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Supplemental Material

Sub-genomic RNA identification in SARS-CoV-2 Genomic Sequencing Data

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Supplemental Files

Supplemental_File_S1.csv

Sheffield Nanopore Canonical ORF Sub-Genomic RNA Counts.
All canonical ORF counts for all 1155 samples.

Supplemental_File_S2.csv

Sheffield Nanopore Noncanonical Sub-Genomic RNA Counts
All noncanonical ORF counts for all 1155 samples.

Supplemental_File_S3.csv

Downsampling of Sheffield Nanopore Data
All canonical ORF counts for all samples used in the downsampling experiment.

Supplemental_File_S4.csv

Technical Replicates of Sheffield SARS-CoV-2 isolates
All canonical ORF counts for all samples used in the replicates experiment.

Supplemental_File_S5.csv

Canonical Sub-Genomic RNA Counts From *In Vitro* Illumina Metagenomic Data
Periscope counts for Illumina metagenomic data.

Supplemental_File_S6.csv

Canonical Sub-Genomic RNA Counts From *In Vitro* ARTIC Nanopore Data
Periscope counts for ONT ARTIC *in vitro* data.

Supplemental_File_S7.csv

Glasgow Nanopore Canonical ORF Sub-Genomic RNA Counts
Canonical periscope counts for Glasgow ONT ARTIC data.

Supplemental_File_S8.csv

Sheffield Sample Technical Information
Run information for the samples in the study for PCA analysis.

Supplemental_File_S9.csv

Sheffield Sample Diagnostic Test E Gene Cycle Threshold
E cycle threshold values for samples in the study.

Supplemental_File_S10.csv

Glasgow Nanopore Noncanonical Sub-Genomic RNA Counts
Noncanonical sgRNA counts for Glasgow ONT ARTIC data.

Supplemental_File_S11.csv

Noncanonical Sub-Genomic RNA Counts From Illumina Bait Capture Clinical Samples
Noncanonical sgRNA counts for Glasgow bait capture Illumina data.

Supplemental_File_S12.csv

Noncanonical Sub-Genomic RNA Counts From *In Vitro* Illumina Metagenomic Data
Noncanonical sgRNA counts for the *in vitro* dataset sequenced using an Illumina metagenomic approach.

Supplemental_File_S13.csv

Noncanonical Sub-Genomic RNA Counts From *In Vitro* ARTIC Nanopore Data
Noncanonical sgRNA counts for the *in vitro* dataset sequenced using an ONT ARTIC approach.

Supplemental_File_S14.csv

Sub-Genomic RNA Counts From Clinical SARS-CoV-2 Samples Using Illumina Bait Capture
Canonical periscope counts for Glasgow ONT ARTIC data.

Supplemental_File_S15.csv

Read Lengths of a Subset of Reads Classified as Sub-Genomic or Genomic
Read lengths for a representative sample classed into either sgRNA or gRNA.

Supplemental_File_S16.csv

Variant Allele Frequencies in Sub-Genomic or Genomic RNA Fractions
Counts of bases at each variant position.

Supplemental_File_S17.csv

Consensus Genome Coverage of Sheffield SARS-CoV-2 ARTIC Nanopore Data
Genomic coverage of consensus sequences.

Supplemental_File_S18.txt

All R markdown code used in generation of figures contained within this manuscript. We suggest you download our github repository to get all of the required tables and images to re-create this analysis. https://github.com/sheffield-bioinformatics-core/periscope-publication/

Supplemental_File_S19.csv

ENA Accession number conversion
Translation from ENA accession (ERS…..) to SHEF or CVR identifier.

Supplemental_File_S20.tar.gz

periscope source code.

Common column names contained within these files:

Column Name	**Contents**
gRNA_count	Raw count of genomic reads
sgRNA_HQ_count, sgRNA_LQ_count, sgRNA_LLQ_count	Raw count of sub-genomic reads classified as high quality, low quality or low low quality
gRPHT	Genomic reads per 100,000 mapped reads
sgRPTg_HQ, sgRPTg_LQ, sgRPTg_LLQ	High, low and low low quantity sub-genomic RNA reads per 1000 genomic
sgRPTg_ALL	Sum of all sub-genomic reads normalized per 1000 genomic
sgRPHT_HQ, sgRPHT_LQ, sgRPHT_LLQ	High, low and low low quantity sub-genomic RNA reads per 100,000 mapped reads
sgRPHT_ALL	Sum of all sub-genomic reads normalized per 100,000 mapped
nsgRNA_HQ_count, nsgRNA_LQ_count, nsgRNA_LLQ_count	Raw count of noncanonical sub-genomic reads classified as high quality, low quality or low low quality
nsgRPTg_HQ, nsgRPTg_LQ, nsgRPTg_LLQ	High, low and low low quantity noncanonical sub-genomic RNA reads per 1000 genomic
nsgRPTg_ALL	Sum of all noncanonical sub-genomic reads normalized per 1000 genomic
nsgRPHT_HQ, nsgRPHT_LQ, nsgRPHT_LLQ	High, low and low low quantity noncanonical sub-genomic RNA reads per 100,000 mapped reads
nsgRPHT_ALL	Sum of all noncanonical sub-genomic reads normalized per 100,000 mapped
sgRPTL	Sub-genomic reads per 1000 reads of local coverage

Periscope README

Below we have provided instructions for the installation, execution and interpretation of periscope.

Requirements

periscope runs on MacOS, unix and unix subsystem for windows 10.

You will need:

- conda
- Your raw FASTQ files from the ARTIC protocol
- Periscope installation

In our hands periscope takes around 1-5 minutes per million reads on a single core on a Dell XPS core i9 with 32GB ram and 1TB SSD.

Installation

```
git clone https://github.com/sheffield-bioinformatics-core/periscope.git
&& cd periscope
conda env create -f environment.yml
conda activate periscope
python setup.py install
```

Execution

```
conda activate periscope

periscope
    --fastq-dir <PATH_TO_DEMUXED_FASTQ> (ont only)
    OR
    --fastq <FULL_PATH_OF_FASTQ_FILE(s)> (space separated list of fastq files,
you MUST use this for Illumina data)
    --output-prefix <PREFIX>
```

```
    --sample <SAMPLE_NAME>
    --artic-primers <ASSAY_VERSION; V1,V2,V3 or 2kb>
    --resources <PATH_TO_PERISCOPE_RESOURCES_FOLDER>
    --technology <SEQUENCING TECH; ont or illumina>
    --threads <THREADS_FOR_MAPPING>
```

Output Files

Filename	**Description**
`<OUTPUT_PREFIX>.fastq`	A merge of all files in the FASTQ directory specified as input.
`<OUTPUT_PREFIX>_periscope_counts.csv`	The counts of genomic, sgRNA and normalization values for *known* ORFs
`<OUTPUT_PREFIX>_periscope_amplicons.csv`	The amplicon by amplicon counts, this file is useful to see where the counts come from. Multiple amplicons may be represented more than once where they may have contributed to more than one ORF. (ONT only)
`<OUTPUT_PREFIX>_periscope_novel_counts.csv`	The counts of genomic RNA, sgRNA and normalization values for *noncanonical* ORFs
`<OUTPUT_PREFIX>.bam` & `<OUTPUT_PREFIX>.bam.bai`	Minmap2 or BWA-MEM mapped reads and index with no adjustments made.
`<OUTPUT_PREFIX>_periscope.bam` & `<OUTPUT_PREFIX>_periscope.bam.bai` & `<OUTPUT_PREFIX>_sorted_periscope.bam` & `<OUTPUT_PREFIX>_sorted_periscope.bam.bai`	This is the original input BAM file and index created by periscope with the reads specified in the fastq-dir. This file, however, has tags which represent the results of periscope: ● XS is the alignment score ● XA is the amplicon number ● XC is the assigned class (gDNA or sgDNA) These are useful for manual review in IGV or similar genome viewer. You can sort or colour reads by these tags to aid in manual review and figure creation.

Examining Base Frequencies of Called Variants in periscope

This script will take the pass vcf from the ARTIC Network pipeline and examine the periscope BAM file for the bases present at that position. It will split the counts by read class and output a plot showing contribution at each base at each site in the VCF.

```
conda activate periscope

gunzip <ARTIC_NETWORK_VCF>.pass.vcf.gz

<PATH_TO_PERISCOPE>/periscope/periscope/scripts/variant_expression.py \
    --periscope-bam <PATH_TO_PERISCOPE_OUTPUT_BAM> \
    --vcf <ARTIC_NETWORK_VCF>.pass.vcf \
    --sample <SAMPLE_NAME> \
    --output-prefix <OUPUT_PREFIX>
```

Filename	**Description**
`<OUTPUT_PREFIX>_base_counts.csv`	Counts of each base at each position
`<OUTPUT_PREFIX>_base_counts.png`	Plot of each position and base composition

Supplemental Methods

Glasgow Amplicon Sequencing

Sequencing libraries were prepared according to the ARTIC nCoV-2019 sequencing protocol version 2, described in detail at https://artic.network/ncov-2019. For amplicon generation cDNA was synthesised from extracted RNA using SuperScript IV (Thermo Scientific, Part Number 18090200) and random hexamer primers (Part number N8080127). PCR amplicons (approximately 400 bp) were generated from this cDNA using Q5® Hot Start High-Fidelity 2X Master Mix (New England Biolabs, Part Number M0494L), nCoV-2019 PrimalSeq sequencing primers (Version 3) and 25-35 cycles of the ARTIC nCoV-2019 recommended thermocycling conditions. The PCR amplicons were purified using Agencourt AMPure XP for PCR Purification (Beckman Coulter, Part Number A63881), following the manufacturer's guidelines and quantified using the Qubit dsDNA HS Kit (Thermo Scientific, Part Number Q32854). Generated amplicons were then used to prepare either Oxford Nanopore or Illumina sequencing libraries.

Oxford Nanopore Sequencing

End repair of amplicons (200 fmol) was carried out using the NEBNext Ultra II End repair /dA-tailing Module (New England Biolabs, Part Number E7546L), then 20 fmol of end prepped amplicon was barcoded using the Native Barcoding Expansion 1-12 (PCR free) kit (Oxford Nanopore Technologies, Part Number EXP-NBD104) and NEBNext® Ultra™ II Ligation Module (New England Biolabs, Part Number E7595L). Barcoded amplicons were pooled together and purified using Agencourt AMPure XP for PCR Purification (Beckman Coulter, Part Number A63881), following the manufacturer's guidelines and quantified using the Qubit dsDNA HS Kit (Thermo Scientific, Part Number Q32854). Adapter mix II (Oxford Nanopore Technologies, Part Number EXP-NBD104) was ligated to the barcoded amplicons using the Ligation Sequencing kit (Oxford Nanopore Technologies, SQK-LSK109) and

NEBNext Quick Ligation Module (New England Biolabs, Part Number E6056L). The final library was purified using Agencourt AMPure XP for PCR Purification (Beckman Coulter, Part Number A63881), following the manufacturer's guidelines and quantified using the Qubit dsDNA HS Kit (Thermo Scientific, Part Number Q32854). Approximately 50 fmol of the sequencing library pool was loaded onto a flow cell (R9.4.1) (Oxford Nanopore Technologies, Part Number FLO-MIN106D) where sequencing was conducted in MinKNOW version 19.12.6 and raw FAST5 files were basecalled using Guppy version 3.2.10 in high accuracy mode using a minimum quality score of 7.

Illumina

Generated amplicons were used to prepare Illumina sequencing libraries using the Kapa LTP Library Platforms kit (Kapa Biosystems, Part Number KK8232). Briefly, the amplicon fragments were end repaired and the protocol followed through to adapter ligation. At this stage the samples were uniquely indexed using the NEBNext Multiplex Oligos for Illumina 96 Unique Dual Index Primer Pairs (New England Biolabs, Part Number E6442S), with 5-15 cycles of PCR performed. All amplified libraries were quantified by Qubit dsDNA HS Kit and run on the Agilent 4200 Tapestation System (Agilent, Part Number G2991AA) using the High Sensitivity D5000 Screentape (Agilent, Part Number 5067-5592) and High Sensitivity D5000 Reagents (Agilent, Part Number 5067-5593). Sequencing of libraries was carried out on Illumina's MiSeq system (Illumina, Part Number SY-410-1003) with more than 75% bases presenting a Q score superior to 30.

Glasgow Bait Capture & Subsequent Illumina Sequencing

A mild DNase treatment was done using DNase 1 (Thermo Fisher Scientific, Part Number AM2222) and samples were purified using RNA Clean AMPure XP Beads (Beckman Coulter,

A63987). cDNA was synthesised using SuperScript III (Thermo Scientific, Part Number 18080044) and NEBNext Ultra II Non-Directional RNA Second Strand Synthesis Module (New England Biolabs, Part Number E6111L). Reagents from the Illumina Nextera Flex for Enrichment (Cat. No. 20025523 and 20025524) were used for library preparation and targeted enrichment. Briefly, The cDNA was tagmented and followed through to PCR with the indexed primers. 12 cycles of PCR were used as per protocol recommendations using the IDT for Illumina Nextera DNA Unique Dual Indexes Set A (Illumina Part Number 20027213). The amplified libraries were quantified by Qubit dsDNA HS Kit and run on the Agilent 4200 Tapestation System (Agilent, Part Number G2991AA) using the High Sensitivity D5000 Screentape (Agilent, Part Number 5067-5592) and High Sensitivity D5000 Reagents (Agilent, Part Number 5067-5593), to guide sample pooling. Baits from Illumina (Respiratory Virus Oligos Panel V2, Part Number 20044311) were hybridized with the sample pool overnight at 62oC, as recommended by Illumina. After capture and wash, 12 cycles of PCR were used to amplify captured DNA. The amplified pools were quantified by Qubit dsDNA HS Kit and run on the Agilent 4200 Tapestation System using the High Sensitivity D5000 Assay to determine the size of the pool in base pairs (bp). The sequencing of the enriched pools was carried out on Illumina's MiSeq System (Illumina, Part Number SY-410-1003) using a MiSeq Reagent Kit v3 600 cycle kit (Illumina, Part Number MS-102-3003) with more than 70% bases presenting a Q score superior to 30.

Supplemental Tables

ORF	Samples with >=1 (HQ+LQ Reads)	Percent of Total
E	1046	90.6
M	1109	96.0
N	1124	97.3
ORF10	11	1.0
ORF3a	673	58.3
ORF6	1053	91.2
ORF7a	906	78.4
ORF8	274	23.7
S	1071	92.7

Supplemental Table S1 - sgRNAs detected for each canonical ORF

Raw counts of the number of sgRNAs found across all 1,155 samples of the cohort with 1 or more HQ or LQ read.

Sample	Genomic RNA	HQ Sub-Genomic	LQ Sub-Genomic
SHEF-C00C0	3793	0	2
SHEF-C045B	911	0	1
SHEF-C046A	1340	0	1
SHEF-C0840	1829	1	0
SHEF-C09F2	2462	0	1
SHEF-C58A5	4611	1	0
SHEF-C722D	1536	0	1
SHEF-C8408	4237	0	1
SHEF-CF595	5173	1	0
SHEF-D179A	2768	1	0
SHEF-D227A	3802	0	1

Supplemental Table S2 - Samples with predicted sgRNA for ORF10

Samples with any HQ or LQ evidence of ORF10 sgRNA (amplicons 97 and 98). Twelve reads in total across the whole cohort putatively support a sgRNA for ORF10 .

Read Start Position	Contributing Amplicon	Total Genomic Read Count	Total HQ sgRNA Read Count	Total LQ sgRNA Read Count	Total sgRNA Read Count
25744	84,85	6019	120	33	153
25745	85	1389	4	5	9
25746	85	1389	1	2	3
25748	85	1389	2	1	3
25749	85	1389	1	0	1
25754	85	1389	2	0	2
25755	85	1389	1	0	1
25732	85	1389	0	1	1
25735	85	1389	0	1	1
25742	85	1389	0	1	1
25753	85	1389	0	1	1
25766	85	1389	0	1	1

Supplemental Table S3 - Noncanonical sgRNA at position 25,744 in sample SHEF-C0118 has strong support

ORF	Contributing Amplicon	Total Genomic Read Count	Total HQ sgRNA Read Count	Total LQ sgRNA Read Count	Total sgRNA Read Count
S	71,72	4939	44	31	75
ORF3a	83,84	8147	3	2	5
E	86,87,88	11183	12	7	19
M	87,88	6590	192	90	282
ORF6	89	564	60	24	84
ORF7a	90,91	5580	0	16	16
N	93,94	8928	500	251	751
Noncanonical sgRNA	**84,85**	**6019**	**131**	**46**	**177**

Supplemental Table S4 - Canonical sgRNA in SHEF-C0118

Showing only those ORF sgRNAs with supporting Reads.

Read Start Position	Contributing Amplicon	Total Genomic Read Count	Total HQ sgRNA Read Count	Total LQ sgRNA Read Count	Total sgRNA Read Count
10639	35,36	4698	103	15	118
10640	35	3583	0	1	1
10641	35	3583	2	3	5
10642	35,36	4698	1	4	5
10643	36	1115	1	0	1
10644	35	3583	1	0	1
10645	35	3583	2	2	4
10647	35	3583	0	1	1

Supplemental Table S5 - Noncanonical sgRNA at 10,639 in SHEF-CE04A has strong support

Counts of reads supporting the sgRNA at 10,639 in SHEF-CE04A.

Sample	Read Start Position	Total Genomic Read Count	Total HQ sgRNA Read Count	Total LQ sgRNA Read Count
CVR2185	5785	12689	3	0
CVR2187	5785	20464	1	2
CVR2191	5785	5479	1	0
CVR2231	5785	7561	2	0
CVR2234	5785	8369	1	0
CVR2239	5785	12261	2	0
CVR2243	5785	21022	1	0
CVR2251	5785	5907	1	1
CVR2265	5785	13147	0	1
CVR2306	5785	8442	4	1
CVR2319	5785	4785	2	0
CVR2322	5785	5926	1	0
CVR2484	5785	3096	7	0
CVR3941	5785	10157	2	0
CVR3943	5785	5521	0	1

Supplemental Table S6 - Noncanonical sgRNA at 5,785 in Glasgow Nanopore samples

Fifteen samples in the Glasgow ONT ARTIC dataset have evidence for a noncanonical sgRNA at 5,785

Sample	Read Start Position	Total Genomic Read Count	Total HQ sgRNA Read Count	Total LQ sgRNA Read Count
CVR2131	10639	9802	3	1
CVR2133	10639	312	1	0
CVR2166	10639	11494	1	1
CVR2185	10639	4653	1	2
CVR2187	10639	9927	3	0
CVR2190	10639	1607	1	0
CVR2191	10639	2107	1	0
CVR2247	10639	11675	1	4
CVR2265	10639	6284	7	1
CVR2306	10639	3537	4	1
CVR2319	10639	1772	5	2
CVR2322	10639	2114	1	2
CVR3940	10639	5916	1	0

Supplemental Table S7 - Noncanonical sgRNA at 10,639 in Glasgow Nanopore samples

Thirteen samples from the ONT ARTIC Glasgow dataset have evidence for a noncanonical sgRNA at 10,639.

ORF	Contributing Amplicon	Total Genomic Read Count	Total HQ sgRNA Read Count	Total LQ sgRNA Read Count	Total sgRNA Read Count
S	71,72	4945	64	16	80
ORF3a	84,85	6990	8	1	9
M	87	4456	176	43	219
ORF6	89	1527	0	0	0
N	93	8083	116	38	154
Noncanonical sgRNA	**35,36**	**4698**	**110**	**26**	**136**

Supplemental Table S8 - Canonical sub-genomic RNA in SHEF-CE04A

Showing only those ORF sgRNAs with supporting Reads

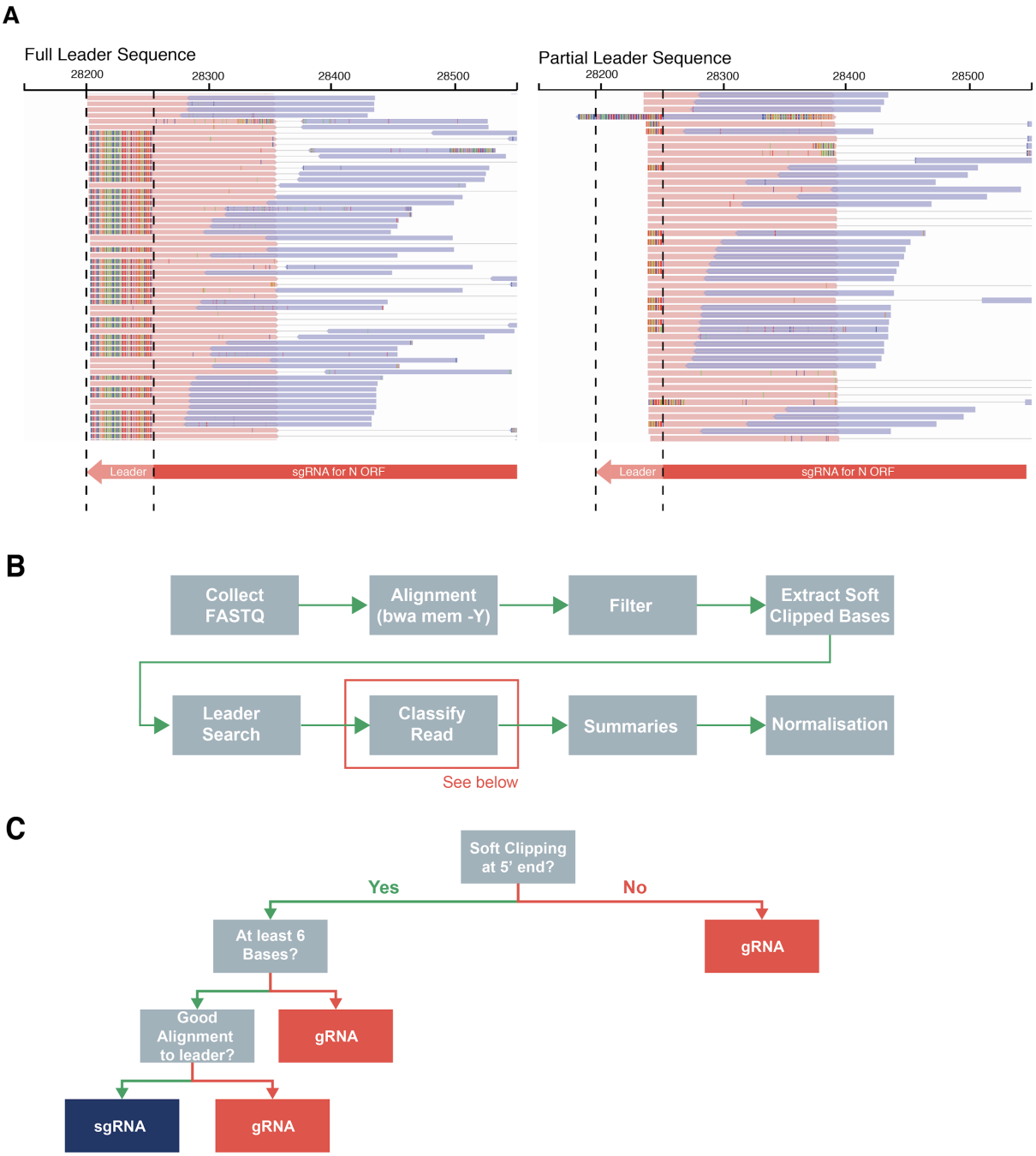
Virus	SNP	Amino Acid Change	
		Gene	Mutation
PHE2	A2618G	nsp2	I605V
	C8782T	nsp14	I150T
	T18488C	S	T95I
	C21846T	E	L37H
	T23605G	ORF 8	L84S
	T26354A	N	P365S
	T28144C	ORF 10	I13M
	C29366T		
	A29596G		
GLA1	C3037T	nsp12	P323L
	C14408T	S	D614G
	A23403G	E	V5A
	A24388T		
	T26258C		
GLA2	C3037T	nsp12	P323L
	C14408T	nsp15	V35A
	T19724C	S	N439K
	C22879A	S	D614G
	A23403G	ORF 10	V6F
	G29573T		

Supplemental Table S9 - Changes with respect to MN908947.3 in viral isolates used in *in vitro* SARS-CoV-2 infection model

Supplemental Figures

Supplemental Figure S1 - Length of reads in SHEF ONT ARTIC data broken down by periscope assigned class in a representative sample (SHEF-BFDBE)

At each of the amplicons responsible for the production of sgRNA supporting reads we examined the size of the two classes of reads. Genomic RNA is between 400 and 600bp, and in most cases sgRNA is shorter at around 200-300bp.

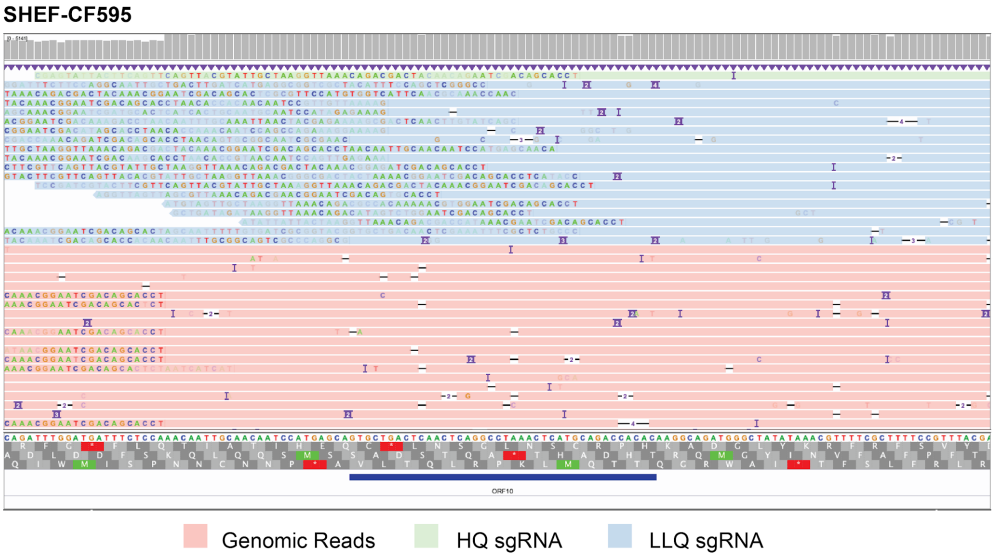


Supplemental Figure S2 - SARS-CoV-2 Illumina Data

A. Bait based capture and metagenomic sequencing of SARS-CoV-2 genomes by illumina methods results in variable lengths of leader sequence included in the read. **B.** Overall workflow for analysis of Illumina data is very similar to that of ARTIC Nanopore data, but adjusted in light the phenomenon described in (**A**). **C.** Classification of reads from illumina data involves extracting soft clipped bases from the 5' end of reads and performing a local

alignment of these to the leader sequence. Only those reads that have >= 6 bases soft-clipped are considered for leader matching to reduce false positives.

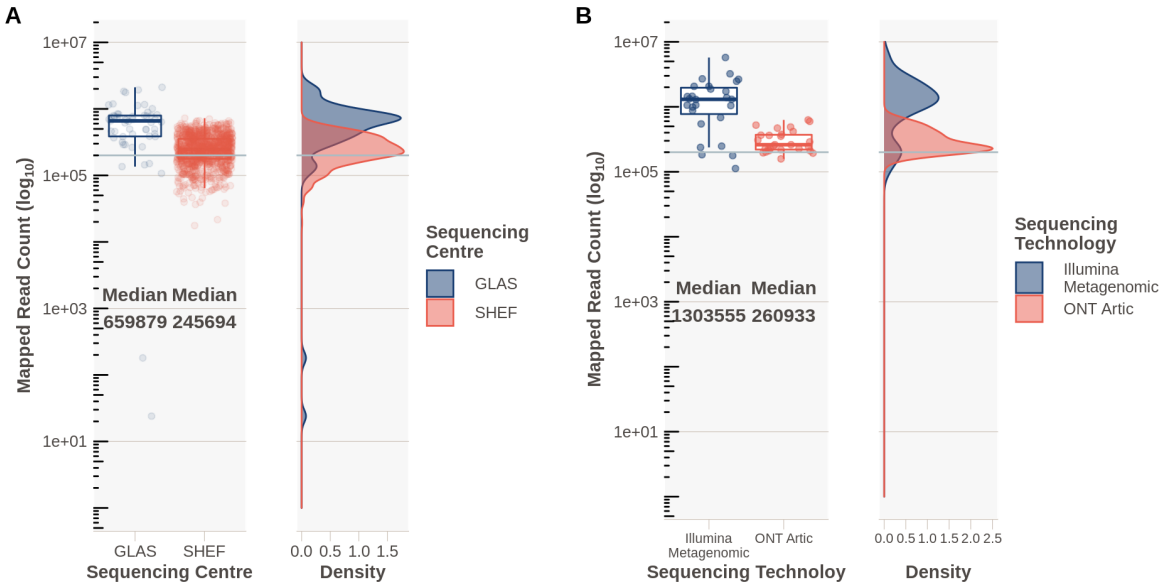
A



B

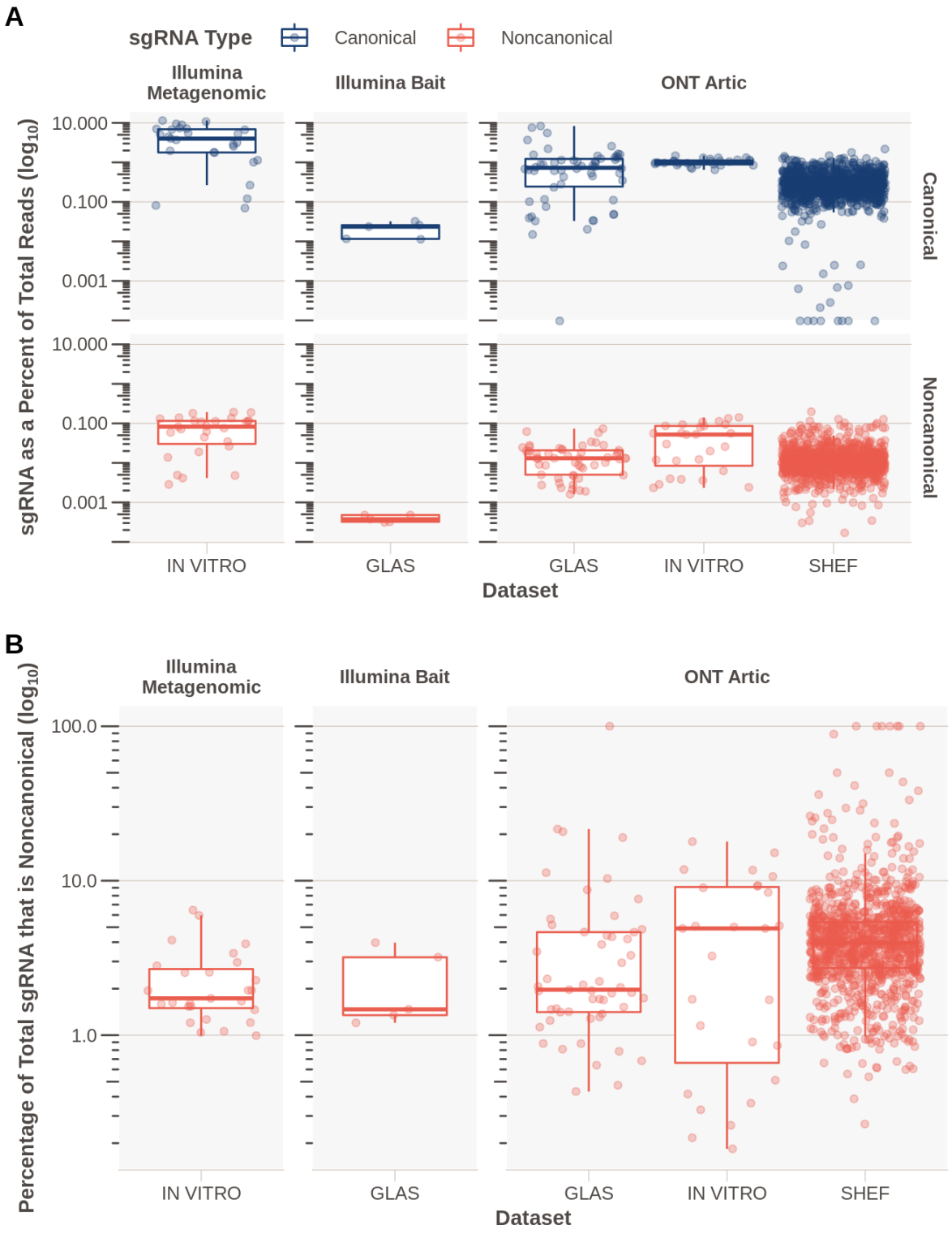
Supplemental Figure S3 - Manual review of periscope BAM files for ORF10

A. SHEF-CF595 has 1 read classified as HQ sgRNA at the predicted ORF10 leader junction (light green). It is clear from this IGV screenshot that this read does not contain a valid leader sequence. **B.** All HQ and LQ sgRNA reads, 12 in total, aligned with Minimap2 to a reference consisting of ORF10 and leader sequence, 3 reads failed to map. Two reads could be bona fide ORF10 sgRNAs (Highlighted in the black box with a good match to the leader) from samples SHEF-C0840 and SHEF-C58A5.



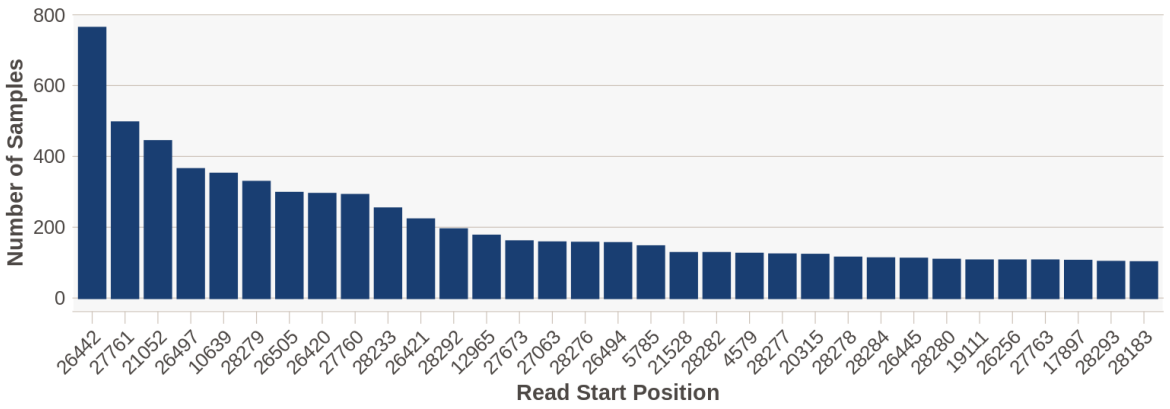
Supplemental Figure S4 - Mapped read counts

Reads were mapped with Minimap2 (ONT) or BWA-MEM (Illumina) to MN908947.3, sorted with SAMtools and mapped reads counted with pysam. Grey horizontal line represents 200,000 mapped reads. Right plot in each panel is a density distribution of the points in the left panel. **A.** Data from SARS-CoV-2 clinical samples, sequenced with ONT Artic at Sheffield and Glasgow. **B.** Mapped read counts from SARS-CoV-2 *in vitro* infection models for Illumina metagenomic and ONT Artic data.



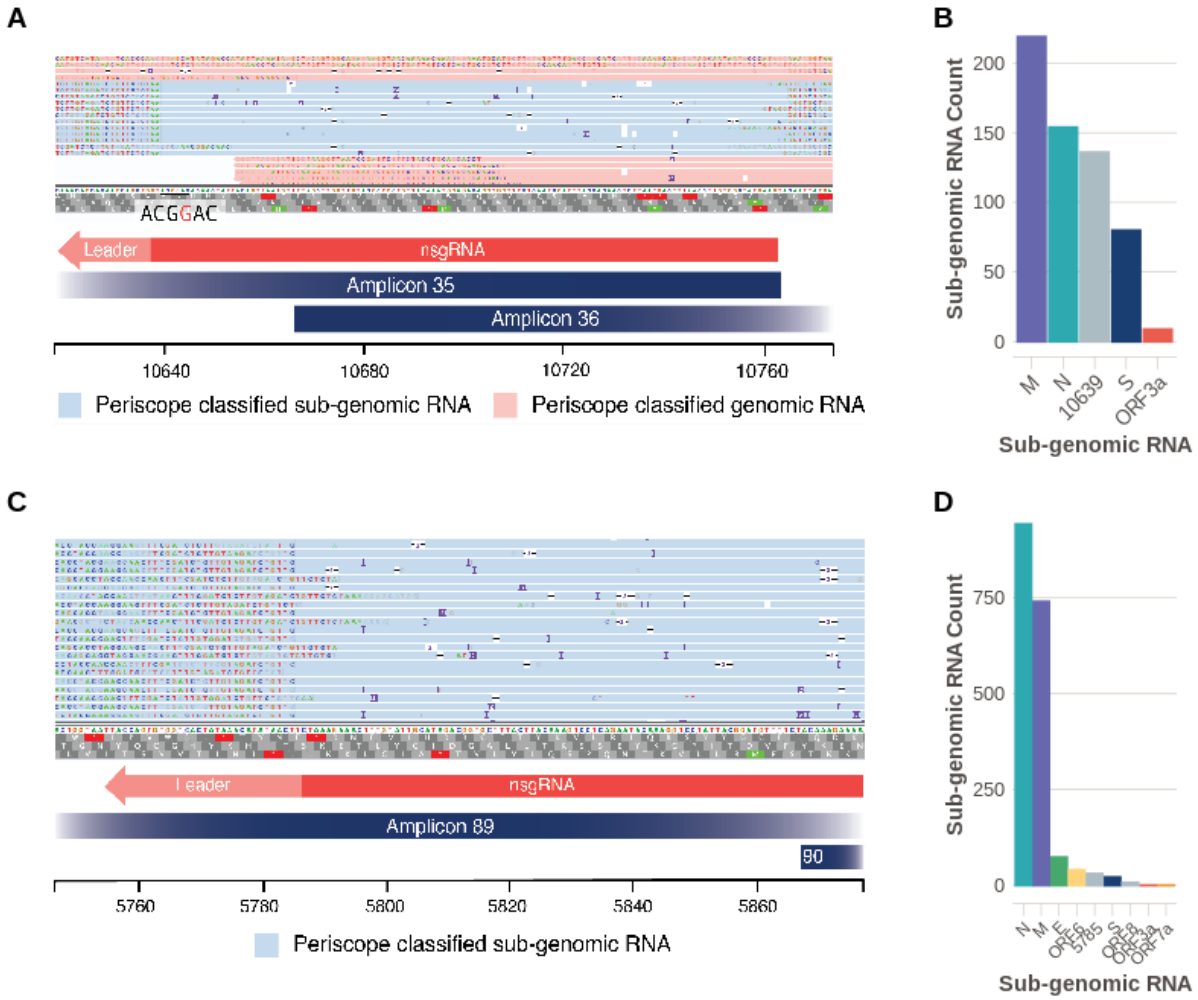
Supplemental Figure S5 - Sub-Genomic RNA Proportions

A. Total sgRNA reads split by canonical and noncanonical as a proportion of the total mapped reads in all featured datasets. **B.** Proportion of total sgRNA that is noncanonical.



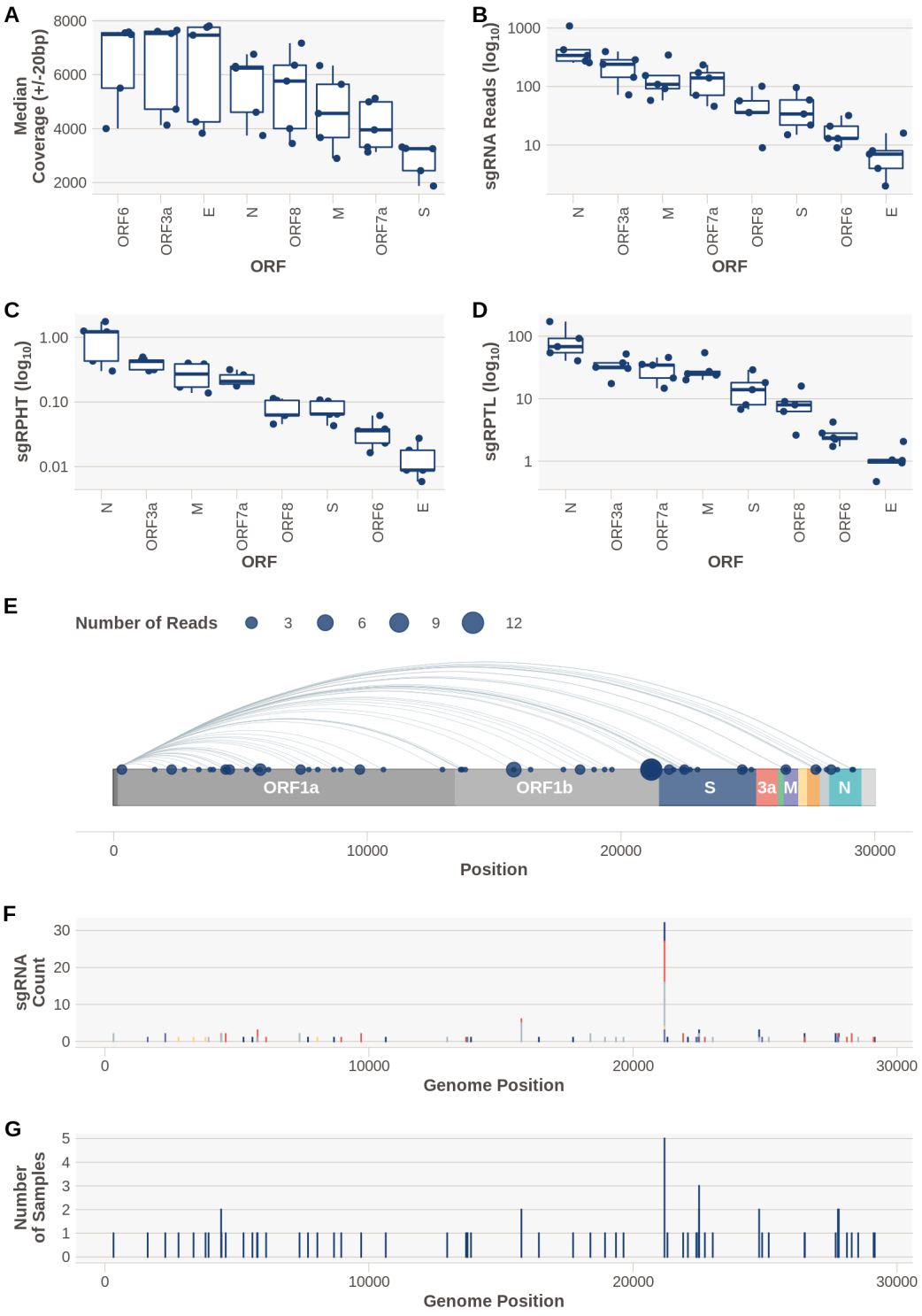
Supplemental Figure S6 - Number of samples with at the most frequently represented noncanonical sub-genomic RNAs

The number of samples each noncanonical sgRNA was found in. This is an exact position match, and includes sgRNAs that could be just outside the +/-20 of the TRS-B site. Sites with support in > 100 samples shown.



Supplemental Figure S7 - Highly expressed noncanonical sgRNAs at 10,369 and 5,785

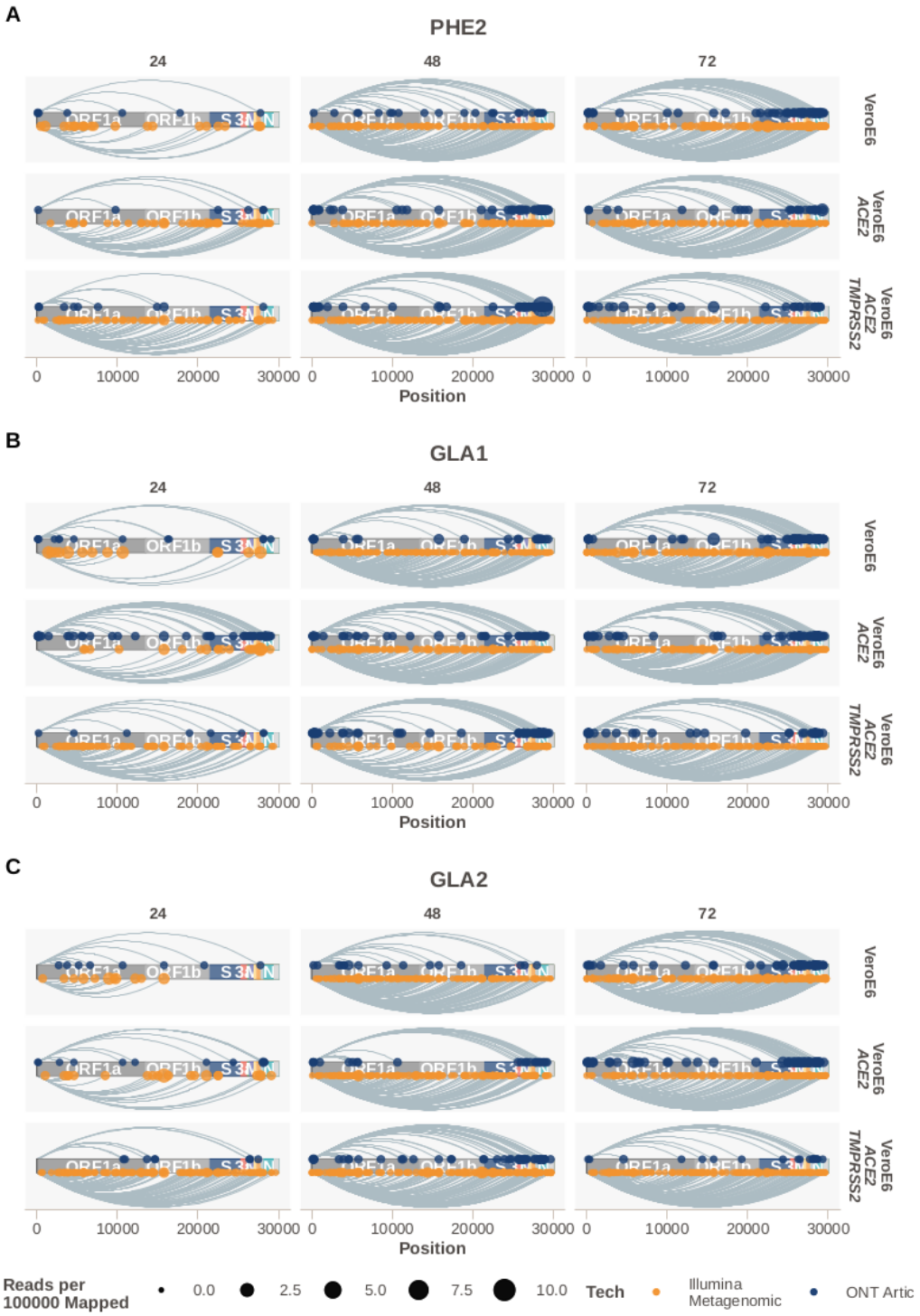
A. Noncanonical sgRNA with strong support in SHEF-CE04A at 10,369 is also supported in additional 377 samples. In close proximity to the leader is a sequence which could be considered a “weak” TRS; `ACGAAC` -> `ACG`**`G`**`AC` . **B.** Raw sgRNA levels (HQ&LQ) in SHEF-CE04A show high relative amounts of this noncanonical sgRNA at 10639. (ORFs with sgRNA evidence shown) **C.** Noncanonical sgRNA at 5,785 (SHEF-BFD90 shown for illustration) found in 226 samples. **D**. Count of HQ&LQ raw sgRNAs in SHEF-D02E5.



Supplemental Figure S8 - sgRNA Levels in bait capture Illumina samples (n=5)

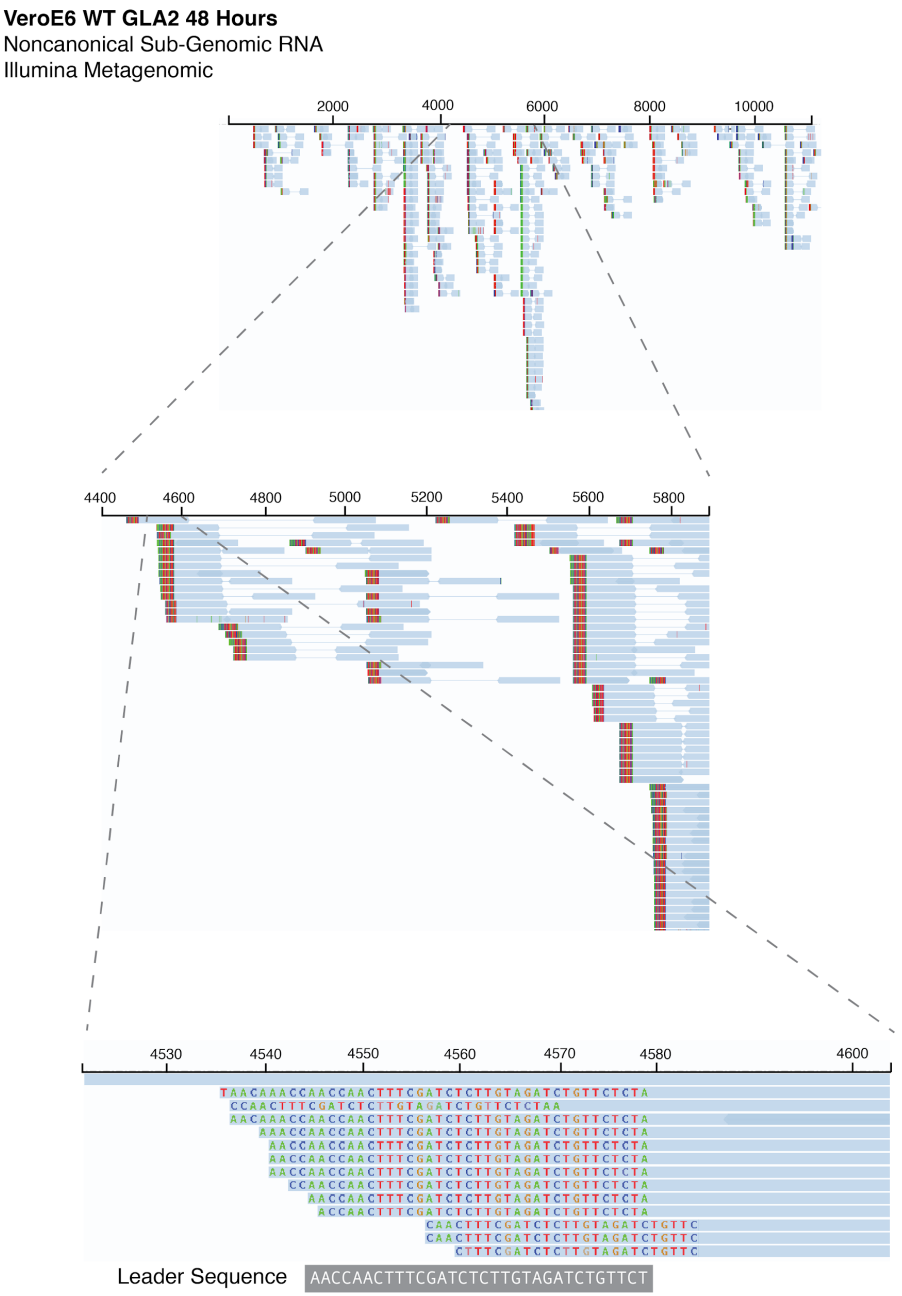
A. Total coverage around canonical ORF TRS-B sites (Supplemental File S14). **B.** Number of raw canonical sgRNA reads, no reads were found supporting ORF10. **C**. sgRNA reads normalized per 100,000 mapped reads showing that N is the most highly expressed sgRNA in this dataset. **D.** Normalizing sgcounts to the local coverage (per 1000 reads +/- 20bp

around TRS-B). **E.** Noncanonical sgRNA detected in this dataset (Supplemental File S11). Size of point represents the total number of reads across all samples (n=5). **F.** Histogram showing the data in E, coloured by sample. **G.** Histogram of the number of samples each noncanonical sgRNA was detected in.



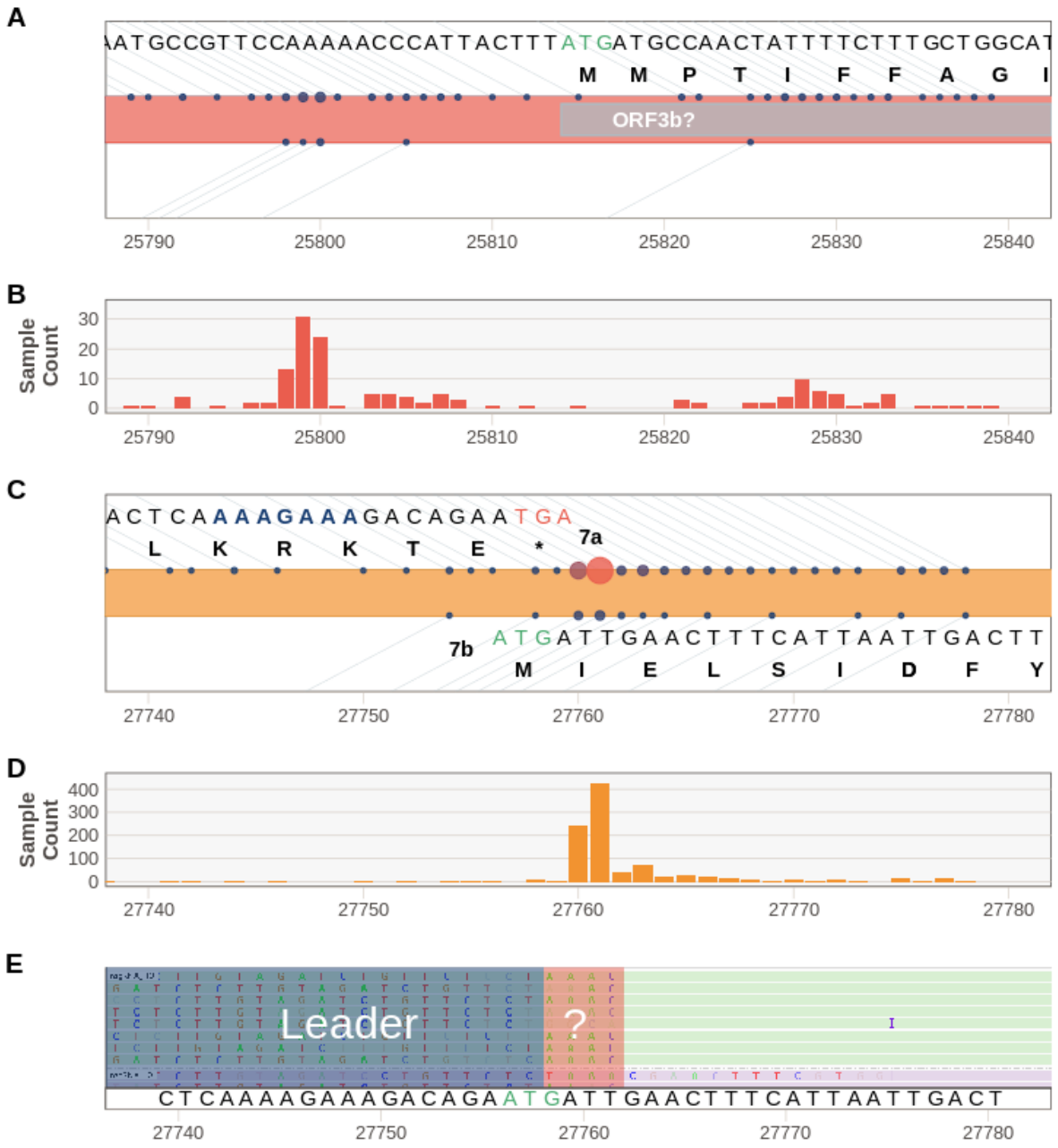
Supplemental Figure S9 - Noncanonical sub-genomic RNA detected in an vitro infection model

Results for WT VeroE6 and *ACE2* & TMPRSS2 VeroE6 cells shown for viral isolates PHE2 (**A**), GLA1 (**B**), and GLA2 (**C**). Size of the point represents the number of reads per 100,000 mapped reads.



Supplemental Figure S10 - Read level detail of Noncanonical sub-genomic RNA in Illumina metagenomic sequencing of an *in vitro* infection model

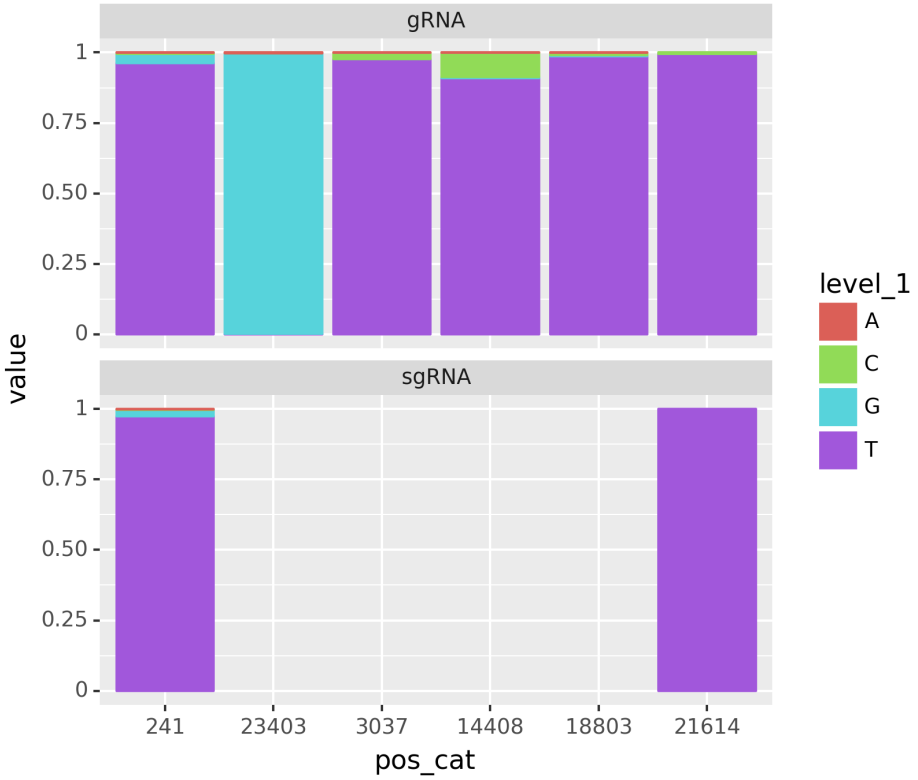
IGV plots showing reads classified as noncanonical sgRNA by periscope in VeroE6 cells, after 48 hours of infection with SARS-CoV-2 (GLA2). Non-canoncial sgRNA can be seen throughout this region. These reads all contain leader sequence at their 5' end, as illustrated by the zoomed in panels.



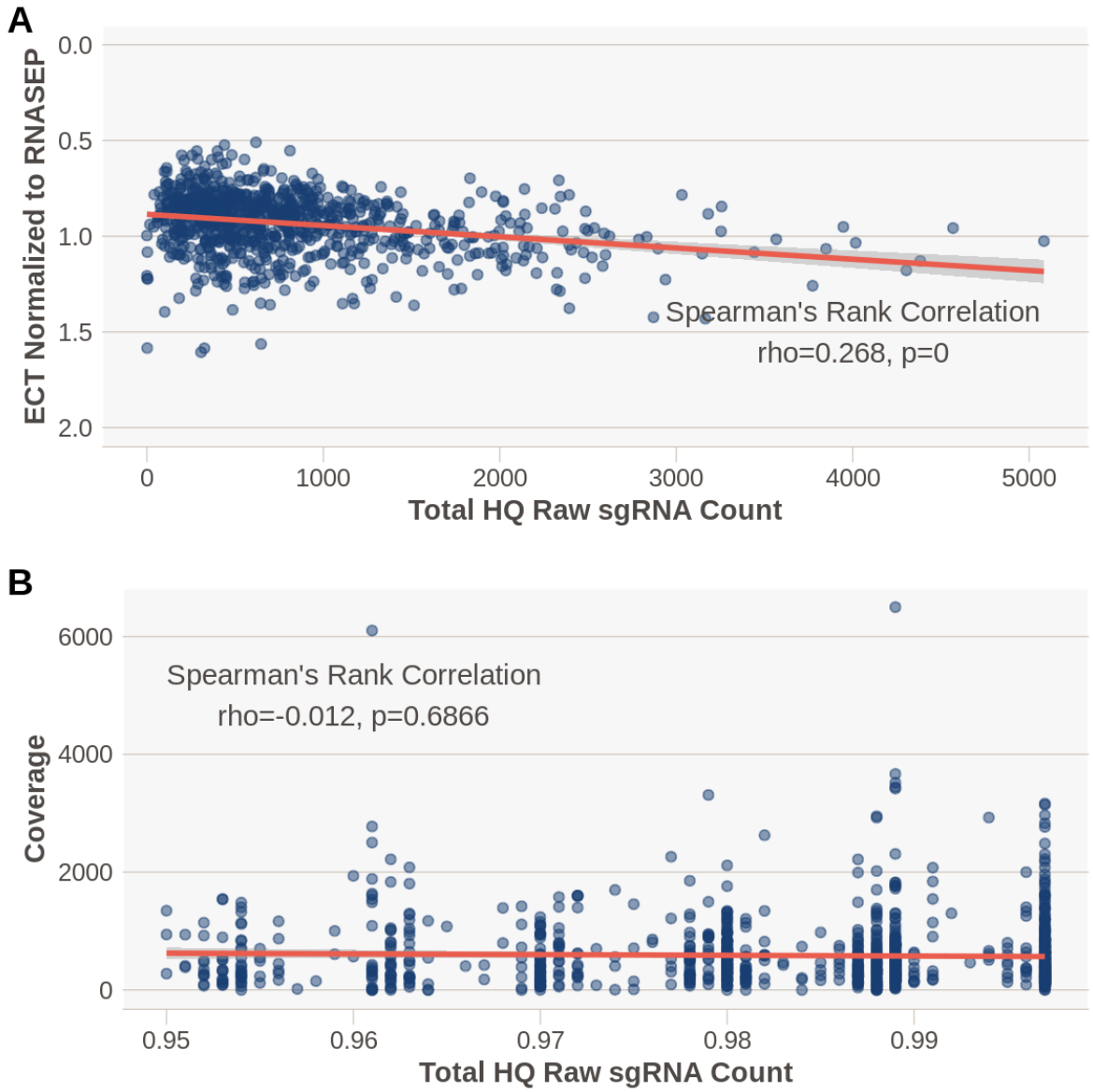
Supplemental Figure S11 - Noncanonical sub-genomic RNAs (High quality) that could represent ORF3b and ORF7b

A. All HQ noncanonical sgRNA between 25,790 and 25,840, Sheffield top, Glasgow bottom. It has been suggested a short 22 amino acid ORF 3b protein is a potent modulator of the interferon response (Konno et al. 2020) represented here in grey. **B.** Histogram of the number of samples from Sheffield with evidence of a noncanonical sgRNA at that position (HQ). **C.** Noncanonical sgRNA between 27,740 and 27,780, Sheffield top, Glasgow bottom. Protein sequence shown for the C terminus of 7a and N terminus of 7b shown at the top and

bottom respectively. Blue text indicates predicted TRS-B site for this ORF(Yang et al. 2020). **D.** Histogram of the number of samples from Sheffield with evidence of sgRNA at that position (HQ). **E.** Raw reads supporting the sgRNA at 27,761 showing the leader sequence, a mismatch of `AAAC`, followed by the genomic sequence for ORF7b. This shows that these sgRNAs do not include the predicted `ATG` for ORF7b.



Supplemental Figure S12 - Example of periscope output for variant analysis



Supplemental Figure S13 - Normalized E Ct and consensus coverage are not correlated with the raw amount of sub-genomic RNA detected

A. Total raw HQ sgRNA counts are not correlated with normalized E Ct (E Ct/RNAseP). Y-axis reversed for ease of understanding as higher normalized E Ct = lower viral load. **B.** Total raw HQ sgRNA counts are not correlated with consensus genome coverage.