In-sample purification using Inactivir™ Viral Inactivation Buffer

Purpose

The aim of the application note is to validate and quantify an 'in-sample purification' method using the LSG VIB-002 and VIB-005 viral inactivation buffers. This principle of this is to use silica-based magnetic-beads to extract the nucleic acids (DNA or RNA) within a large sample volume (tested with 4 ml sample).

For testing purposes the recovery of the synthetic RNA EURM-19 reference standard for SARS nCoV-19 in a pseudo-sample also containing a mix of human and bacterial material was measured. This models recovery of viral RNA within the variety of eukaryotic and prokaryotic material that might be present in a real-world sample.

We have previously validated that the VIB-002 and VIB-005 inactivation buffers will stabilise and preserve viral RNA in such samples over an extended period with samples stored at both room temperature (21 °C) for up to 5 days (120 hours) and for a period of up to 10 weeks when stored at -20 °C[1]. This means the samples will not degrade over extended sampling periods if required.

The VIB-002 buffer is a second-generation REACH-compliant guanidine-based buffer, analogous to the buffer developed and validated by Burton et al for inactivation and testing of Ebola[2]. VIB-005 is a novel buffer with an emulsion-based inactivation system containing a stabilised iodide chaotrope. This has the advantage over guanidine-based buffers of being compatible with bleach decontamination methods. The Inactivir™ buffers have been tested by PHE and accepted as both inactivating SARS nCoV-19 and stabilising the released RNA for future analysis[3].

To allow in-tube recovery of nucleic acids, the VIB-002 and VIB-005 buffers require addition of 33% ethanol (absolute or 95% denatured) to facilitate direct extraction from magnetic silica beads present in solution. This method allows both immediate inactivation of material that enters the buffer, but also concentration and extraction in a single step.

Results

Extractions were completed from 4 ml duplicate pseudo-samples of VIB-002 or VIB-005 supplemented by 33% absolute ethanol, either with or without magnetic beads to allow in-tube purification. In the 'standard' extraction procedure, mimicking general Covid sample testing, duplicate 200 µl samples were taken and magnetic beads were added and taken through a standard purification process. For in-tube purification, beads were magnetically extracted from the 4 ml sample. These were then taken through standard washing and extraction steps. Using the standard 200 µl extraction protocol recovery was compared with and

[1] See LSG application note 'Inactivir™ Viral Inactivation Buffers Stability and Storage of samples. https://lifescienceproduction.co.uk/wp-content/uploads/2021/09/Inactivir-Buffers-Stability-and-Storage.pdf
[2] Burton et al. *Journal of virological methods*, (2017) https://doi.org/10.1016/j.jviromet.2017.09.020
[3] See PHE reports HCM/CoV2/084/v1 and HCM/CoV2/085/v1. Available on Request

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without the addition of poly-A carrier RNA, which is commonly used in RNA extraction kits to improve sample recovery. After the initial in-tube extraction, 200 µl samples of the supernatant were taken to measure residual RNA levels. All samples were tested in duplicate by QPCR to measure viral RNA recovery. The Ct values determined for each extraction are shown in Figure 1 with analysis in Table 1.

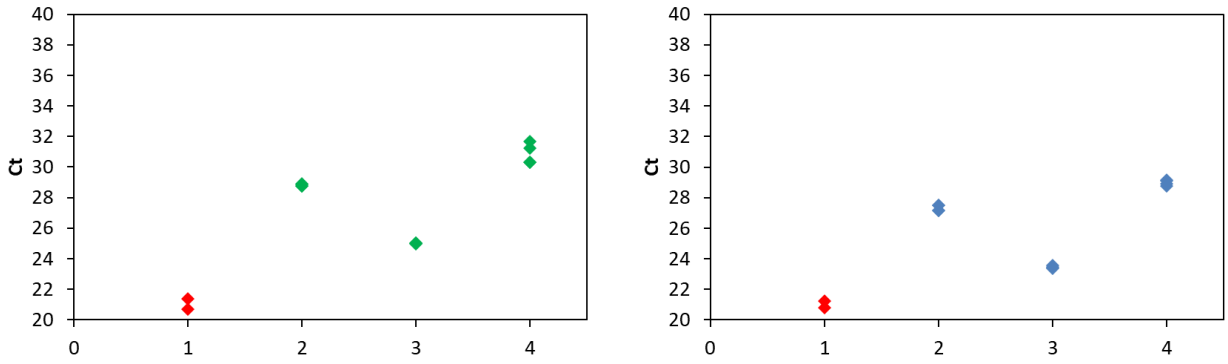

Fig1: Ct values of samples determined using CDC N2 primer / probe combination for SARS nCoV-2 RNA. The left panel shows results with VIB-002 (green) and right VIB-005 (blue). Samples in each are 1: Viral RNA control, 2: Extraction using 200 µl from 4 ml sample with carrier RNA**,** 3: Direct extraction from 4 ml sample. 4: Recovery from 200 µl of supernatant from direct extraction.

Table1: Average Ct values for samples

	RNA control	200 µl Extract	4 ml Extract	Δ Ct Ext	Residual Ct	Δ Ct Res
VIB-002	21.0	28.8	25.0	3.8	30.9	2.1
VIB-005	21.0	27.3	23.5	3.8	29.0	1.7

The results in Fig 1 show both the RNA control replicates have an average Ct of 21.0, which conforms with the expectation of the EURM-19 standard with minimal run to run variation. There is a 3.8 Ct difference in values for the extraction from 200 µl from a 4 ml sample and the in-tube extraction from the entire 4 ml sample for both buffers. This equates to a 14.4 fold ($2^{3.8}$) increase in recovery, equating to around 72% of the total available RNA in comparison to the standard samples (14.4 fold increase from 20 x sample volume).

Residual RNA in the supernatants was measured by sampling from the supernatants. This shows an approximate 2 Ct reduction, this shows around 25% (2^{-2}) residual RNA detectable. This agrees closely with the estimated recovery from the increase in sensitivity.

The extraction data using VIB-005 show a consistently lower Ct value (more sensitive) across all assays. The reason for this is unclear.

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Conclusions

In-tube purifications using magnetic silica beads result in recovery of around 72% of all viral RNA available within a 4 ml volume of 4 ml pseudo-samples. This is confirmed by both a 3.8 Ct (14.4 fold) improvement in sensitivity of detection of viral RNA and a reduction of around 2 Ct (4 fold) in the residual viral RNA in the supernatant of the extracted sample.

This demonstrates that both buffers are suitable for in-tube purification of samples and give a good recovery even from an order of magnitude more sample than normally used for viral RNA extraction.

Methods

Pseudo-samples were made to replicate the environment of typical sampled viral RNA. These contained both human and bacterial cells, replicating the range of eukaryotic and prokaryotic as well as viral nucleic acids that would be expected in a real-world sample. The EURM-19 reference standard Covid RNA used is a synthetic single-strand RNA encoding parts of the Covid genome routinely used for detection[4]. Samples were made in duplicate of 25 µl of a 1/1000 dilution of Covid-19 reference material EURM-019 plus 50µl of Hela cells (~$10^{\wedge 6}$ ml^{-1}) in Phosphate Buffered Saline (PBS) and 50 µl of *E. coli* (~1 OD stock). To this sample 4 ml of the VIB under test mixed with 33% absolute ethanol was added. Other experiments (not shown) have demonstrated that ethanol is essential for RNA recovery.

Samples were purified using a 'home made' kit based on a published extraction procedure outlined by the Baker lab and used by Addenbrookes Hospital, Cambridge, Uk[5]. This protocol has previously been validated to produce similar results to extractions from the commercial Qiagen™ QIAamp™ Viral RNA Mini Kit and the Applied Biosystems™ MagMAX™ Viral RNA isolation kit.

Samples were processed initially by either taking 200 µl volumes and adding 20 µl of magnetic beads, incubating for 5 minutes before magnetic isolation or by magnetically isolating the pre-loaded beads from the entire volume. Having initially isolated the beads, the same steps followed. The beads were washed with first with 250 µl of 3 M Sodium Acetate (pH 5.2) wash buffer and then twice with 350 µl 70% ethanol, with the beads isolated magnetically after each wash. The beads were then allowed to air dry before extracting in 50 µl of 10mM Tris pH 7.2 for 2 min, with the purified RNA collected from the magnetically isolated beads.

For detection of the RNA the CDC published N2 primer / probe combination[6] was used with a FAM / BHQ-1 quencher combination for the probe (Sigma Aldrich). Samples were amplified using PCR Biosystem qPCRBIO Probe 1-Step Go master mix. For each tube 5µl of sample was added to a total 20 µl reaction volume with

[4] https://crm.jrc.ec.europa.eu/p/EURM-019
[5] bioRxiv preprint https://doi.org/10.1101/2020.04.14.041319
[6] https://www.cdc.gov/coronavirus/2019-ncov/lab/rt-pcr-panel-primer-probes.html

the addition of N2 primers to 250 nM and N2 probe to 125 nM. Samples were amplified using a Corbett Research Rotor Gene 6000. The protocol was 50 °C for 10 minutes for reverse transcription, followed by 95 °C for 2 mins and 40 cycles of 95 °C 5 sec, 60 °C 20 sec with read on the FAM channel.

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