PCR*opsis*™ Reagent RVD Facilitates RNA Extraction-free Amplification of Gene Targets from SARS-CoV-2

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Abstract:
Viral RNA extraction is unnecessary and presents a burdensome step towards amplification of SARS-CoV-2 gene targets. Here we outline and validate an extraction-free protocol that facilitates amplification of viral RNA targets from specimens in compatible universal viral transport mediums. This approach replaces viral RNA extraction with a 10-minute incubation utilizing PCR*opsis*™ Reagent RVD. The extraction-free protocol resulted in a limit of detection of 11.13 genome copies / RT-qPCR reaction and was able to correctly identify 100% of tested clinical samples.

Methods:

Materials:
- Heat inactivated 2019 Novel Coronavirus (ATCC: VR-1986HK)
- Heat inactivated remnant clinical samples
- Nuclease-free water
- Low-binding pipette tips
- BD™ Universal Viral Transport (UVT) medium
- MedSchenker™ SmartTransport
- Entopsis: PCR*opsis*™ Universal Viral Transport, PCR*opsis*™ Reagent RVD
- Qiagen: QIAamp Viral RNA Kit
- Stellar Scientific: Thin-walled qPCR tubes
- IDT: SARS-CoV-2 (2019-nCoV) CDC qPCR Probe Assay
- Promega: 1-Step GoTaq® RT-qPCR Systems
- Chai: Open qPCR Thermocycler

Limit of Detection (LoD) Study:
1. Dilute 1 part heat inactivated SARS-CoV-2 with 9 parts viral transport medium, and mix thoroughly.
2. Thoroughly mix 50 µL Reagent RVD with 50 µL of diluted viral samples in a thin walled tube (0.2 ~ 0.6 mL) and cap tubes.

3. Heat mixture of Reagent RVD / viral sample for 10 minutes at 95˚C and let cool at room temperature for ~10 seconds before continuing.
4. Mix thoroughly.
5. Add 5 µL of heated Reagent RVD / viral sample to 15 µL of RT-qPCR mix.
    a. RT-qPCR mixture:
        i. GoTaq® qPCR Master Mix, 2X: 10 µl
        ii. 1X GoScript™ RT Mix for 1-Step RT-qPCR (50X): 0.4 µl
        iii. IDT primer / probe: 1.5 µl
        iv. Nuclease-Free Water: 3.1 µl
        v. Reagent RVD / viral sample: 5 µL
6. Run samples on qPCR Thermocycler for 45 cycles.
    a. Reverse Transcription: 45˚C for 15 minutes, then 95˚C for 2 minutes
    b. DNA Amplification: 95˚C 30 seconds, 55˚C 30 seconds, 72˚C 30 seconds for 45 cycles

Studies with clinical samples:

1. Thoroughly mix 50 µL Reagent RVD with 50 µL of clinical sample in a thin walled tube (0.2 ~ 0.6 mL) and cap tubes.
2. Heat mixture of Reagent RVD / viral sample for 10 minutes at 95˚C and let cool at room temperature for ~10 seconds before continuing.
3. Mix thoroughly.
4. Add 5 µL of heated Reagent RVD / viral sample to 15 µL of RT-qPCR mix.
    a. RT-qPCR mixture:
        i. GoTaq® qPCR Master Mix, 2X: 10 µl
        ii. 1X GoScript™ RT Mix for 1-Step RT-qPCR (50X): 0.4 µl
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        iv. Nuclease-Free Water: 3.1 µl
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5. Run samples on qPCR Thermocycler for 45 cycles.
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Viral RNA extraction:

RNA was extracted from samples using Qiagen QIAamp Viral RNA Kit according to the manufacture's protocol.

Results:

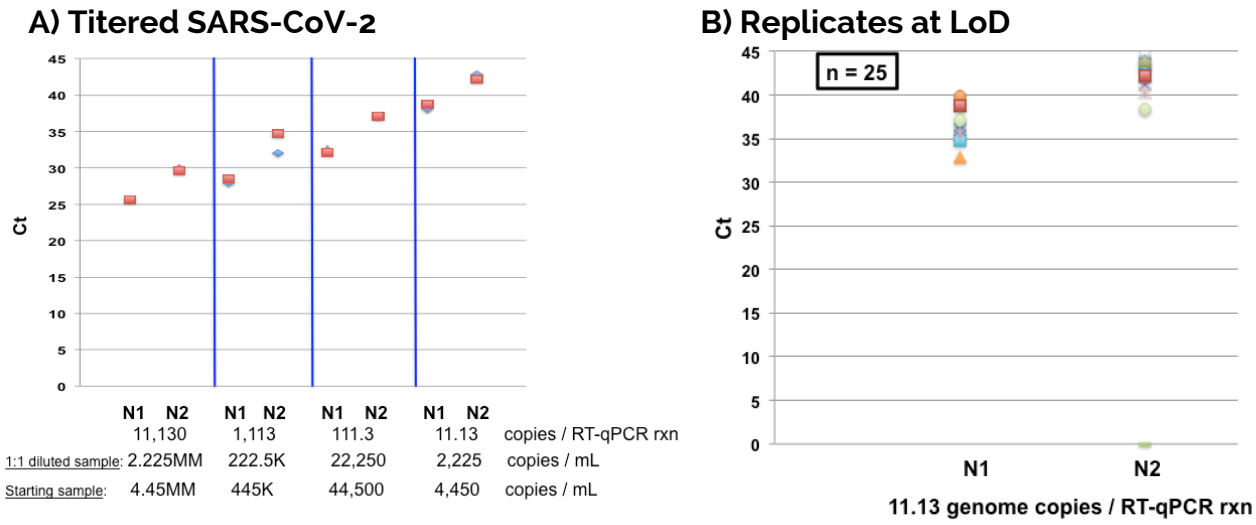

Figure 1. Reagent RVD facilitates viral RNA extraction-free amplification of N1 and N2 sites on heat inactivated SARS-CoV-2 with a LoD of 11.13 genome copies / RT-qPCR reaction. Both gene sites were amplified from diluted viral samples containing 11,130 to 11.13 genome copies / RT-qPCR reaction. B) Both gene sites were amplified >95% of the time in replicate studies; thus demonstrating a limit of detection (LoD) of 11.3 genome copies / RT-qPCR reaction. SARS-CoV-2 was diluted in 3 separate viral transport mediums to demonstrate the compatibility of Reagent RVD with mediums from different manufacturers (Becton Dickinson, MedSchenker and Entopsis).

	Unprocessed Sample	Traditional RNA Extraction	Entopsis PCR*opsis* Reagent RVD
Correctly called Coronavirus (-) Samples	100%	100%	100%
Correctly called Coronavirus (+) Samples	65%	100%	100%

Figure 2. Clinical samples (n=55) processed with PCR*opsis*™ Reagent RVD and traditional RNA extraction followed by RT-qPCR identified 100% of SARS-CoV-2 negative and positive samples. Clinical samples that were previously confirmed as positive (n=21) and negative (n=34) for SARS-CoV-2 by a FDA authorized RT-qPCR protocol were used for this study. Unprocessed samples failed to identify 35% of positive samples. A sample is considered positive when amplification is observed for N1, N2 and RP gene targets.

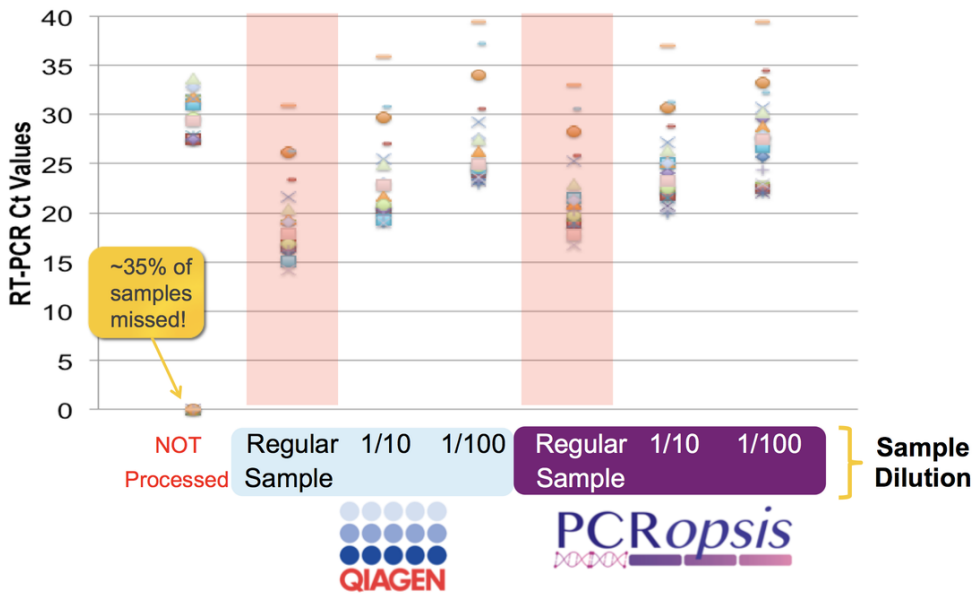

Figure 3. Amplification of the SARS-CoV-2 N1 gene using Reagent RVD or RNA extraction + RT-qPCR resulted in comparable Ct values from 21 previously confirmed SARS-CoV-2 positive samples. This was also observed when the samples were diluted 1/10 or 1/100 in BD™ UVT. 35% of SARS-CoV-2 positive samples not processed with Reagent RVD or RNA extracted, followed by RT-qPCR, were incorrectly identified as negative.

Conclusions & Discussion:

- Reagent RVD + RT-qPCR resulted in amplification of both N1 and N2 gene sites in all samples containing 11,130 to 11.13 genome copies / RT-qPCR reaction of SARS-CoV-2 with Cts <45 (Figure 1A). Here we see viral titer dependent amplification of relevant genes.

- Replicate studies (n=25) at 11.13 genome copies / RT-qPCR reaction of SARS-CoV-2 demonstrate amplification of both N1 and N2 in 96% of tested samples (Figure 1B). These studies indicate that Reagent RVD combined with RT-qPCR has a **LoD of 11.13 genome copies / RT-qPCR reaction** based on available data.

- Successful replicate studies at 11.13 genome copies / RT-qPCR reaction of SARS-CoV-2 were performed using universal viral transport medium from Becton Dickinson, MedSchenker and Entopsis. Thus demonstrating the compatibility of Reagent RVD with transport mediums from different manufacturers. Reagent RVD is expected to perform well with similar transport mediums from numerous manufacturers. However, Reagent RVD was found to not be compatible with inactivating transport mediums containing guanidinium thiocyanate (data not shown). Moreover, Reagent RVD performs sub-optimally when plain phosphate buffered saline (PBS), Hank's Balanced Salt Solution

(HBSS) + 2% Fetal Bovine Serum (FBS) or water with veal infusion broth + BSA + antibiotics is used to transport specimens (data not shown).

- Reagent RVD + RT-qPCR was able to correctly identify all 55 tested clinical samples (Figure 2), both positives (n=21) and negative (n=34) samples. In addition, samples processed with Reagent RVD or RNA extraction resulted in comparable Ct values for the N1 SARS-CoV-2 gene site (Figure 3). Ct values for the N2 gene site are generally higher (3~5 Ct) for Reagent RVD processed samples compared to the same samples processed with RNA extraction (data not shown).

- The data supports the replacement of viral RNA extraction with Reagent RVD for the amplification of SARS-CoV-2 gene targets from samples transported in compatible universal viral transport mediums. Reagent RVD may similarly be able to facilitate extraction-free amplification of gene targets from various other microorganisms.