An improved method for RNA isolation-free detection of SARS-CoV-2 RNA by Reverse Transcription – qPCR

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Introduction

The current COVID-19 pandemic has underscored the relevance of molecular testing for epidemiologic surveillance and containment and has set under unprecedented strain the diagnostic testing capacity of health care systems. To date, the most sensitive and broadly accepted assay to diagnose SARS-CoV-2 infection involves collection of nasopharyngeal swab samples, RNA isolation and reverse transcription coupled with quantitative PCR amplification (RT-qPCR). Typically, RNA isolation from swab samples is performed using silica-based (spin) mini-column or magnetic bead technology and, thus, contributes significantly to duration, costs and material waste of the whole process. Furthermore, availability and production of these materials have become rate limiting for testing capacity[1]. In order to expedite the RNA isolation step and minimize the risk of sample cross-contamination, column or magnetic separations are carried out with automated workstations that constitute upfront costs for testing facilities. To obviate these drawbacks, considerable efforts have been made to establish alternative procedures that avoid RNA isolation[2–11]. These procedures inevitably lead to higher limits of detection, likely due to the relative lower concentration of target RNA and the presence of substances that inhibit the RT-qPCR assay in crude lysates. However, mathematical modelling showed that frequency and sample-to-answer reporting time are more relevant than sensitivity for surveillance effectiveness[12]. This underlines the necessity to strike the best trade-off among sensitivity, capacity and turnaround. Thus, procedures that maximize speed of execution and throughput, yet maintaining high levels of sensitivity, are highly desirable.

Methods

RT-qPCR was performed with various commercial master mixes for one-step reverse transcription and qPCR amplification on a qTOWER^3 G (analytik jena). Reactions were set up in final volumes of 20 and 25 µl, including 5 and 8 µl of sample, respectively. N1 and N2 primer/probe sets from baseclick were as described in the CDC Real-Time RT-PCR Diagnostic Panel for Emergency Use Authorization (CDC-EUA)[13] and were used at 500 µM forward and reverse primers and 125 µM probe. E and ORF1a primer/probe sets and their usage conditions are proprietary. Reverse transcription was at 50-52°C for 15 minutes followed by an initial denaturation of 20 seconds at 95°C degrees and 45 cycles of 95°C for 3 seconds and 60°C for 30 seconds. Results were analyzed and visualized with the qPCRsoft software version 4.2. *In vitro* transcription and transfection of HEK293T cells with mRNA were performed as previously described[14]. For isolation of total human RNA, frozen pellets of THP-1 cells were directly lysed in monophasic lysis reagent[15] and processed as previously described[16,17].

Results

We devised a simple sample preparation procedure involving a short incubation with a viral extraction reagent (VER) followed by heat treatment. The latter step inactivates components of the

VER to allow the subsequent enzymatic reactions and further helps releasing viral RNA from viral particles and infected cells. The crude lysate so obtained is then used directly in the RT-qPCR assay. To optimize sample treatment conditions, infected samples were simulated by adding a known amount of *in vitro* transcribed mRNA coding for the N protein to Hank's balanced salt solution (HBSS; without phenol red, calcium and magnesium) containing 5 µg/ml of total human RNA from THP-1 cells as carrier and generating serial dilutions in the same solution. Figure 1 shows RT-qPCR results from such samples that were either left untreated or subjected to VER/heat treatment under optimized conditions (incubation with VER for 3 minute at 56°C, followed by 10 minutes at 95°C). Amplification of untreated and VER/heat-treated samples was essentially indistinguishable for target RNA concentrations over three orders of magnitude.

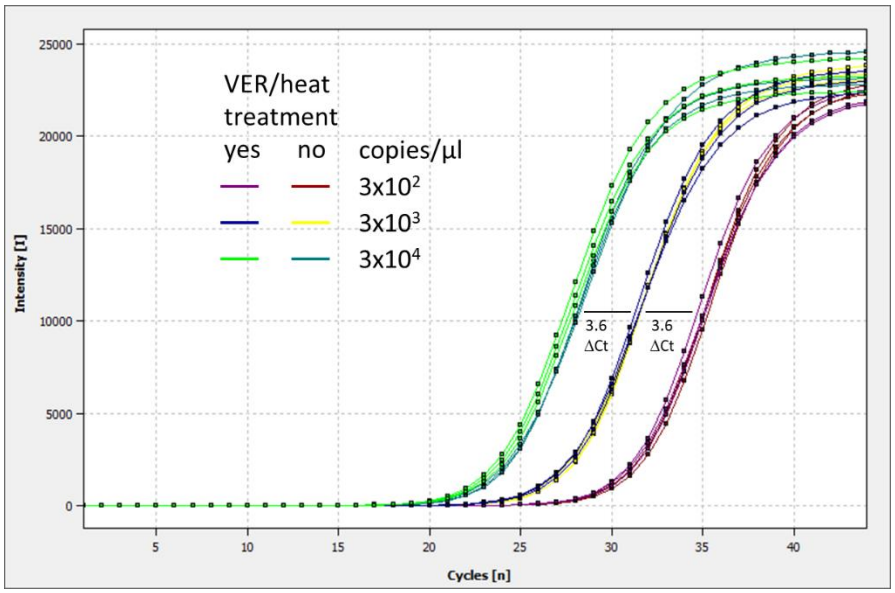

Figure 1. Combined VER and heat treatment does not affect the ability of target RNA to be reverse transcribed and amplified. Plot of probe fluorescence intensity over cycle number shows amplification of target RNA in VER/heat-treated and untreated samples over three orders of concentration magnitude.

To test whether the VER/heat treatment can efficiently release viral RNA from infected cells we simulated the latter by transfecting HEK293T cells with *in vitro* transcribed mRNA coding for the N protein. Four hours after transfection the cells were harvested and washed six times in ice cold HBSS to dilute residual untransfected RNA. The cells were then resuspended at $2x10^6$/ml and further 3 serial ten-fold dilutions (down to $2x10^3$/ml) were generated in HBSS and treated with VER/heat as above. These samples were then subjected to RT-qPCR with both N1 and RNase P primer/probe sets. The latter set targets a transcript coding for the RPP30 subunit of the RNase P complex and serves as reference for amplification of an endogenous cellular transcript. Figure 2 shows amplification profiles with decreasing Ct values for cell densities from $2x10^5$/ml to $2x10^3$/ml for both targets as expected. However, little (N1) or no difference (RNase P) could be seen for amplification of undiluted and 1:10 diluted samples. This would not be expected if residual untransfected RNA were the main source of template (as Ct values are not so low to expect saturation of RT-qPCR reagents, especially for the RNase P primer/probe set) and indicates a saturation effect on the proficiency of the VER/heat treatment in releasing target RNA templates from within the cells, an effect likely due to out-titration of the VER at high cell density as well as degradation or entrapment of RNA in cell debris. We conclude

that the VER method efficiently releases RNA from within cells up to cell densities well above the typical range for swab samples.

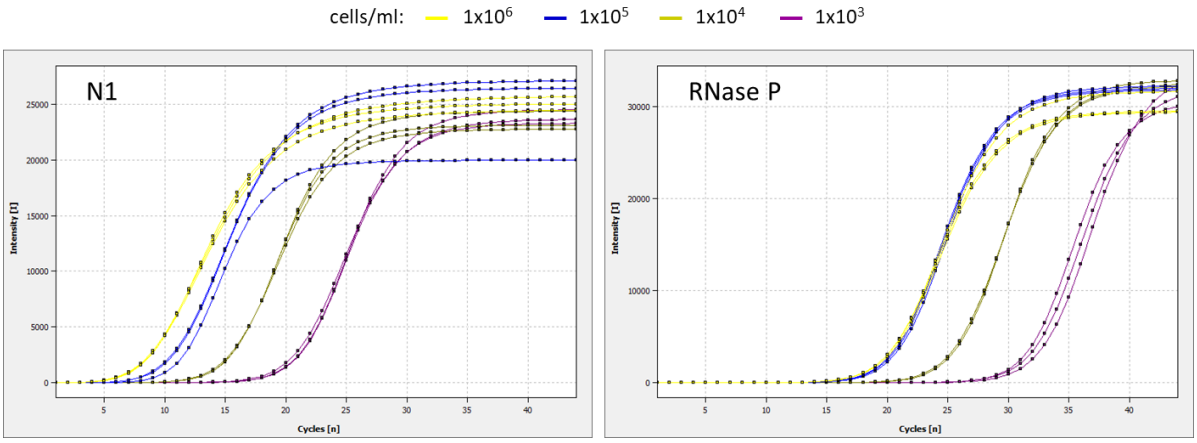

Figure 2. VER/heat treatment releases target RNA from transfected cells. Amplification plots as in Fig. 1 from VER/heat treated serial dilutions of transfected HEK293T cell suspensions.

We then screened a few primer/probe sets as well as RT-qPCR master mixes and SARS-Cov-2 specific RT-qPCR assays to select highly efficient ones on the basis of their lower limit of detection (LLoD). This was performed using serial dilutions of corresponding *in vitro* transcribed mRNA standards at known concentrations, including transcripts coding for the N (as described above; Fig. 1) and the E proteins. Consistent with the specifications from the CDC-EUA[13], N1 and N2 primer/probe sets yielded similar results (not shown). We further compared N2 and E primer/probe sets using three and two commercially available RT-qPCR master mixes, respectively, here designated as L, T and M. This revealed an increasing sensitivity gradient among the master mixes in the sequence order L, T and M (Fig. 3).

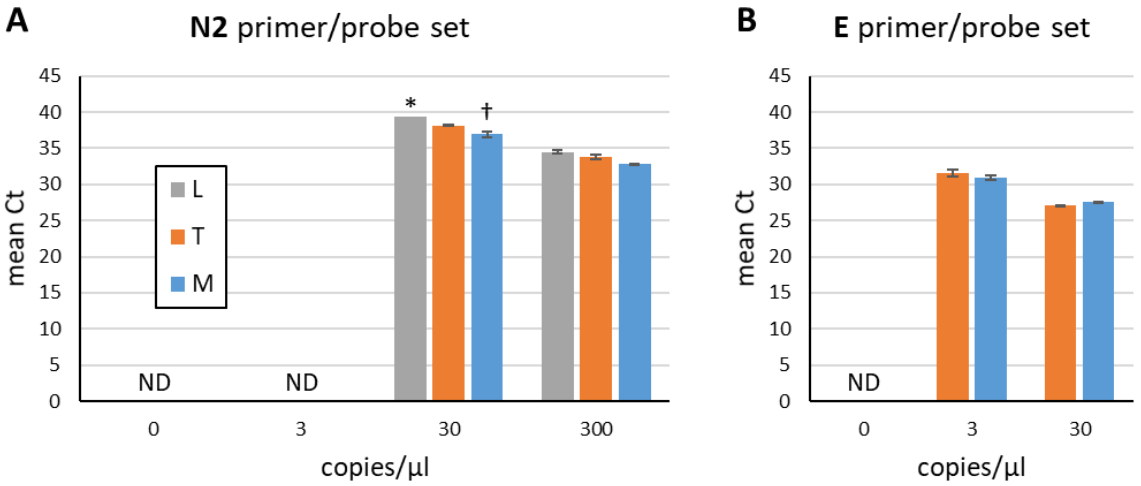

Figure 3. Comparison of detection performance with different RT-qPCR master mixes and primer/probe set combinations. Mean Ct values and standard deviations from triplicate RT-qPCR reactions with N2 (A) and E (B) primer/probe sets are plotted for the indicated concentrations of RNA target standard. * and † denote cases were only one and two out of triplicate reactions yielded a Ct value. ND = not detectable.

Also, in our hands, the proprietary primer/probe set provided with the M assay kit and targeting the mRNA coding for the Envelope protein (E) afforded sharply higher sensitivity than the N1 and N2 sets, with a LLoD of at least 3 copies/µl for E (Fig. 3B; lower concentrations were not tested). In contrast, the N sets failed to detect their target RNA at the same concentration with any of the master mixes (Fig. 3A for N2).

Finally, the two most sensitive master mixes were selected to test the VER method on ten swab samples that had been previously validated as positive with an independent RT-qPCR assay using isolated RNA as template. Among these samples, eight swabs were eluted in liquid Amies medium (i.e., without charcoal), while for two of them Universal Transport Medium (UTM) was used (both from Copan). For each swab sample, RNA was extracted from an aliquot with magnetic bead-based isolation on a Maxwell RCS 48 instrument (Promega; hereafter referred to as RNA isolates), while a 100 µl aliquot was treated with VER and heat as described above (VER lysate). Volumes of RNA preparations and VER lysates corresponding to the same amount of original sample were reverse transcribed and amplified with master mixes T and M, using the same primer/probe sets targeting the E and ORF1a sequences. Ct values obtained from RNA isolates with the M master mix (Fig. 4) were consistently lower by an average 0.8 and 0.7 for the detection of E and ORF1a, respectively.

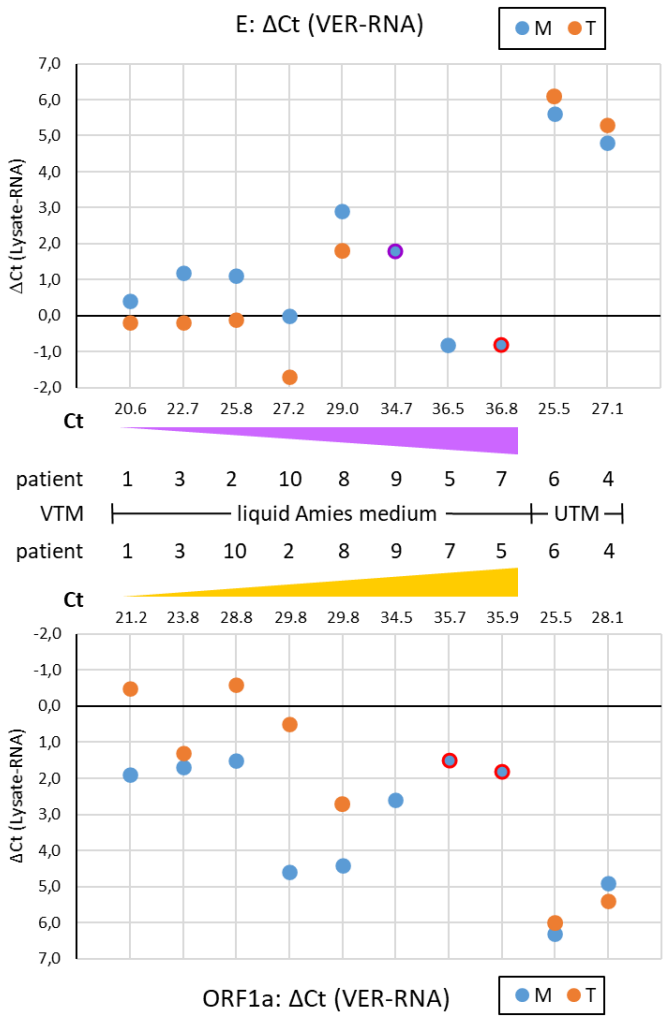

Figure 4. A highly sensitive RT-qPCR assay shows only marginal difference in detection performance between VER lysates and corresponding RNA isolates over a wide range of viral loads. Detection performance was compared from patient swab samples using two highly sensitive RT-qPCR master mixes, T and M, in combination with two VTM as well as E and ORF1a primer/probe sets (higher and lower plots, respectively). The difference in Ct values (ΔCt) between VER lysate and RNA isolate as template from the same samples is plotted. Ten swabs from infected patients (1-10) were eluted with either liquid Amies medium (patients 1-3, 5 and 7-10) or Universal Transport Medium (UTM; patients 4 and 6) Samples eluted with the different transport media are separately ordered according to increasing Ct value obtained with the M master mix. Data points circled with a purple and red line denote cases where Ct values were returned only from two or one of triplicate reactions, respectively.

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However, for each of the primer/probe sets they were proportionally concordant across samples. In Figure 4 the difference between Ct values from VER lysates and RNA isolates is plotted for both primer/probe sets and both master mixes, with samples eluted with the two transport media separately order according to increasing Ct value from isolated RNA. As we showed that the treatment with VER and heat does not affect the ability of isolated RNA to be amplified (Fig. 1), we assume that most of the difference in the sensitivity of detection between VER lysates and corresponding RNA isolates is due to the presence of substances in former that inhibit the enzymes of the RT-qPCR assay. The head-to-head comparison in Figure 4 indicates that, while in general the T master mix is more robust to inhibition by sample-born components, it fails to detect viral RNA at a lower viral load relative to the M master mix. This analysis also shows a major difference in performance between transport media in combination with the VER method, with liquid Amies medium clearly leading to lower difference in detection efficiency at comparable Ct values from RNA isolates. With regard to this, we note that liquid Amies medium does not contain proteins, while UTM contains bovine serum albumin[18–21].

Conclusions

We have developed a rapid and inexpensive RNA isolation-free method for sample processing before RT-qPCR that involves treatment with a lysis reagent and heat. Using synthetic transcripts as standard templates, we show that our method leads to a LLoD at least as low as 3 copies/µl, which is comparable with the specifications reported by the CDC-EUA. Combined with careful selection of commercial RT-qPCR assays, our VER method affects only marginally the detection sensitivity of SARS-CoV-2 RNA in swab samples compared to standard RNA isolation. We also show that the robustness of detection with crude VER lysates partly depends on the transport medium used for swab elution, with liquid Amies medium, which is free of proteins, giving best results. Therefore, we do not recommend the use of UTM or other transport media containing bovine serum albumin or fetal bovine serum[21–23].

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