et al*., 2020). SARS-CoV-2 receptor is the angiotensin-converting enzyme 2 (ACE2), which although being detected in the upper respiratory tract samples, indicating nasopharynx as a site of replication (Qi *et al*., 2020; Zhao *et al*., 2020), has the highest expression in the brush border of intestinal enterocytes (Qi *et al*., 2020; The Human Protein Atlas, 2020). Viral RNA has been found, for instance, in rectal swabs even after the nasopharyngeal testing became negative, implying infection of the gastrointestinal tract (Holshue *et al*., 2020; Wang *et al*., 2020; Xiao *et al*., 2020a).

SARS-CoV-2 RNA has been detected worldwide in raw wastewater and in some cases in treated wastewater, which could imply potential environmental transmission via the water cycle (Gonzalez *et al*., 2020; Medema *et al*., 2020; Randazzo *et al*., 2020; Westhaus *et al*., 2021; Monteiro *et al*., 2022). Historically, the gold standard for the isolation of infectious viral particles is cell culture, using distinct mammalian cell lines such as VERO, VERO E6 or BGM. However, the use of cell culture to determine the presence of SARS-CoV-2 in wastewater is hindered by several aspects: i) viable SARS-CoV-2 has rarely been isolated from the feces of infected patients despite the high levels of RNA detected, which suggests that the virus is already inactivated when excreted (Kim *et al*., 2020; Wölfel *et al*., 2020); ii) low throughput and significant costs of a cell culture system; iii) the need for availability of a biosafety level 3 (BSL-3)

laboratory for the isolation of SARS-CoV-2 (CDC, 2021); iv) the need to concentrate large volume of wastewater for the detection of viruses, therefore co-concentrating contaminants that are difficult to remove prior to inoculation of samples in cell culture systems, thus impairing virus isolation. Due to these limitations, it is necessary to explore other approaches, namely based on molecular methods such as reverse transcription quantitative PCR (RT-qPCR). However, due to the nature of these techniques, they inform only on the presence of nucleic acids, providing no information on the infectivity. Such feature is not the most relevant when the main interest is to use wastewater-based epidemiology (WBE) for early warning purposes, but it is important to understand if the water cycle plays an important role in further disseminating SARS-CoV-2, namely to wastewater treatment plant workers and/or other individuals that might come into contact with contaminated water, such as bathers and other surface water users.

The virus envelope and capsid protect the viral genome from the external influence of nucleases exerted upon RNA, while the spike protein determines the ability of the virus to bind with high efficiency and stability to ACE2. In light of the current knowledge, the integrity of the envelope, and particularly the spike protein, is crucial for infectivity and for the virus' ability to establish infection in humans. In the last decade, methods based on nucleases (DNase or RNase) and on viability dyes have been tested as pretreatment to infer infectivity through qPCR in different matrices (Lamhoujeb *et al*., 2008; Nowak *et al*., 2008; Schielke *et al*., 2011; Monteiro and Santos, 2018; Puente *et al*., 2020; Leifels *et al*., 2021). The underlying principle is that the viral genome (e.g. RNA) in a given matrix may be degraded by nucleases. If the viral envelope or capsid is degraded (and the viral ligand to human receptors becomes impaired), then nucleic acids become exposed and susceptible to cleavage by endonucleases and, thus,

amplification by PCR is greatly affected. If there are integer viruses, then the endonucleases will not come into contact with the nucleic acids that remain protected. Such pretreatments to infer infectivity using exposure and degradation of nucleic acids as proxy are extremely relevant in different contexts, including for environmental surveillance and for food and water safety assessments.

In this study, we aimed to develop a specific viability RT-qPCR for the selective detection of infectious SARS-CoV-2 in secondary-treated wastewater and then apply this methodology to infer the infectivity of over 80 secondary-treated wastewater samples collected for a 32-week period in 2020, during the first two waves of the COVID-19 pandemic in Portugal. Porcine epidemic diarrhea virus (PEDV), a member of the *Alphacoronavirus* genus in the *Coronaviridae* family, was used as a model surrogate for SARS-CoV-2. Enzymatic reaction and a viability dye (monoazide dye; Reagent D) were used to infer infectivity through RT-qPCR. The optimized viability RT-qPCR was then applied to secondary-treated wastewater to evaluate the infectivity of detected SARS-CoV-2, thus helping to assess the potential risk exerted by the presence of this virus in treated wastewater and along the water cycle.

2. Materials and Methods

2.1. Sampling sites and sample collection

Secondary-treated wastewater (*n* = 89) samples were collected over a 32-week period, between April 27thand December 2nd 2020, from five wastewater treatment plants (WWTP) located in the North of Portugal (Vila Nova Gaia (GA) and Serzedelo (SE)) and in Lisboa e Vale do Tejo (LVT; Alcântara (AL), Beirolas (BE), and Guia (GU)) region (Fig. S1).

Twenty-four-hour composite samples were collected using an automated sampler (ISCO, US). Samples were transported refrigerated to the laboratory, within 8 h of collection and processed immediately upon arrival to the laboratory, as described in Monteiro *et al.* (2022).

2.2. SARS-CoV-2 analysis of secondary-treated wastewater samples

Five litre of secondary-treated wastewater were concentrated using hollow-fiber filters inuvai R180 (inuvai, a division of Fresenius Medical Care, Germany), with a molecular weight cut-off of 18.8 kDa. Samples were eluted in 300 ml of 1X phosphate buffered saline (PBS) containing 0.01% sodium polyphosphate (NaPP; Sigma-Aldrich, US) and 0.01 Tween 80/0.001% antifoam and precipitated overnight with 20% polyethylene-glycol (PEG) 8000 (Sigma-Aldrich, US). Samples were then centrifuged at 10,000 xg for 30 min and resuspended in 5 ml 1X PBS, pH 7.4 (Blanco *et al*., 2019). Samples were kept at -80 (± 10) ºC until further processing.

2.3. PEDV viral strain and infectivity assay

The PEDV strain CV777 (kindly provided by Dr. Gloria Sanchez, IATA-CSIC, Spain) was propagated in VERO cells (Puente *et al*., 2020). Briefly, VERO cells cultured in Dulbecco's Modified Eagles's Medium (DMEM, ThermoFisher Scientific, US) supplemented with 100 units/ml of penicillin (Lonza, Swiss), 100 units/ml of streptomycin (Lonza, Swiss), 0.25 mg/ml amphotericin B (Lonza, Swiss) and 10% heat-inactivated fetal bovine serum (FBS; Biological Industries, Israel), were assayed as complete confluent monolayers in 24-well plates (Corning, US). Briefly, ten-fold dilutions of PEDV were prepared in DMEM supplemented with 10 µg/µl trypsin (trypsin 1:250; SAFC, Sigma-Aldrich, US) and 100 µl per well were inoculated. Following 2h

post-infection, 100 µl of media (DMEM supplemented with 0.3% tryptose phosphate broth (TPB, Sigma, US), 100 units/ml of penicillin, 100 units/ml of streptomycin, 0.25 mg/ml amphotericin B, and 10 µg/µl trypsin) was added. Plates were incubated at 37 (± 1) ºC in a 5% CO_2 incubator and monitored for cytopathic effects (CPE) for 4 days. CPE are morphological changes in cells caused by a viral infection. After visual observation of cells for detection of CPE, the infectivity was calculated by determining the 50% tissue culture infectious dose ($TCID_{50}$) using the Spearman-Karber method (Hierholzer and Killington, 1996).

2.4. Nuclease and viability dye pretreatment on purified PEDV RNA

Nucleic acid extraction was performed in DNA LoBind microcentrifuge tubes (Eppendorf, Germany) using the QIAamp DNA stool mini kit (Qiagen, Germany) according to the manufacturer's instructions. Viral RNA was eluted in a final volume of 100 µL.

Pierce Universal nuclease for cell lysis (Thermo Fischer Scientific, US) and viability dye (Reagent D, Biotecon, Germany) were tested primarily on purified PEDV RNA. Reagent D contains a light sensitive substance that upon exposure to visible light binds covalently to nucleic acids and prevents their amplification via PCR. This reagent is provided already reconstituted by the manufacturer and was used in accordance with the manufacturer's instructions. Briefly, to each sample, Reagent D was added in a proportion of 1:4 (v:v), the mixture incubated in the dark for 5 min at room temperature and the dye photoactivated for 15 min using a photoactivation system (PhAST Blue; GenIUL, Spain) (Fig. 1). For the enzymatic pretreatment, 50 units of Pierce universal nuclease for cell lysis, a genetically engineered endonuclease that degrades single-stranded, double-stranded, linear, and circular DNA and RNA and is

effective for cell lysis over a wide range of temperatures and pH, was added to each sample and incubated for 15 min at room temperature (Fig. 1).

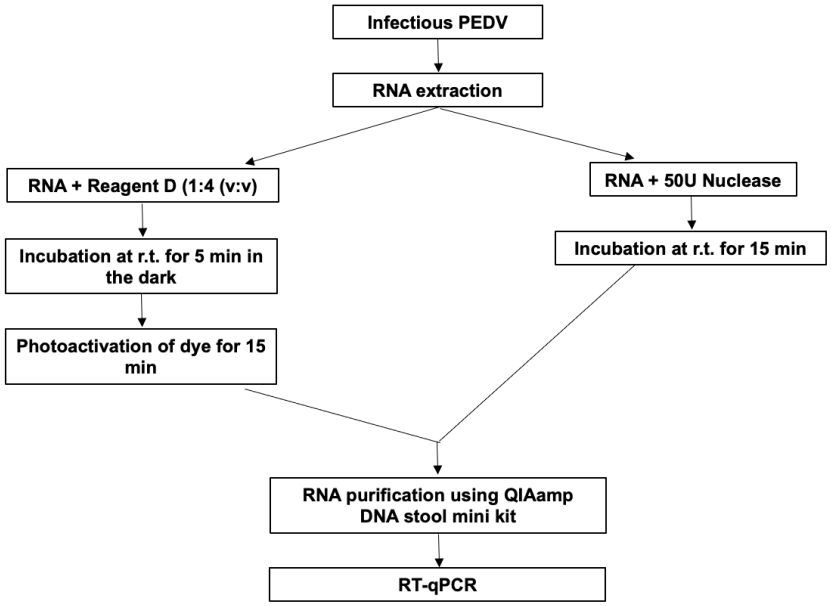

Fig. 1. Schematics for the pretreatments (viability dye and nuclease) applied to purified PEDV RNA

Following pretreatment, a new RNA purification step using the QIAamp DNA stool mini kit (Qiagen, Germany) was conducted as previously described to remove potential interference of the enzyme and the viability dye in the following steps.

Each experiment was performed in triplicate and a purified PEDV RNA sample without pretreatment was included as a positive control.

2.5. Viability pretreatments to infer infectivity of heat-inactivated PEDV by RT-qPCR

Nuclease and viability dyes were additionally tested on heat-inactivated PEDV. PEDV suspensions were divided into two categories: i) non-treated infectious viral particles; and ii) heat-treated viral particles by incubation for 15 min at 72 ºC (heat-inactivated).

The heat-inactivated samples were subjected to the above-described pretreatments before RT-qPCR (Fig. 2).

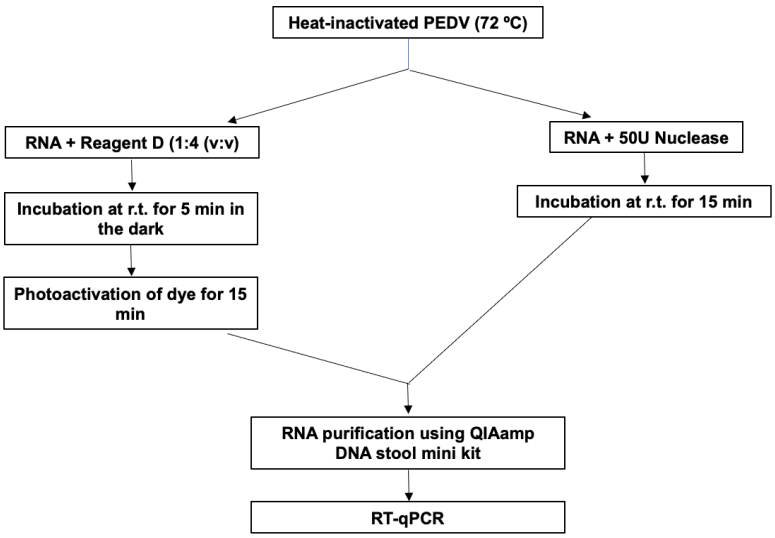

Fig. 2. Schematics for the pretreatments (viability dye and nuclease) applied to heat-inactivated PEDV

The experiments were conducted in triplicate and three controls were added: i) infectious virus with viability pretreatments; ii) infectious virus without pretreatment; and iii) heat-inactivated virus without pretreatment. All experiments were conducted in DNA LoBind microcentrifuge tubes. Following viability pretreatments, samples were extracted as described previously and quantified by RT-qPCR.

2.6. Artificial contamination of secondary-treated wastewater

Heat-inactivated PEDV suspensions (100 μL, final concentration ~ 10^4 $TCID_{50}$/mL) were spiked into 5 mL of concentrated secondary-treated wastewater from two distinct WWTP: SE and GA. SE WWTP has the particularity of receiving a large input of industrial influent, namely from the tannery industry. It has been shown previously that having a high input of industrial wastewater impaired the detection of SARS-CoV-2

from raw wastewater (Bernd *et al*., 2021) given that such wastewater generates a large amount of liquid waste constituted by pollutants such as organic and inorganic matter, total dissolved solids as well as a variety of synthetic compounds which can difficult the concentration and the final detection of the virus. Therefore, and taking into account such characteristics, SE WWTP was chosen to test the potential use of pretreatments to determine infectivity through RT-qPCR. The spiked secondary-treated wastewaters were subjected to the two viability pretreatments as described above. All experiments were conducted in DNA LoBind microcentrifuge tubes. Three controls were included: i) infectious viruses spiked into secondary-treated wastewater subjected to pretreatment; ii) infectious viruses spiked into secondary-treated wastewater without pretreatment; and iii) heat-inactivated viruses spiked into secondary-treated wastewater without pretreatment. Following pretreatment, samples were purified as described previously. Experiments were performed in triplicate.

2.7. SARS-CoV-2 infectivity in secondary-treated wastewater

SARS-CoV-2 RNA positive concentrated secondary-treated wastewater samples were tested for infectivity using VERO E6 cells, which are commonly used to isolate and propagate SARS-CoV-like viruses since they support viral replication to high titers. Cells were cultured in DMEM containing 10% FBS. Plates with freshly grown VERO E6 cells were inoculated with 1 ml volume from each secondary-treated wastewater sample following sterilization through a 0.22 µm polyvinylidene fluoride (PVDF) filter (Pall, UK) (Tartera *et al*., 1992). Samples were incubated for 1 h, the supernatant was removed, rinsed twice with phosphate-buffered saline (PBS) and 20 ml of fresh culture medium (DMEM supplemented with FBS, 50 units/ml penicillin, 50 units/ml streptomycin, and 2 mM L-glutamine (Sigma, US)) was added to the samples. Plates

were incubated at 37 (± 1) ºC for 5 days, inspected for CPE and the $TCID_{50}$ was calculated according to the Spearman-Karber method. Negative controls (PBS) were included in each test batch.

Concentrates from secondary-treated wastewater were analyzed with and without viability pretreatment (previously tested on PEDV as previously described) to evaluate the usefulness of the viability RT-qPCR for SARS-CoV-2. To 200 µl of secondary-treated wastewater concentrated sample were added 600 µl of Reagent D (Biotecon, Germany) or 50 units of Pierce universal nuclease. Incubations and photoactivation of Reagent D were performed as described previously. Experiments were performed in triplicate.

2.8. Extraction and quantification of PEDV and SARS-CoV-2

Viral RNA from concentrated untreated secondary-treated wastewater (200 µl), Reagent D pretreatment concentrates (800 µl) and nuclease pretreatment concentrates (200 µl) was extracted using the QIAamp DNA stool mini kit, according to the manufacturer's instructions, with final elution in 100 µl. Molecular detection of PEDV and SARS-CoV-2 was performed in an Applied Biosystems 7300 Real-Time PCR (Applied Biosystems, US) using the AgPath-ID One-Step RT-PCR kit (Thermo Fischer Scientific, US), with primers and probes described by Zhou *et al*. (2017) and Corman *et al*. (2020) (Supplementary Table 1). For SARS-CoV-2, E_Sarbeco, RdRp, and N_Sarbeco were amplified as described by Monteiro *et al.* (2022). The 25 µl final volume reaction mixture consisted of 12.5 µl of 2X RT-PCR buffer, 1 µl of RT-PCR enzyme mix, 800 nM of each primer, 200 nM of probe, 5 µl of sample, with the final volume completed with nuclease-free water. PCR inhibition was evaluated by determining the concentration of PEDV and SARS-CoV-2 in the 10- and 100-fold

sample dilutions. Cycle threshold differences (ΔCt) ≥ 3.50 between crude extracts and 10-fold dilution and between 10-fold dilution and 100-fold dilutions, were considered amplification inhibition free. Thermal cycling conditions were as follows: i) PEDV: reverse transcription for 10 min at 45 ºC, initial denaturation for 10 min at 95 ºC, followed by 45 cycles of 15 s at 95 ºC and 1 min at 60 ºC; ii) SARS-CoV-2: reverse transcription for 10 min at 45 ºC, initial denaturation for 10 min at 95 ºC, followed by 45 cycles of 15 s at 95 ºC and 1 min at 58 ºC. Reactions were considered positive only if the cycle threshold was below 40 cycles (Medema *et al*., 2020; F. Wu *et al*, 2020). Analysis of PEDV was performed qualitatively, therefore, positive and negative controls were added with each reaction. Quantification of E_Sarbeco and RdRp assays was performed through calibration curves using 10-fold dilutions of nCoV-ALL-Control plasmid (Eurofins Genomics, Germany), ranging from 1.94 to 1.94 x 10^6 and 1.00 to 1.00 x 10^6 GC per reaction respectively. Quantification of N_Sarbeco assay was performed using 2-fold and 10-fold dilutions (ranging between 2.00 to 2.00 x 10^4 GC per reaction) of the Amplirun SARS-CoV-2 RNA control (Vircell, Spain). Negative controls (extraction and RT-qPCR assay) were also performed using DNase/RNase free distilled water, following the same conditions as the samples.

2.9. Statistical analysis

Data analysis was conducted with GraphPad Prism (GraphPad Software, US). Each experiment was conducted in triplicate, and each sample was analyzed in duplicate. Normality test of the dataset was conducted using the Shapiro-Wilk test and the equality of variances was determined using the Levene's test. Kruskal-Wallis test (KW statistics) was conducted to compare differences between each test and pairwise comparison was performed with Dunn's test. Mann-Whitney test was used to compare

between infectious and heat-inactivated PEDV, and between infectious and heat-inactivated PEDV following pretreatments (nuclease and Reagent D). Data was considered significant with values of $p < 0.05$.

3. Results

3.1. Viability of purified PEDV RNA

Pierce Universal nuclease for cell lysis and Reagent D were initially screened for their potential to discriminate between infectious and non-infectious viral particles in purified PEDV RNA extracts (Fig. 3). Treatment with Pierce Universal nuclease for cell lysis was able to completely remove the amplification signal (Ct) from purified PEDV RNA (mean removal $\Delta Ct \geq 21.3$).

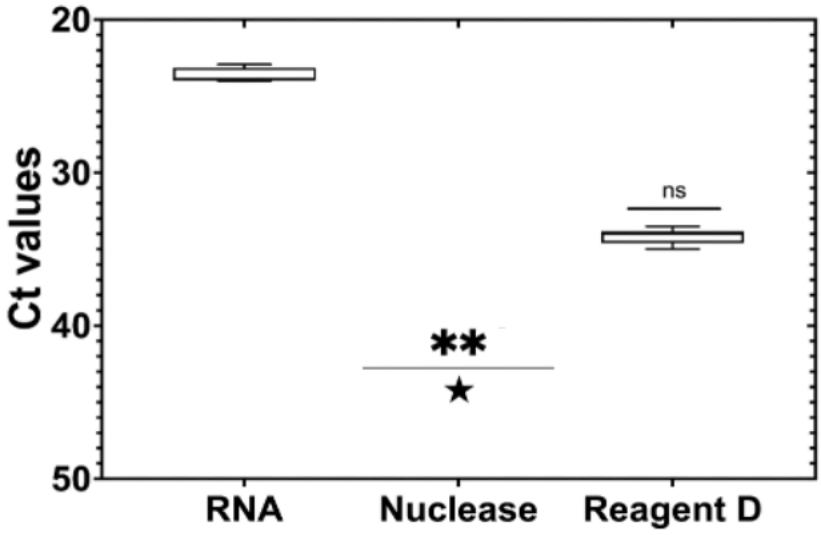

Fig. 3. Number of cycles (Ct) as a function of different viability treatments applied to purified PEDV RNA. Asterisks represent statistically significant differences. ** p = 0.001; ns, no significant difference. Star (★) represent undetected results.

Applying Reagent D on purified PEDV RNA prior to RT-qPCR decreased on average the PCR signal by 10.5 Ct. Differences between control and the tested viability

304 treatments were statistically significant ($p = 0.001$). Dilutions of 1:10 and 1:100 did not
305 show inhibitory effects on RT-qPCR for both pretreatments.
306
307 3.2. Efficiency of viability pretreatments on heat-inactivated PEDV
308 Infectious and heat-inactivated (72 ºC for 15 min) PEDV were treated with Pierce
309 Universal nuclease for cell lysis and Reagent D before extraction and quantification by
310 RT-qPCR (Fig. 4). Differences were obtained between infectious and heat-inactivated
311 viral particles ($p < 0.05$), with mean ΔCt of 1.98.
312 Pretreatment of infectious PEDV viral particles with nuclease significantly decreased
313 the RT-qPCR signal by an average of 3.51 Ct ($p < 0.05$), whereas Reagent D reduced
314 the signal, on average, by more than 2.50 Ct ($p = 0.40$). The difference in detection by
315 RT-qPCR between heat-inactivated PEDV and nuclease-treated inactivated PEDV
316 was 1.22 Ct. No differences between nuclease-treated infectious and heat-inactivated
317 PEDV were detected by RT-qPCR ($p = 0.31$). Reagent D was able to significantly
318 decrease the RT-qPCR signal of heat-inactivated PEDV (mean ΔCt = 11.2; $p < 0.05$).
319 Statistically significant differences were determined between infectious and heat-
320 inactivated PEDV pretreated with Reagent D (mean ΔCt = 10.65; $p < 0.05$). No
321 inhibitory effect was detected for both pretreatments.

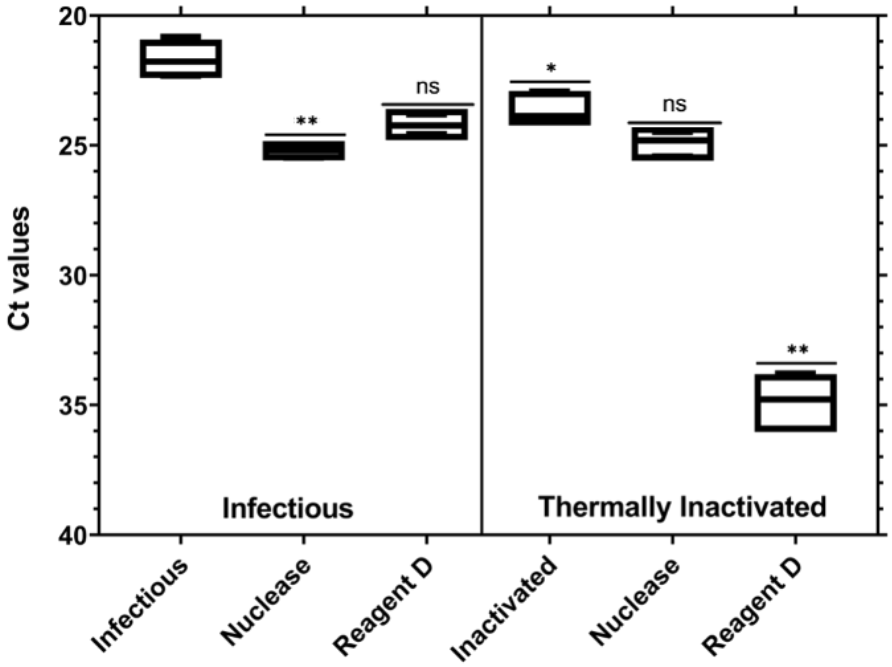

Fig. 4. Number of cycles (Ct) as a function of different viability treatments applied to purified infectious and thermally inactivated PEDV. Asterisks represent statistically significant differences. *$p < 0.05$; **$p = 0.001$; ns, no significant difference.

3.3. Performance of viability RT-qPCR in spiked secondary-treated wastewater

Secondary-treated wastewater was spiked with heat-inactivated PEDV (72 ºC for 15 min) and subjected to pretreatment with nuclease and Reagent D prior to RT-qPCR to determine the influence of the matrix on the viability pretreatments. Viability pretreatments were tested in two very distinct secondary-treated wastewaters: GA and SE. GL WWTP receives mainly municipal wastewater with an average flow of 66,700 m^3/day, and secondary-treated wastewater presented high turbidity. On the other hand, SE WWTP in addition to municipal wastewater, receives a large volume of industrial wastewater from the tannery industry which by itself represents an additional challenge, but the turbidity levels were lower.

Results from GA showed a RT-PCR signal reduction of heat-inactivated PEDV treated with nuclease when compared to the inactivated spiked control (4.59 Ct), but the difference was not statistically significant (p = 0.45) (Fig. 5). Conversely, treating

spiked GA secondary-treated wastewater with Reagent D strongly decreased the RT-qPCR signal (ΔCt > 20.0; $p < 0.05$).

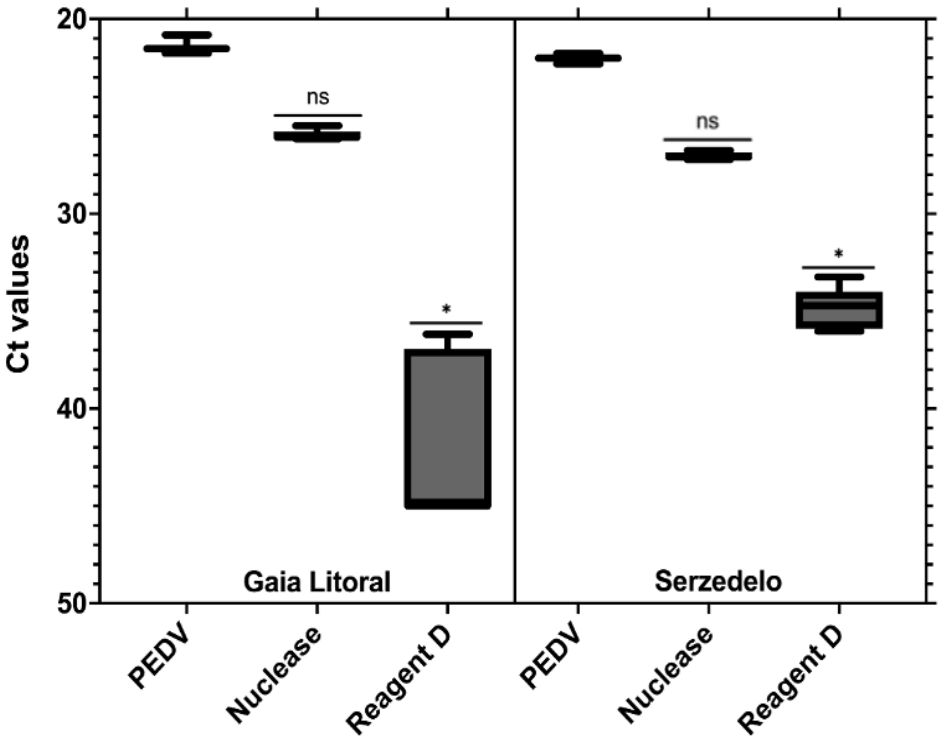

Fig. 5. Number of cycles (Ct) as a function of different viability treatments applied to heat-inactivated PEDV spiked into two secondary-treated wastewater, GA and SE. Asterisks represent statistically significant differences. *$p < 0.05$; ns, no significant difference.

Similarly, data for PEDV spiked SE secondary-treated wastewater showed that Reagent D pretreatment performed at a higher level than nuclease (ΔCt = 12.8 and ΔCt = 5.00, respectively). Results for Reagent D differed significantly from the control ($p < 0.05$). Smaller variability was detected for the SE secondary-treated wastewater pretreated with Reagent D compared to GL, possibly due to the higher turbidity of the latter, which may have affected the performance of the dye, therefore increasing the variability of the results. Inhibition was not detected in the RT-qPCR assays, as indicated by molecular results of dilution testing.

3.4. Presence of SARS-CoV-2 RNA in secondary-treated wastewater using RT-qPCR

Throughout the 32-week study, a total of 89 secondary-treated wastewater samples were collected and tested for the presence of SARS-CoV-2 RNA, using the three assays described by Corman *et al*. (2020): E_Sarbeco, RdRp, and N_Sarbeco. SARS-CoV-2 RNA was present, as determined by RT-qPCR, in 30% (27/89) of the samples, with concentrations ranging from 1.71 x 10^2 in SE to 1.18 x 10^4 GC/L in BE (Fig. 6). From the 27 positive samples, 18 were positive for a single assay, 8 were positive for two assays (E_Sarbeco and RdRp) and a single sample was positive for all three assays.

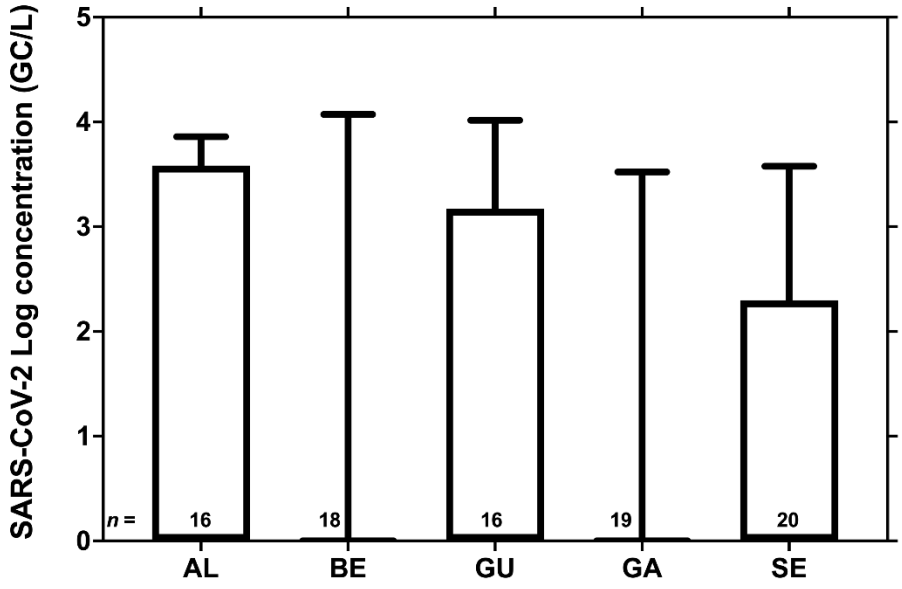

Fig. 6. SARS-CoV-2 RNA concentration in the secondary-treated wastewater from LVT WWTP (AL- Alcântara; BE – Beirolas; GU – Guia) and the WWTP from the North region of Portugal (GA – Gaia Litoral; SE – Serzedelo), from April to December 2020. Boxes, 25th and 75th percentile; lines within the boxes, median; whiskers, lowest and highest SARS-CoV-2 RNA concentration; *n*, number of samples from each WWTP.

As the number of COVID-19 cases in the country increased by the end of our sampling period, an increase was also registered in the percentage of positive samples for this matrix (Fig. S2).

3.5. Evaluation of SARS-CoV-2 infectivity in secondary-treated wastewater by viability RT-qPCR

SARS-CoV-2 presence in secondary-treated wastewater was evaluated, in a first approach, using RT-qPCR alone. Nonetheless, RNA detection does not necessarily imply infectious potential and correspondent health risk, as discussed previously (Kim *et al*., 2020; Wölfel *et al*., 2020). Following confirmation of the presence of SARS-CoV-2 RNA in the tested effluents, we evaluated the infectivity potential in three ways: i) cell culture using Vero E6 cells; ii) enzymatic degradation of nucleic acids using Pierce Universal nuclease; and iii) viability dyes using Reagent D. Analysis of SARS-CoV-2 RNA positive secondary-treated wastewaters by cell culture provided negative results for infectivity. Secondary-treated wastewaters detected by viability RT-qPCR results are shown in Fig. 7. This representation includes all the positive samples obtained at each site. Remarkably, Reagent D and nuclease pretreatment were able to completely remove the amplification signals obtained by RT-qPCR in all samples.

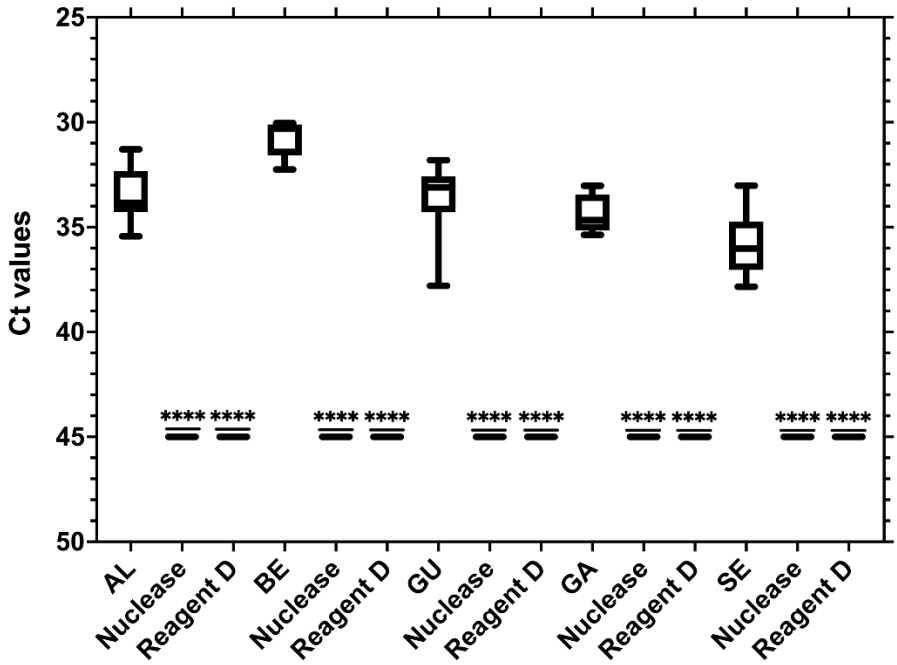

Fig. 7. Number of cycles (Ct) as a function of different viability treatments applied to SARS-CoV-2 in secondary-treated wastewater. This representation includes the median Cts obtained for all positive samples at each location. Locations: AL – Alcântara (n=7), BE – Beirolas (n=3), GU – Guia (n=7), GA – Gaia Litoral (n=4), SE – Serzedelo (n=6). Asterisks represent statistically significant differences. ****ρ < 0.0001.

Results for both viability RT-qPCR methods showed average Ct reductions of more than 9.00 with respect to RT-qPCR alone. The RT-qPCR pretreatments were able to completely remove the signal in all samples, with average decreases varying between 9.17 for SE and 14.22 for BE.

4. Discussion

The urgent situation the world has been facing for the last year and a half requires more in-depth research into enveloped viruses, including on the transmission and viral fate in the environment. Better analytical tools are thus necessary for monitoring potential routes of transmission. Although SARS-CoV-2 is preferentially transmitted via respiratory droplets (Qi *et al*., 2020; Zhao *et al*., 2020), excretion of the viruses in the feces have been confirmed in a high proportion of infected individuals (Holshue *et al*., 2020; Wang *et al*., 2020; Xiao *et al*., 2020a). Nonetheless, studies looked mainly

412 at the presence of SARS-CoV-2 RNA in feces for two main reasons: (i) this is a
413 problematic matrix due to the high concentration of microorganisms which may affect
414 the performance of virus culture in cell lines; and (ii) SARS-CoV-2 isolation should be
415 conducted at least in a BLS-3 laboratory using BSL-3 good practices (CDC, 2021) and
416 most environmental laboratories do not have such facilities and protocols in place. At
417 least two studies have investigated the infectivity of SARS-CoV-2 detected in the
418 stools of infected patients with contradictory findings. While Xiao *et al*. (2020b) were
419 able to detect infectious viral particles in the stools of an infected patient, using the
420 Vero E6 cell line, Wölfel *et al*. (2020) were unable to isolate infectious viral particles,
421 using the same cell line, in two separate laboratories, despite the high viral RNA load
422 detected by RT-qPCR.
423 Due to the presence of SARS-CoV-2 in the feces of infected individuals, a WBE
424 approach has been put in place in many locations of the world, with SARS-CoV-2 RNA
425 being detected in raw wastewater. However, only a few studies have investigated the
426 presence of SARS-CoV-2 RNA in treated wastewaters (Randazzo *et al*., 2020;
427 Westhaus *et al*., 2021), with both studies confirming the presence of SARS-CoV-2
428 RNA in treated effluents. From these studies only Westhaus *et al*. (2021) looked at the
429 potential presence of infectious viral particles in treated wastewater, using the CaCo-
430 2 cell line. In agreement with the results from Wölfel *et al*. (2020), the authors were
431 incapable of isolating infectious viral particles. In our study, SARS-CoV-2 RNA was
432 detected in 30% of the secondary-treated wastewater samples in concentrations up to
433 10^4 GC/L, with the presence of RNA not implying immediate risks to public health.
434 Following detection by RT-qPCR and to determine possible health risks across the
435 water cycle, positive samples were tested in cell culture and using viability RT-qPCR
436 techniques based on enzymatic and viability dyes. Our results suggest that SARS-

CoV-2 detected in treated wastewater appears to be non-infectious. It is important to refer that until now, only a few studies described the usage of pretreatments to infer SARS-CoV-2 infectivity through RT-qPCR (Cuevas-Ferrando *et al*., 2021; Polo *et al*., 2021; Wurtzer *et al*., 2021). Polo *et al*. (2021) was capable of fully eliminating the RT-qPCR signal by using a combination of PMAxx with a surfactant in clam and sediment samples, a similar result to that obtained in our study. Wurtzer *et al*. (2021) was capable to differentiate between total viral genome and protected RNA by using a viability dye. On the other hand, in the study by Cuevas-Ferrando *et al*. (2021) PMAxx, although showing significant reduction in the signal from 8 replicates of purified SARS-CoV-2 RNA using the E gene (one of the targets used in our study), treatment of the viral RNA with a platinum compound ($PtCl_4$), produced increased results. The authors have also shown that, regardless of the concentration of the platinum compound used, a complete removal of the RT-qPCR signal was achieved in samples with an initial low viral concentration (Ct values ≥ 30), which agrees with our results as all secondary-treated wastewater samples were detected in Ct values above 30. A study by Cuevas-Ferrando *et al*. (2020) on the use of viability dyes and platinum compounds to determine infectivity of PEDV by RT-qPCR has shown that PMAxx followed more closely the inactivation rates of PEDV at different temperatures determined by cell culture and that the combination of PMAxx with a surfactant (Triton X-100) sharply improved the results from the viability RT-qPCR. The authors have found similar results to those of our study, with PMAxx performing at a higher level than the other tested viability RT-qPCR.

However, it should be noted that the application of pretreatments to infer infectivity through RT-qPCR may be impaired in situations where disinfection with UV light occurs. The impact of free chlorine and UV_{254} in Phi6, an enveloped bacteriophage,

has shown that UV_{254} inactivates Phi6 primarily by reacting with the genome (Ye *et al*., 2018). To be able to work, viability dyes must first enter the cell, and therefore it is necessary that damage to the envelope occur. Many publications have already shown, for non-enveloped viruses, that viability dyes are not effective at removing the signal of UV-inactivated viruses (Karim *et al*., 2015; Leifels *et al*., 2015).

A recent publication showed that SARS-CoV-2 RNA continued to be detected even when infectious SARS-CoV-2 was below the detection limit of the cell culture assay (Bivins *et al*., 2020). Times for 90% reduction (T_{90}) of viable SARS-CoV-2 in frozen untreated wastewater at room temperature varied between 1.5 and 2.1 days. The authors showed that, at high titers, SARS-CoV-2 could be detected for the entire 7-day duration (10^5 $TCID_{50}$ mL^{-1}), and at low titers (10^3 $TCID_{50}$ mL^{-1}) detection fell below the limit of detection after only 72 h, with both virus titers being highly improbable to be found in real world scenarios. Nonetheless, in the study by Bivins *et al*. (2020), the authors might have extended the survivability of the virus due to several experimental design choices made: i) the study was conducted in frozen/thawed wastewater that may have altered the microbiota usually contributing to the inactivation of viruses in water due to proteolytic activity (Gerba *et al*., 1978; Kim and Unno, 1996; John and Rose, 2005; Gundy *et al*., 2009; Ye *et al*., 2016); ii) the study was performed in a single wastewater from a single WWTP in a laboratory setting, therefore excluding the contribution of factors that are known to promote varying inactivation rates including the pH, mixing conditions, and suspended solids (Ye *et al*., 2016; Aquino de Carvalho *et al*., 2017). A recent systematic review and meta-analysis of the persistence of coronavirus and surrogates in water determined a 99% reduction of approximately 2 days in wastewater at room temperature (Silverman and Boehm, 2020). Likewise, a meta-analysis concluded that the persistence of different enveloped viruses varied

widely for comparable conditions being highly dependent upon virus type, matrix composition and temperature (Aquino de Carvalho *et al*, 2016). Moreover, the authors concluded that differences in persistence in water are also dependent on the virus strain.

Considering data on the persistence of SARS-CoV-2 and other enveloped viruses in raw wastewater, the residence times in sewage systems (in the range of hours), and in the WWTP (varying between 24 - 48h depending on the WWTP treatment line), SARS-CoV-2 detected in secondary-treated wastewater should already be mostly non-infectious, a premise supported by the results from our study, either by using cell culture or viability RT-qPCR.

5. Conclusion

To our knowledge, this is the first study applying viability RT-qPCR to infer SARS-CoV-2 infectivity in secondary-treated wastewater. Our study highlights the potential of viability RT-qPCR as a suitable, scalable and easy approach to infer infectivity of SARS-CoV-2 in the water cycle, with potential use in environmental applications used for risk analyses and prevention/control contingency plans as well.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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668

Figure 1

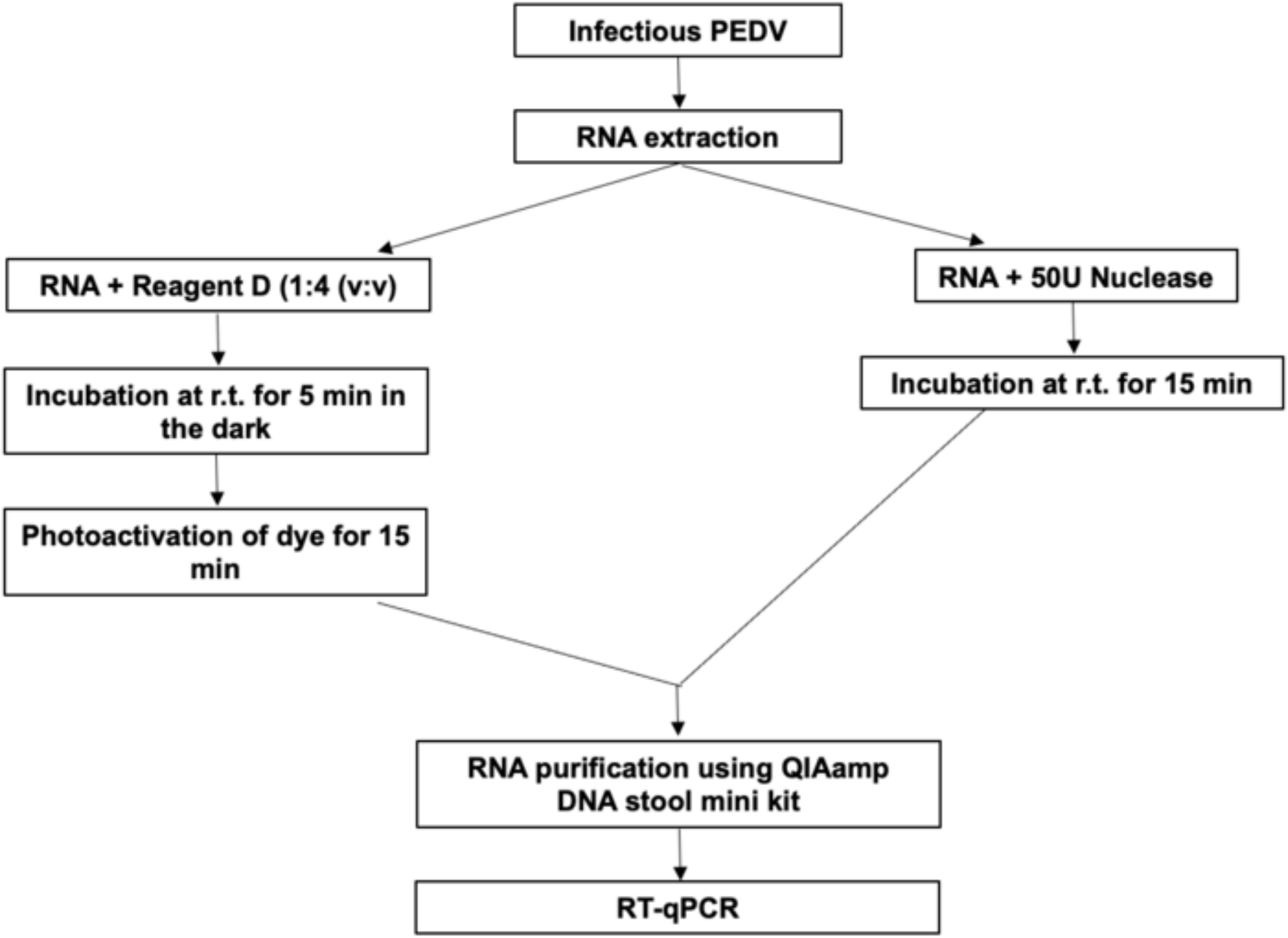

Figure 2

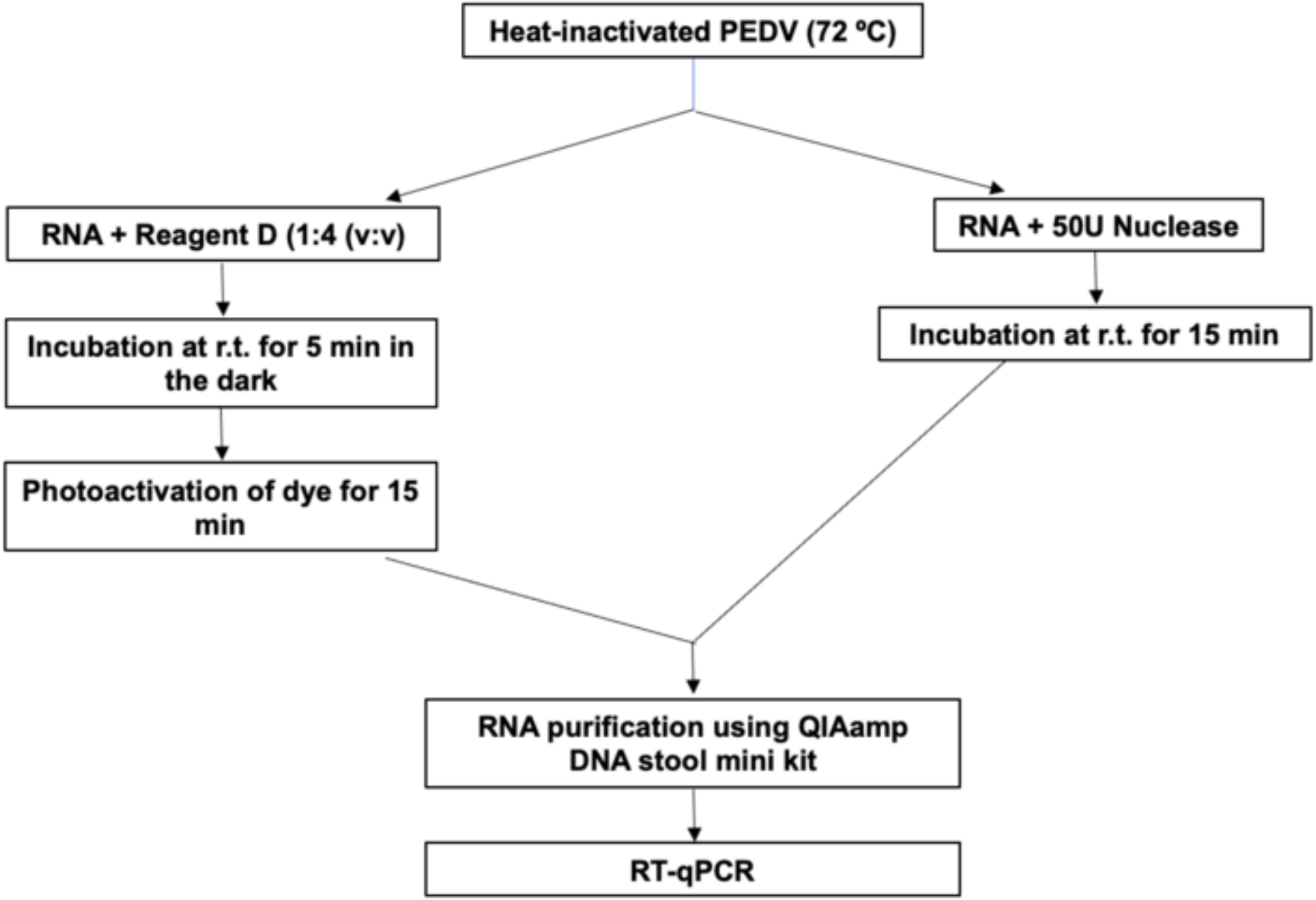

Figure 3

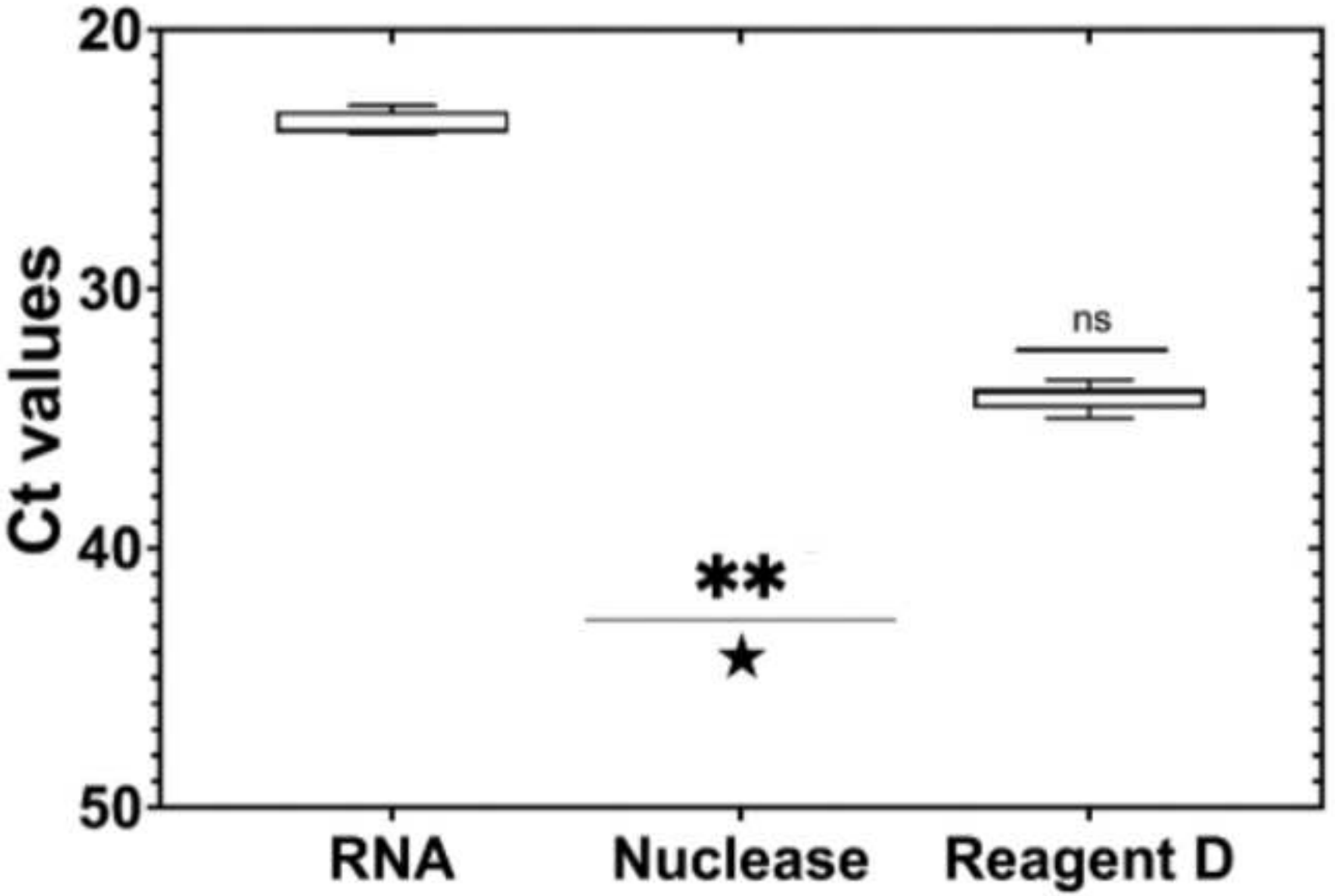

Figure 4

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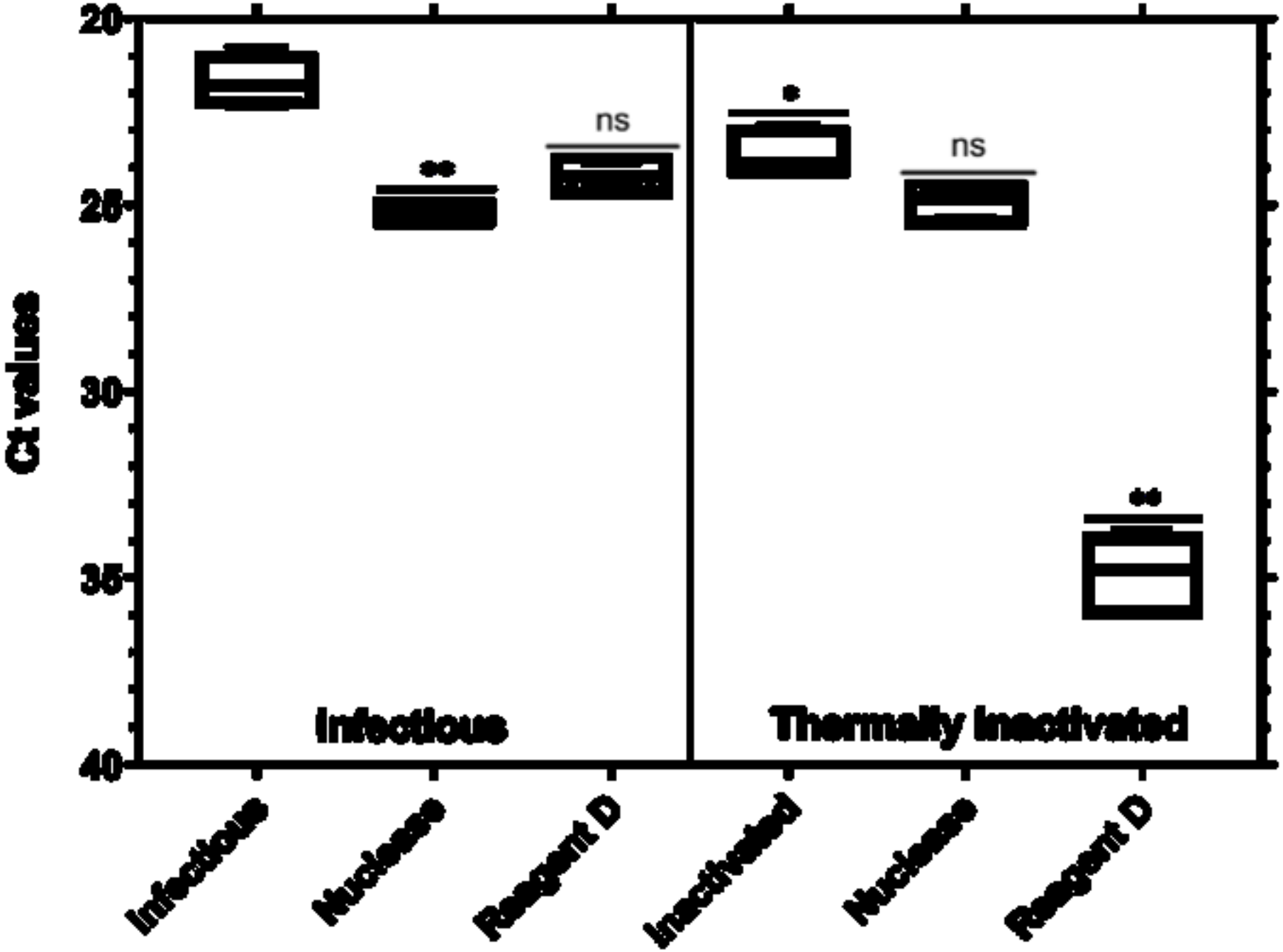

Figure 5

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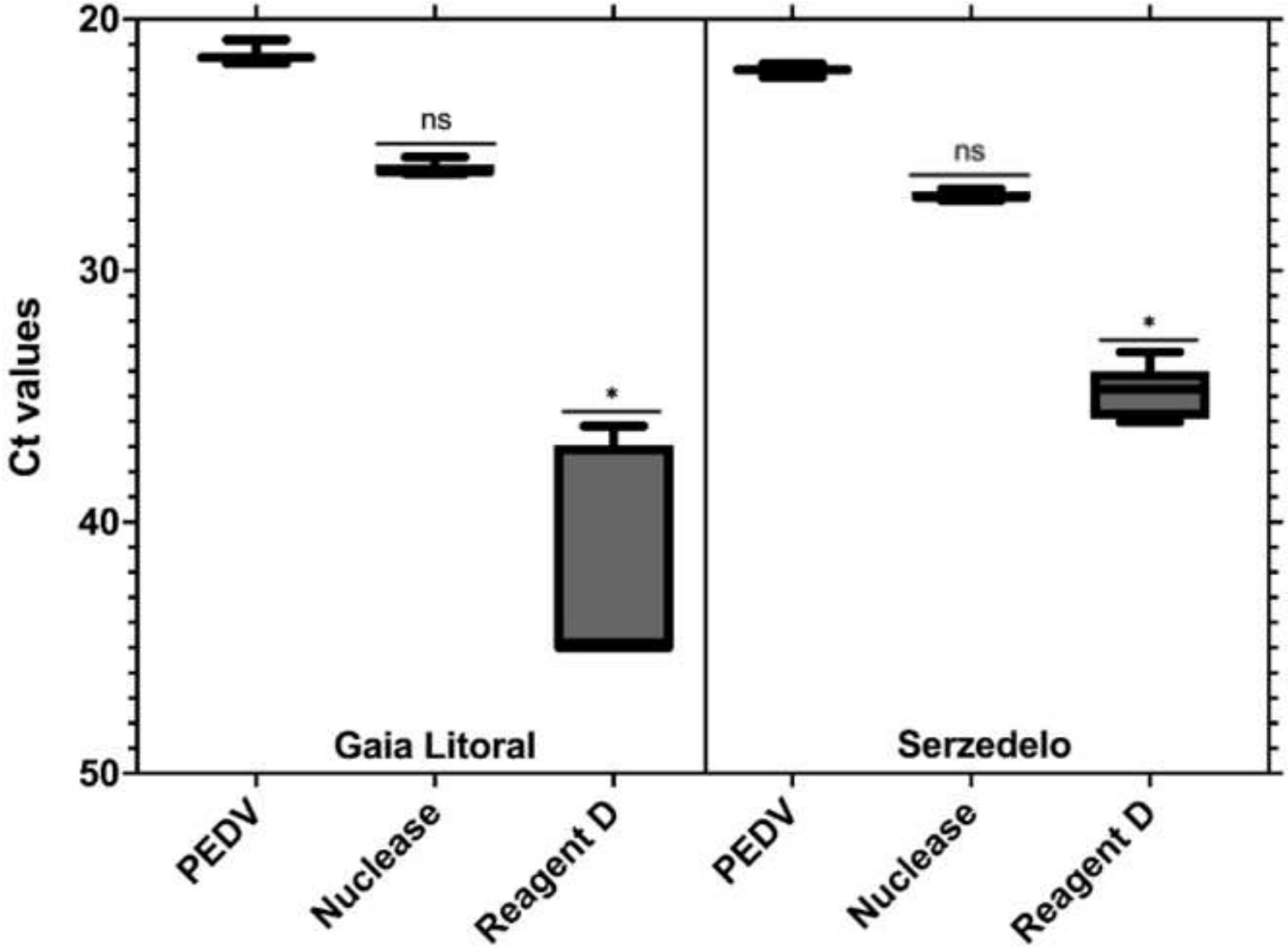

Figure 6

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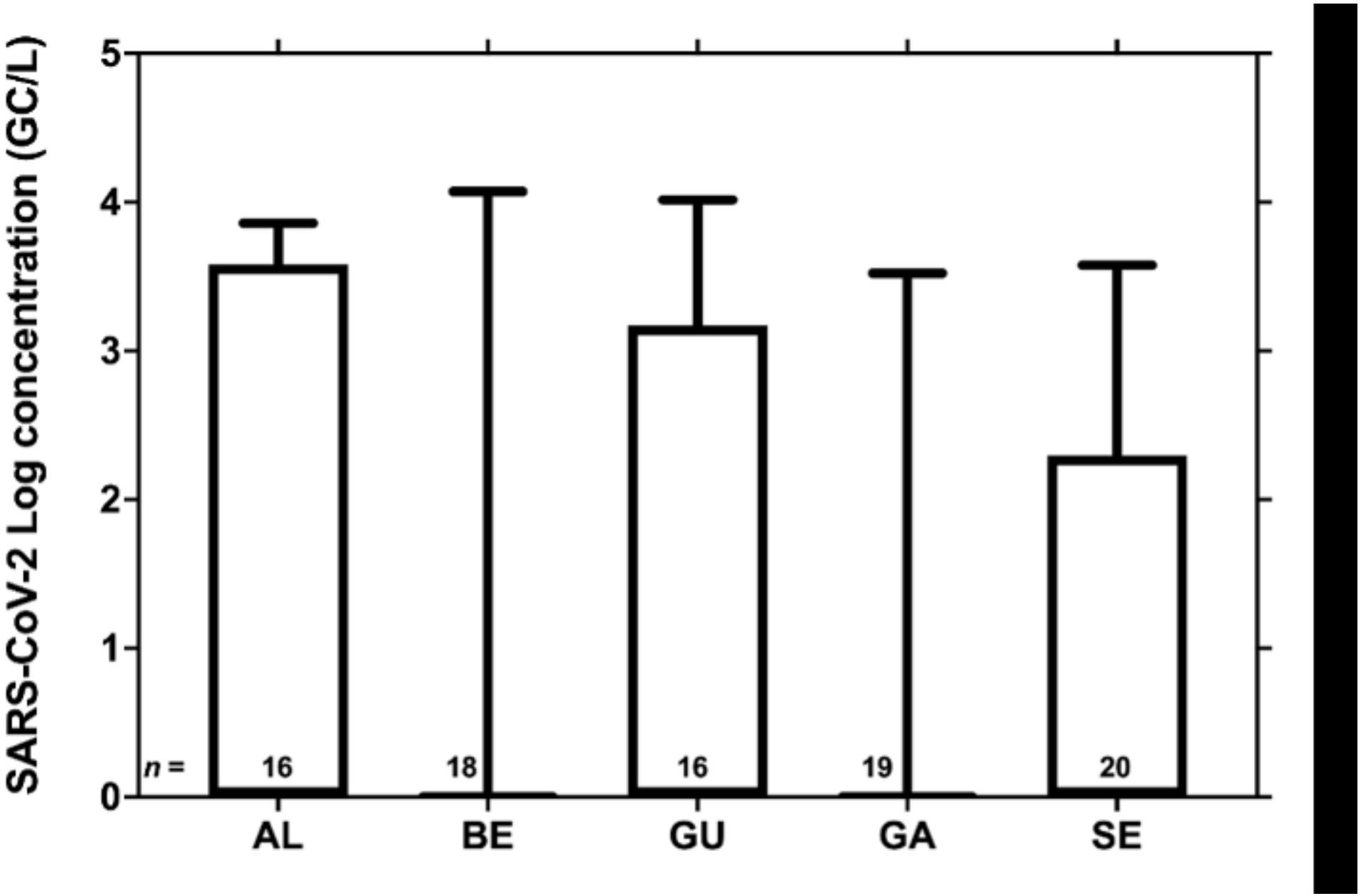

Figure 7

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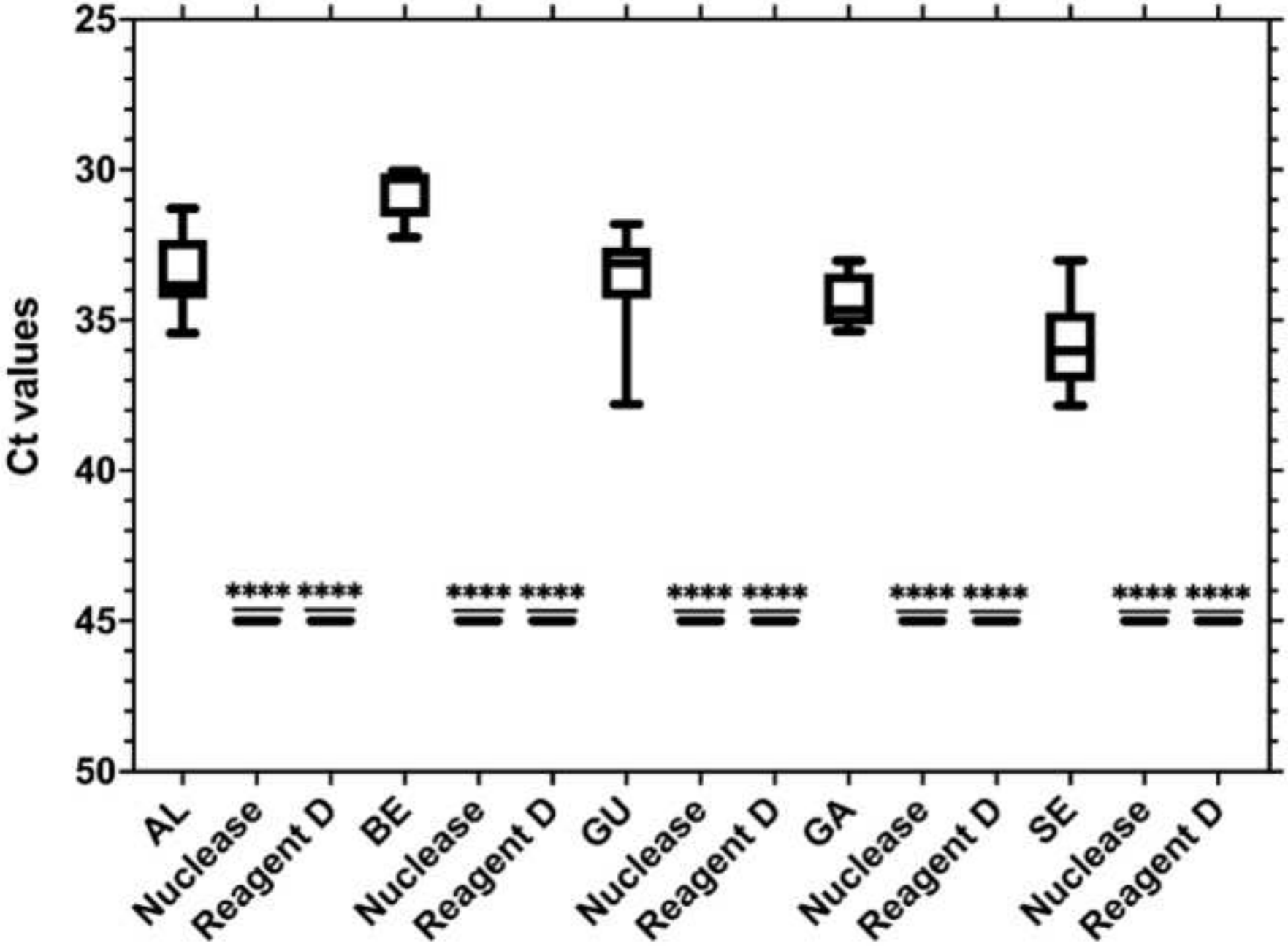

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

CrediT authorship contribution statement

Sílvia Monteiro: conceptualization, methodology, software, validation, formal analysis, investigation, writing – original draft, writing – review and editing, visualization. **Daniela Rente**: investigation. **Mónica V. Cunha**: conceptualization, funding acquisition, methodology, formal analysis, writing – review and editing. **Tiago A. Marques**: formal analysis, writing – review and editing. **Eugénia Cardoso**: review and editing, sampling. **João Vilaça**: review and editing, sampling. **Nuno Brôco**: project administration, funding acquisition, review and editing; **Marta Carvalho**: project administration, funding acquisition, review and editing; **Ricardo Santos**: conceptualization, methodology, resources, formal analysis, writing – review and editing.