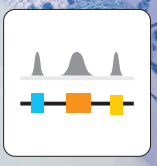
Pathogen detection and characterization with Revelo™ RNA-Seq High Sensitivity.

Application Note



UNCOVER SARS-CoV-2 FROM DEGRADED SAMPLES

INTRODUCTION

Studying the SARS-CoV-2 genome can provide valuable insights into its evolution and transmission, as well as aiding surveillance and vaccine development efforts to help control the COVID-19 pandemic. The virus can be detected by RT-PCR in nasal or throat swab samples from patients with COVID-19, but it is detailed, sequence-based analysis that provides an understanding of the nature and mutation of the virus and the resulting disease state. Next generation sequencing (NGS) offers a comprehensive view of the complete genome, providing information about viral origins, pathogenesis, genetic variations, immune responses and phylogenetics.

Revelo RNA-Seq High Sensitivity is a whole transcriptome solution that provides several key benefits for NGS library preparation, offering a single-day sample-to-sequencer workflow for rapid processing. This upfront amplification method effectively addresses the challenge of ultralow input to increase detection sensitivity, particularly in asymptomatic carriers [1]. Our Single Primer Isothermal Amplification (SPIA®) technology has been used in hundreds of peer reviewed publications to enable access to low input RNA samples from various sources. As shown in Figure 1, the Revelo RNA-Seq High Sensitivity library preparation kit shortens and simplifies the NGS library preparation process using our integrated library quantification technology – NuQuant®. The resulting high quality output provides a perfect starting point for researchers studying SARS-CoV-2 and other infectious pathogens.

This application note describes effective use of the Revelo RNA-Seq High Sensitivity library preparation kit for NGS applications to identify and sequence SARS-CoV-2 from COVID-19 positive patient samples.

MATERIALS AND METHODS

Materials

1. Starting material: 500 pg to 5 ng total RNA extracted from SARS-CoV-2 positive nasal swab samples (Discovery Life Sciences)
2. Revelo RNA-Seq High Sensitivity, Human rRNA suppression with SPIAboost™, UDI, 96 reactions (Tecan: Core Module #30201359; Adaptor Plate (UDI) #30184203; SPIABoost Human #30201372)
3. Library preparation kit from Competitor T

Experimental design and protocol

The experiment was designed to identify the detection sensitivity of the Revelo RNA-Seq High Sensitivity library preparation kit. RNA-Seq libraries were prepared from six total RNA samples – with varying viral copy numbers and sample integrities (see Table 1 below) – according to the kit manufacturers' guidelines. In addition, two negative control samples were processed with Revelo RNA-Seq High Sensitivity only. The final libraries were quantified using NuQuant (for Revelo RNA-Seq High Sensitivity libraries) or KAPA™ qPCR (for the Competitor T kit libraries). Libraries were then pooled and sequenced on Illumina MiniSeq® (2 x 75 bp paired-end run) and aligned reads were normalized to 1 million reads for downstream data analysis. Custom dynamic read trimming was performed using cutadapt (https://cutadapt.readthedocs.io/en/stable/), and reads were aligned using STAR to hg19 and SARS-CoV-2 (MN908947.3) concatenated genome sequence.

Input total RNA

It is important to evaluate the quality of the input material prior to library preparation. The quality of total RNA extracted from nasal swabs was established by electrophoresis using an Agilent BioAnalyzer system, which reports the RNA Integrity Number (RIN) on a scale from 1 to 10 - with 1 being the most degraded [2].

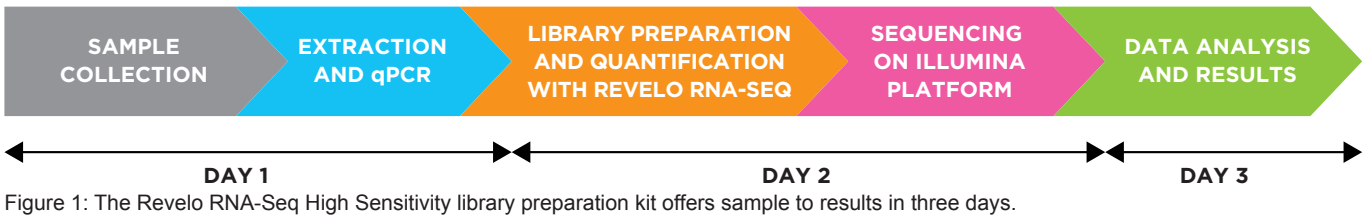


Figure 1: The Revelo RNA-Seq High Sensitivity library preparation kit offers sample to results in three days.

The results indicated high levels of degradation for a majority of the samples, with RIN values from 2.2 to 5.9, and a majority with a RIN of less than 3.0 (Figure 2). The Revelo RNA-Seq High Sensitivity library preparation kit has been optimized to ensure high quality libraries from these types of degraded samples.

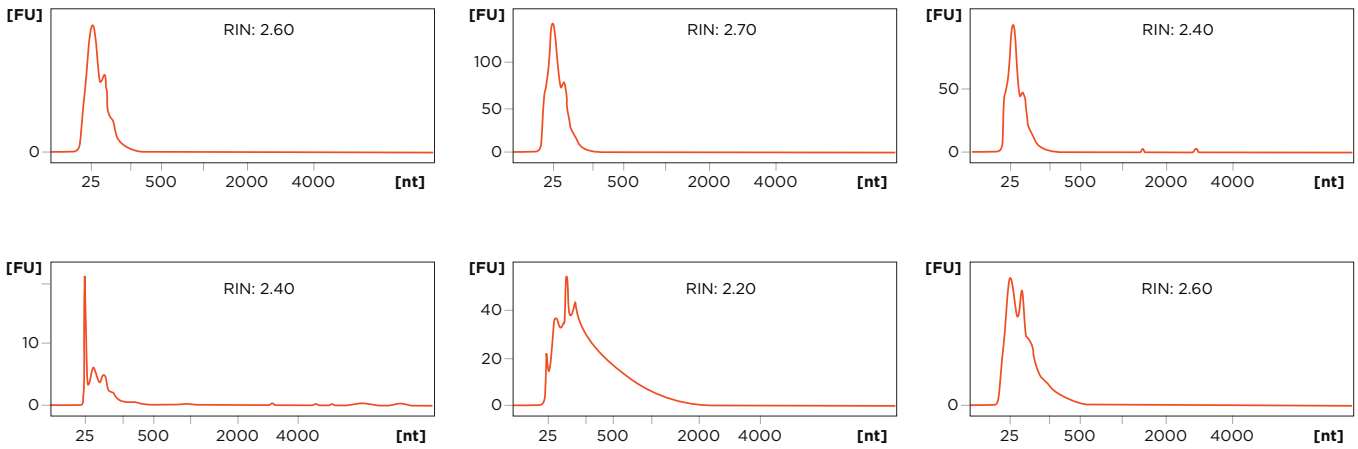


Figure 2: BioAnalyzer traces indicating the sample quality for total RNA samples extracted from nasal swabs.

RT-qPCR:

The total RNA from all samples was tested for SARS-CoV-2 using the Abbott RealTi*m*e SARS-CoV-2 assay [3]. The viral loads (copy number per µL of total RNA) and average Ct values are indicated in Table 1. The two SARS-CoV-2 negative samples processed using only the Revelo RNA-Seq High Sensitivity were true negatives, and so the results are not shown.

CoV Sample ID	SARS-CoV-2 copies per µL	Avg Ct value
Sample 1	741	32
Sample 2	760	32
Sample 3	1,380	31
Sample 4	2,205	30
Sample 5	137,902	23
Sample 6	2,950,950	19

Table 1: SARS-CoV-2 positive samples with a range of viral loads.

RESULTS AND ANALYSIS

The Revelo RNA-Seq High Sensitivity library preparation kit showed significant benefits for library preparation across a range of critical parameters, including:

- Consistent high quality final libraries (Figure 3)
- Effective DNase treatment, eliminating contaminating gDNA (Table 2)
- High viral detection sensitivity at low copy numbers (Table 3 and 4)
- Successful SARS-CoV-2 detection without the need for deeper sequencing (Figure 4)

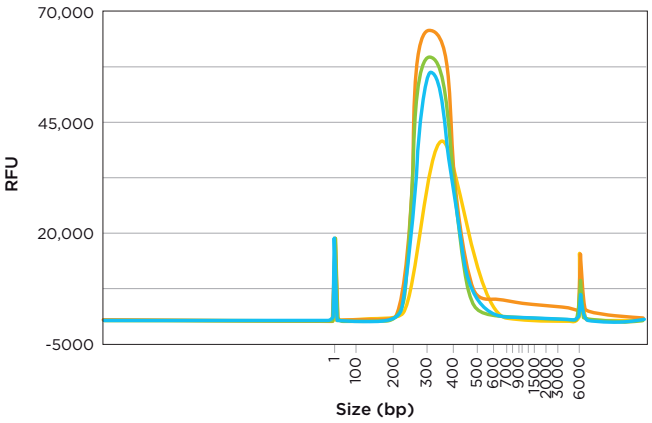


Figure 3: Agilent Fragment Analyzer traces for libraries generated using the Revelo RNA-Seq High Sensitivity library preparation kit.

As seen in Figure 3, the Revelo RNA-Seq High Sensitivity library preparation kit enables the generation of robust libraries with consistent fragment length from 500 pg to 5 ng low integrity samples. The fragment size distribution of 200 to 600 bp is as expected, and is ideal for cluster generation on Illumina sequencer flow cells [4]. Notably, all of the libraries generated using the Revelo RNA-Seq High Sensitivity kit yielded successful sequencing results. Sequencing performance metrics (Clusters %PF, Q30 metrics, total reads per run) for the libraries sequenced on the Illumina MiniSeq platform were as expected (data not shown here) [4].

Contaminating gDNA can interfere with gene expression analysis in RNA-Seq experiments [5]. To avoid erroneous conclusions from the sequencing results, DNase digestion is strongly recommended to eliminate gDNA from total RNA samples. Revelo RNA-Seq High Sensitivity includes an integrated DNase step prior to library preparation that effectively eliminates contaminants, as seen in Table 2. The competitor kit, on the other hand, shows very high levels of gDNA contamination in the sequenced libraries, skewing the results and adversely impacting the detection sensitivity of SARS-CoV-2 from patient samples.

	% exon		% intron		% SARS-CoV-2	
	Revelo	Competitor T	Revelo	Competitor T	Revelo	Competitor T
Sample 1	13.67	3.01	1.89	39.70	0.20	0.00
Sample 2	43.46	2.58	23.16	40.33	0.63	0.00
Sample 3	27.02	2.19	3.88	39.35	0.12	0.00
Sample 4	9.41	2.26	0.86	38.76	0.30	0.00
Sample 5	4.33	2.61	4.59	40.50	84.41	0.00
Sample 6	1.35	2.71	0.59	40.64	92.52	0.09

Table 2: % intron, % exon and % SARS-CoV-2 metrics to identify potential DNA contamination and its effects on SARS-CoV-2 enrichment in sequenced libraries.

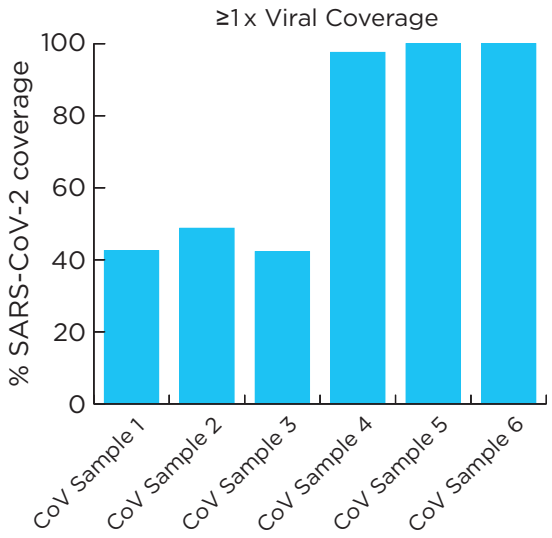


Figure 4: Revelo RNA-Seq High Sensitivity libraries SARS-CoV-2 sequence coverage at ≥1x (Illumina MiniSeq, reads normalized to 1M/sample)

CoV Sample ID	SARS-CoV-2 copies per sample
Sample 1	537
Sample 2	647
Sample 3	1,052
Sample 4	17,638
Sample 5	216,827
Sample 6	7,026,072

Table 3: SARS-CoV-2 copies per sample.

To study the SARS-CoV-2 enrichment and coverage metrics, reads were aligned to hg19 + SARS-CoV-2 concatenated custom reference sequence. The Revelo RNA-Seq High Sensitivity kit consistently maintains ~100 % coverage at ≥1x (>17,000 viral copies), enabling detection of the virus at even 1M read depth (Figure 4). Sequencing depth is critical for both effective detection of the virus and downstream genome analysis pipelines. Results successfully demonstrate the high detection sensitivity of SARS-CoV-2 using Revelo RNA-Seq High Sensitivity, from as low as ~500 viral copies (Table 3). For the competitor kit, viral coverage drops drastically, even when the viral copy number is as high as ~200,000 (Table 4).

CoV Sample ID	Revelo RNA-Seq High Sensitivity				Competitor T			
	Viral coverage			Viral reads	Viral coverage			Viral reads
	≥1x	≥5x	≥10x		≥1x	≥5x	≥10x	
Sample 1	42.55%	24.72%	14.59%	1,293	1.33%	0.00%	0.00%	5
Sample 2	48.75%	31.54%	23.21%	2,797	0.00%	0.00%	0.00%	14
Sample 3	42.24%	19.37%	8.80%	639	0.48%	0.00%	0.00%	13
Sample 4	97.57%	73.13%	40.07%	1,995	4.37%	0.00%	0.00%	33
Sample 5	100.00%	99.99%	99.99%	688,987	13.90%	0.00%	0.00%	37
Sample 6	100.00%	100.00%	99.99%	843,342	99.87%	38.57%	5.81%	717

Table 4: Comparison of viral genome coverage of SARS-CoV-2 using Revelo RNA-Seq High Sensitivity and the competitor kit (Illumina MiniSeq, reads normalized to 1M/sample).

SUMMARY

The Revelo RNA-Seq High Sensitivity library preparation kit is optimized for the detection of rare and low abundance sequences, offering accurate identification of low copy number viral pathogens. The results demonstrate that it is sufficiently sensitive for use on low concentration, low quality, degraded samples with inputs as low as 250 pg. RNA sequencing using the Revelo RNA-Seq High Sensitivity library preparation kit has several key benefits, including:

- Ability to obtain consistent results with low quality, degraded samples
- Effective DNase treatment to eliminate unwanted contaminating gDNA
- High detection sensitivity from samples with low viral titers in high host background
- Human rRNA depletion to enhance informative reads
- Single day, sample-to-sequencer workflow

Revelo RNA-Seq High Sensitivity library preparation kit achieves this exceptional performance by combining an array of novel technologies. This kit includes Unique Dual Indexed Adaptors for increased multiplexing and detection of index hopping, DimerFree® technology for elimination of adaptor dimers, NuQuant for library quantification within minutes, and ready-to-go automation scripts for the DreamPrep™ NGS workstation to enable high throughput applications.

REFERENCES

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2) RNA Integrity Number (RIN) – Standardization of RNA Quality Control'. https://www.agilent.com/cs/library/applications/5989-1165EN.pdf
3) The Abbott RealTime SARS-CoV-2 assay is an Emergency Use Authorization (EUA) test authorized by the U.S. FDA' https://www.molecular.abbott/us/en/products/infectious-disease/RealTime-SARS-CoV-2-Assay
4) www.Illumina.com
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