

# Discovery and Quantification of Long-Range RNA Base Pairs in Coronavirus Genomes with SEARCH-MaP and SEISMIC-RNA

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# Introduction

Across all domains of life, RNA molecules perform myriad functions in development [1], immunity [2], translation [3], sensing [4, 5], epigenetics [6], cancer [7], and more. RNA also constitutes the genomes of many threatening viruses [8], including influenza viruses [9] and coronaviruses [10]. The capabilities of an RNA molecule depend not only on its sequence (primary structure) but also on its base pairs (secondary structure) and three-dimensional shape (tertiary structure) [11].

Although high-quality tertiary structures provide the most information, resolving them often proves difficult or impossible with mainstay methods used for proteins [12]. Consequently, the world's largest database of tertiary structures – the Protein Data Bank [13] – has accumulated only 1,839 structures of RNAs (compared to 198,506 of proteins) as of February 2024. Worse, most of those RNAs are short: only 119 are longer than 200 nt; of those, only 24 are not ribosomal RNAs or group I/II introns. Due partly to the paucity of non-redundant long RNA structures, methods of predicting tertiary structures for RNAs lag far behind those for proteins [14].

The situation is only marginally better for RNA secondary structures. If a diverse set of homologous RNA sequences is available, a consensus secondary structure can often be predicted using comparative sequence analysis, which has accurately modeled ribosomal and transfer RNAs, among others [15]. A formalization known as the covariance model [16] underlies the widely-used Rfam database [17] of consensus secondary structures for 4,170 RNA families (as of version 14.10). Although extensive, Rfam contains no protein-coding sequences (with some exceptions such as frameshift stimulating elements) and provides only one secondary structure for each family, even though many RNAs fold into multiple functional structures [18, 19]). Each family also models only a short segment of a full RNA sequence; for coronaviruses, existing families encompass the 5' and 3' untranslated regions, the frameshift stimulating element, and the packaging signal, which collectively constitute only 3% of the genomic RNA.

Predicting secondary structures faces two major obstacles due to the scarcity of high-quality RNA structures, particularly for RNAs longer than 200 nt (including long non-coding [20], messenger [21], and viral genomic [22] RNAs). First, prediction methods trained on known RNA structures are limited to small, low-diversity training datasets (generally of short sequences), which causes overfitting and hence inaccurate predictions for dissimilar RNAs (including longer sequences) [23, 24]. Second, without known secondary structures of many diverse RNAs, the accuracy of any prediction method cannot be properly benchmarked [21, 25]. For these reasons, and because thermodynamic-based models also tend to be less accurate for longer RNAs [22] and base pairs spanning longer distances [26], predicting secondary structures of long RNAs remains unreliable.

The most promising methods for determining the structures of long RNAs use experimental data. Chemical probing experiments involve treating RNA with reagents that modify nucleotides depending on the local secondary structure; for instance, dimethyl sulfate (DMS) methylates adenosine (A) and cytidine (C) residues only if they are not base-paired [27]. Modern methods use reverse transcription to encode modifications of the RNA as mutations in the cDNA, followed by next-generation sequencing – a strategy known as mutational profiling (MaP) [28, 29]. A key advantage of MaP is that the sequencing reads can be clustered to detect multiple secondary structures in an ensemble [30, 31]. Determining the base pairs in those structures still requires structure prediction [32], although incorporating chemical probing data does improve accuracy [33, 34].

Several experimental methods have been developed to find base pairs directly, with minimal reliance on structure prediction. M2-seq [35] introduces random mutations before chemical probing to detect correlated mutations between pairs of bases, which indicates the bases interact. However, alternative structures complicate the data analysis [36], and detectable base pairs can be no longer than the sequencing reads (typically 300 nt). For long-range base pairs, many methods involving crosslinking, proximity ligation, and sequencing have been developed [37].

These methods can find base pairs spanning arbitrarily long distances – as well as between different RNA molecules – but cannot resolve single base pairs or alternative structures. Detecting, resolving, and quantifying alternative structures with base pairs that span arbitrarily long distances remains an open challenge.

Here, we introduce “Structure Ensemble Ablation by Reverse Complement Hybridization with Mutational Profiling” (SEARCH-MaP), an experimental method to discover RNA base pairs spanning arbitrarily long distances. We also develop the software “Structure Ensemble Inference by Sequencing, Mutation Identification, and Clustering of RNA” (SEISMIC-RNA) to analyze MaP data and resolve alternative structures. Using SEARCH-MaP and SEISMIC-RNA, we discover an RNA structure in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that comprises dozens of long-range base pairs and folds in nearly half of genomic RNA molecules. We show that it inhibits the folding of a pseudoknot that stimulates ribosomal frameshifting [38, 39], hinting a role in regulating viral protein synthesis. We find similar structures in other SARS-related viruses and transmissible gastroenteritis virus (TGEV), suggesting that long-range base pairs involving the frameshift stimulation element are a general feature of coronaviruses. In addition to revealing new structures in coronaviral genomes, our findings show how SEARCH-MaP and SEISMIC-RNA can resolve secondary structure ensembles of long RNA molecules – a necessary step towards a true “AlphaFold for RNA” [14].

# Results

## Workflow of SEARCH-MaP and SEISMIC-RNA

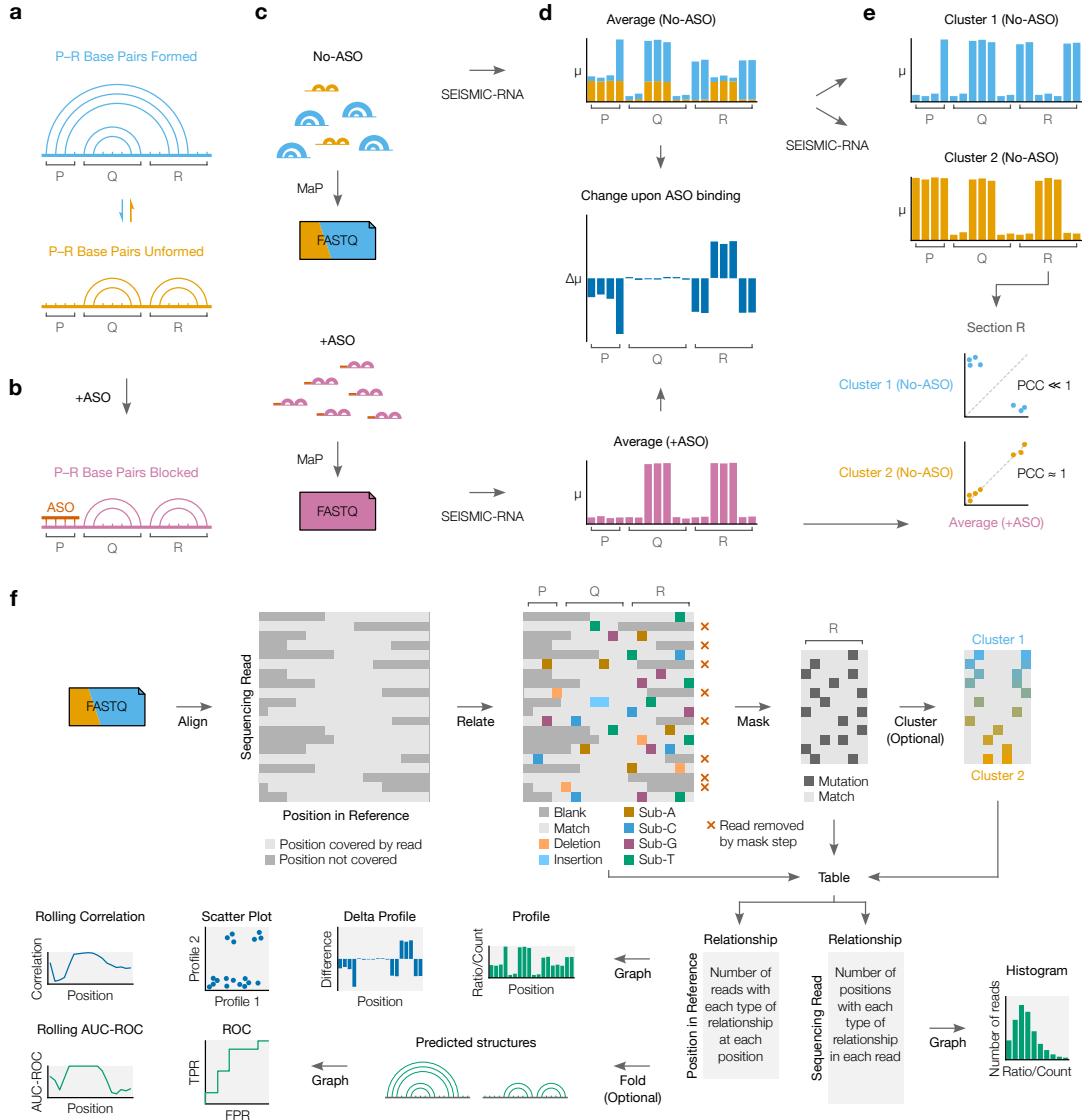


Figure 1: The workflow of SEARCH-MaP and SEISMIC-RNA. (Continued on next page.)

Figure 1: (Continued from previous page.) **(a)** This toy RNA is partitioned into three sections (P, Q, and R) and folds into an ensemble of two structures: one in which base pairs between P and R form and one in which they do not. **(b)** Hybridizing an ASO to P blocks it from base-pairing with R. **(c)** A SEARCH-MaP experiment entails separate chemical probing and mutational profiling (MaP) with (+ASO) and without (no-ASO) the ASO, followed by sequencing to generate FASTQ files. The RNA molecules and FASTQ files use the same color scheme as in (a) and are illustrated/colored in proportion to their abundances in the ensemble. **(d)** Mutational profiles with (+ASO) and without (no-ASO) the ASO, computed as ensemble averages with SEISMIC-RNA. The x-axis is the position in the RNA sequence; the y-axis is the fraction of mutated bases ( $\mu$ ) at the position. Each bar in the no-ASO profile is drawn in two colors merely to illustrate how many mutations at each position come from each structure; in a real experiment, this information would not exist before clustering. The change upon ASO binding indicates the difference in the fraction of mutated bases ( $\Delta\mu$ ) between the +ASO and no-ASO conditions. **(e)** Mutational profiles of two clusters (top) obtained by clustering the no-ASO ensemble in (d) using SEISMIC-RNA, and scatter plots comparing the mutational profiles (bottom) between the +ASO ensemble average (x-axis) and each cluster (y-axis); each point represents one base in section R. The expected Pearson correlation coefficient (PCC) is shown beside each scatter plot. **(f)** The workflow of SEISMIC-RNA. First, sequencing reads (in FASTQ files) are aligned to reference sequence(s). For every read, the relationship to each base in the reference sequence (i.e. match, substitution, deletion, insertion) is determined. In the next step, relationships are called as mutated, matched, or uninformative; and positions and reads failing to meet certain criteria are masked out. Optionally, masked reads can be clustered to reveal alternative structures. The types of relationships at each position and in each read are then counted and tabulated. SEISMIC-RNA can use these tables to predict RNA secondary structures or draw a variety of graphs including mutational profiles, scatter plots, and receiver operating characteristic (ROC) curves.

We illustrate SEARCH-MaP with an RNA comprising three sections (P, Q, and R) that folds into an ensemble of two structures: one in which base pairs between P and R form and one in which they do not (Figure 1a). Searching for base pairs involving section P begins by blocking P with an antisense oligonucleotide (ASO), which ablates the base pairs between P and R (Figure 1b). The RNA is chemically probed separately with (+ASO) and without (no-ASO) the ASO, followed by mutational profiling (MaP) and sequencing, e.g. using DMS-MaPseq [29] (Figure 1c).

SEISMIC-RNA can detect base pairs by comparing the +ASO and no-ASO mutational profiles. Theoretically, each structure has its own mutational profile [40], but the mutational profile of a single structure is not directly observable because all structures are physically mixed during the experiment (Figure 1c, top). Instead, the directly observable mutational profile is of the “ensemble average” – the average

of the structures' (unobserved) mutational profiles, weighted by the their (unobserved) proportions (Figure 1d, top). Because the mutational profile of section R changes when it base-pairs with P, the ensemble averages of R differ between the +ASO and no-ASO conditions (Figure 1d, middle). However, the ASO has little effect on section Q because this section does not base-pair with P (Figure 1d, middle). Therefore, one can deduce that P interacts with R – but not with Q – because hybridizing an ASO to P alters the mutational profile of R but not of Q.

Going one step further, one can resolve the mutational profile where P and R base-pair, even without knowing the exact base pairs. This step uses SEISMIC-RNA to cluster the no-ASO ensemble into two mutational profiles over section R – each corresponding to one structure – and comparing them to the +ASO ensemble average (Figure 1e). Because the ASO blocks the P–R base pairs, the +ASO mutational profile will correlate better with that of the structure where P and R do not base-pair; in this case, cluster 2 correlates better. Therefore, the mutational profile of cluster 1 corresponds to the structure where P and R base-pair.

## **SEARCH-MaP detects and quantifies long-range base pairing in SARS-CoV-2**

Aside from ribosomes, many of the best-characterized functional long-range RNA base pairs occur in the genomes of RNA viruses [41]. Coronaviruses regulate translation of their first open reading frame (ORF1) using programmed ribosomal frameshifting [42]. In the middle of ORF1, a switch called a frameshift stimulation element (FSE) makes a fraction of ribosomes slip backwards into the -1 reading frame. Ribosomes that maintain reading frame terminate at a stop codon shortly after the FSE, while those that frameshift bypass that stop codon and reach the end of ORF1.

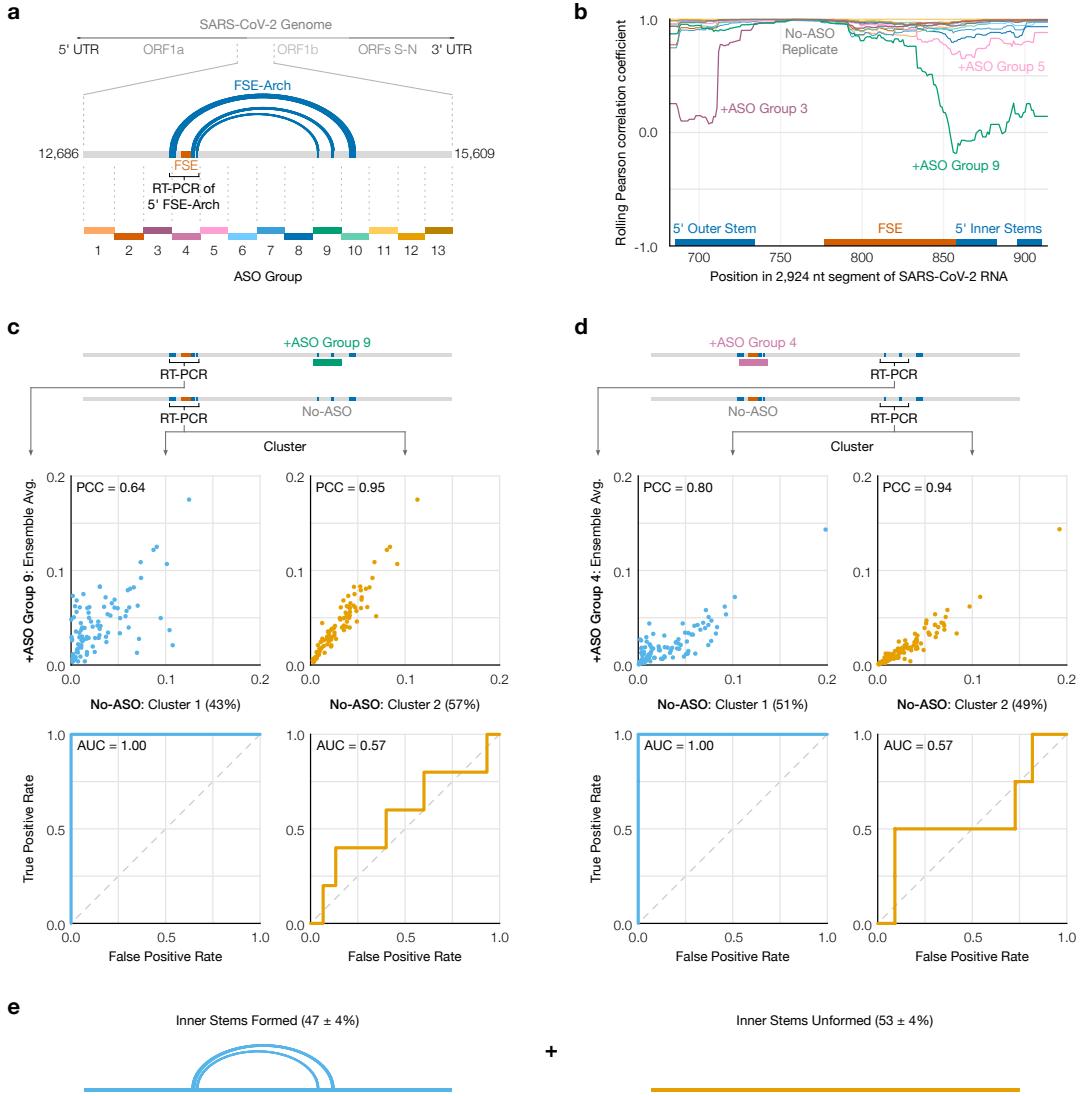
Every coronaviral FSE contains a “slippery site” (UUUAAAC) and a structure characterized as a pseudoknot in multiple species [43, 44, 45]. Indeed, the isolated core of the FSE in SARS-CoV-2 was shown to fold into a pseudoknot with three

stems [39, 46, 47]. However, we discovered that when FSE is in its natural place in the SARS-CoV-2 genome, pseudoknot stem 1 is disassembled while an alternative stem 1 folds [48]. A 283 nt segment of the RNA genome – containing both the FSE and alternative stem 1 – failed to fully mimic the DMS reactivities of the full virus ( $PCC = 0.75$ ). A 2,924 nt segment came closer ( $PCC = 0.93$ ), suggesting that – only in the context of this longer sequence – the FSE adopts yet another structure, presumably long-range base-pairing [48].

We used SEARCH-MaP and SEISMIC-RNA to find the long-range base pairs formed by the FSE. We hypothesized they would match a structure another group had discovered and named the “FSE-arch” [49]. If so, the structure of the FSE would be perturbed by – and only by – ASOs targeting either side of the putative FSE-arch. To investigate, we added (separately) thirteen groups of DNA ASOs to the 2,924 nt segment (Figure 2a). Each group contained up to five ASOs targeting a contiguous 213-244 nt section of the RNA; target sites of adjacent groups abutted without overlapping (Supplementary Table 1). After adding each group of ASOs, we performed DMS-MaPseq [29] with two pairs of RT-PCR primers. With the first pair of primers, flanking the ASO target site (Supplementary Table 2), we confirmed that the DMS reactivities were suppressed – hence the ASO groups bound – except for group 13, for which we obtained no data (Supplementary Figure 1). With the second pair of primers, flanking the 5' side of the FSE-arch, we investigated how its structure was perturbed by each ASO group (Supplementary Figure 2).

To quantify structural changes over the 5' FSE-arch, we calculated the rolling Pearson correlation coefficient (PCC) of the DMS reactivities between each sample and a no-ASO control (Figure 2b). The rolling PCC of a no-ASO replicate remained between 0.93 and 1.00 (mean = 0.97), confirming the DMS reactivities were reproducible. ASO group 9 – targeting both 3' inner stems of the FSE-arch – caused the rolling PCC to dip below 0.5 over both 5' inner stems, exactly as expected if the inner stems of the FSE-arch existed. The only other ASO groups with substantial effects were 3, 4, and 5, which overlapped or abutted the FSE and presumably perturbed short-range base pairs; the outer stem of the FSE-arch (targeted by ASO

group 10) did not apparently form. These results suggest both inner stems of the FSE-arch exist and are the predominant long-range base pairs involving the immediate vicinity of the FSE.



**Figure 2: Search for long-range base pairs involving the SARS-CoV-2 FSE.** (a) The 2,924 nt segment of the SARS-CoV-2 genome containing the frameshift stimulation element (FSE) and putative FSE-arch [49]. The target site for each group of antisense oligonucleotides (ASOs) is indicated by dotted lines; lengths are to scale. (b) Rolling (window = 45 nt) Pearson correlation coefficient (PCC) of DMS reactivities over the 5' FSE-arch between each +ASO sample and a no-ASO control. Each curve represents one ASO group, colored as in (a); groups 4 and 13 are not shown. Locations of the FSE and the outer and inner stems of the 5' FSE-arch are also indicated. (c) (Top) Scatter plots of DMS reactivities over the 5' FSE-arch comparing each cluster of the no-ASO sample to the sample with ASO group 9; each point is one position in the 5' FSE-arch. (Bottom) Receiver operating characteristic (ROC) curves comparing each cluster of the no-ASO sample to the two inner stems of the FSE-arch, with area under the curve (AUC) indicated. (d) Like (c) but over the 3' FSE-arch, and comparing to the sample with ASO group 4. One highly reactive outlier was ignored when calculating PCC (which is sensitive to outliers) but included in the ROC (which is robust). (e) Model of the inner two stems in the ensemble of structures formed by the 2,924 nt segment.

We next sought to determine the fraction of molecules in which the two inner stems of the FSE-arch form. Using SEISMIC-RNA, we clustered reads from the 5' side of the FSE-arch for the no-ASO control and found two clusters with a 43/57% split. To determine if they corresponded to the two inner stems formed and unformed, we compared their DMS reactivities to those after adding ASO group 9, which blocks the two inner stems (Figure 2c, top). Cluster 2 had similar DMS reactivities ( $PCC = 0.95$ ), indicating it corresponds to the stems unformed. Meanwhile, the DMS reactivities of cluster 1 differed ( $PCC = 0.64$ ), suggesting it corresponds to the stems formed.

To further support this result, we leveraged the preexisting model of the FSE-arch [49]. If cluster 1 did correspond to the two inner stems formed, its DMS reactivities would agree well with their structures (i.e. paired and unpaired bases should have low and high reactivities, respectively), while those of cluster 2 would agree less. We quantified this agreement using receiver operating characteristic (ROC) curves (Figure 2c, bottom). The area under the curve (AUC) for cluster 1 was 1.00, indicating perfect agreement with the two inner stems of the FSE-arch; while that of cluster 2 was 0.57, close to no agreement (0.50). This result further supports that clusters 1 and 2 correspond to the two inner stems formed and unformed, respectively.

If the RNA did exist as an ensemble of the two inner stems formed and unformed, the 3' side of the FSE-arch would also cluster into formed and unformed states. To investigate, we performed RT-PCR with primers flanking the 3' side of the inner two stems – both without ASOs and with ASO group 4 (targeting the 5' side of the FSE-arch). We clustered the no-ASO control into two clusters (51/49% split) and found – similar to the previous result – that the DMS reactivities after blocking the 5' FSE-arch with ASO group 4 resembled those of cluster 2 ( $PCC = 0.94$ ) but not cluster 1 ( $PCC = 0.80$ ), while the structure of the two inner stems agreed with cluster 1 ( $AUC = 1.00$ ) but not cluster 2 ( $AUC = 0.57$ ) (Figure 2d). We concluded that the RNA exists as an ensemble of structures in which the two inner stems of the FSE-arch form in  $47\% \pm 4\%$  of molecules (Figure 2e).

## The long-range stems compete with the frameshift pseudoknot in SARS-CoV-2

To determine if the FSE forms other long-range stems, in lieu of the original outer stem of the FSE-arch [49], we modeled a 1,799 nt segment centered on the FSE-arch. Although computationally predicting long-range base pairs is notoriously unreliable [26, 22], we speculated that we could improve accuracy by incorporating the DMS reactivities of cluster 1 on both sides of the FSE-arch (Supplementary Figure 3). For the innermost stem – which we call long stem 1 (LS1) – nine of thirteen structures (69%) predicted using the cluster 1 DMS reactivities contained LS1, compared to five of eleven (45%) using the ensemble average and four of twenty (20%) using no DMS reactivities. For the second-most inner stem (LS2), eight structures (62%) predicted using cluster 1 contained LS2, while none did using average or no DMS reactivities. Thus, the DMS reactivities corresponding to the long-range cluster enabled predicting the long-range stems more consistently, allowing us to refine our model of the long-range stems.

Our refined model based on the long-range cluster (Figure 3) included not only the two inner stems of the FSE-arch – LS1 and LS2a/b – but also two long stems (LS3a/b and LS4) that were not in the original FSE-arch model [49]. The structure also contained the alternative stem 1 (AS1) that we had previously discovered [48]. To our surprise, LS2b, LS3, and LS4 of the refined model collectively overlapped all three stems of the pseudoknot (PS1, PS2, and PS3) that is thought to stimulate frameshifting [38, 39, 47]. Thus, these long stems – if they exist – would be mutually exclusive with the pseudoknot.

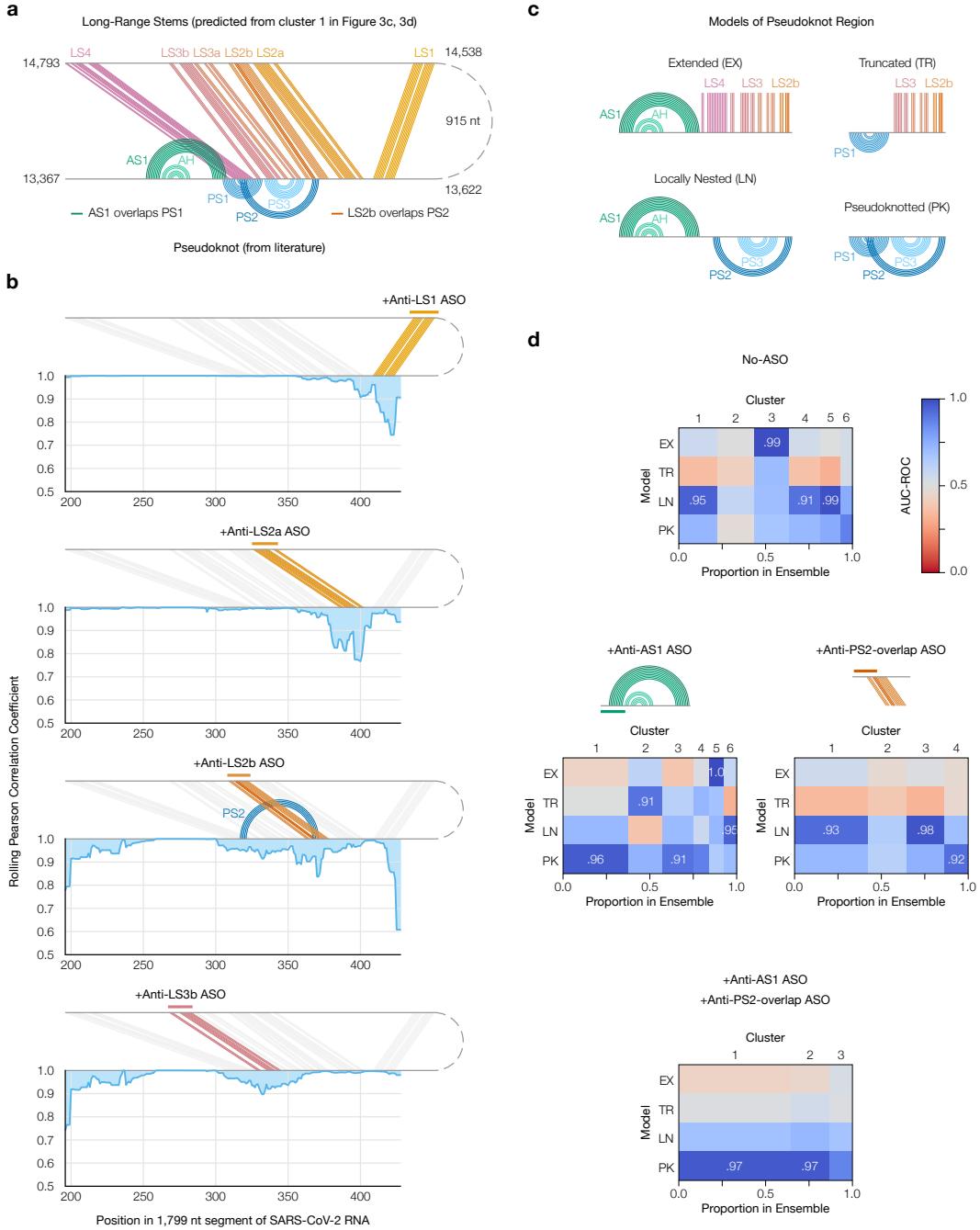
To verify this refined model, we performed SEARCH-MaP on the 1,799 nt segment using 15-20 nt LNA/DNA mixmer ASOs for single-stem precision (Figure 3b, Supplementary Table 3). Each ASO targeted the 3' side of one stem, and we measured the change in DMS reactivities of the FSE. ASOs targeting the 3' sides of LS1 and LS2a perturbed the DMS reactivities in exactly the expected locations on the 5' sides. Binding an ASO to the 3' side of LS2b caused a larger perturbation with

more off-target effects, likely because this stem overlaps with pseudoknot stem 2 (PS2). Blocking LS3b also resulted in a main effect around the intended location, with one off-target effect upstream, suggesting that other base pairs between the pseudoknot and this upstream region may exist. Therefore, stems LS1, LS2a/b, and LS3b do exist – at least in a portion of the ensemble.

We then investigated whether the long-range stems compete with the pseudoknot. If they did, blocking them with ASOs would increase the proportion of the pseudoknot in the ensemble. To test this hypothesis, we first generated four possible models of the FSE structure by combining mutually compatible stems from the refined model (Figure 3c). Then, we clustered the 1,799 nt segment without ASOs up to 6 clusters – the maximum number reproducible between replicates – (Supplementary Figure 4a) and compared each cluster to each structure model using the area under the receiver operating characteristic curve (AUC-ROC) over the positions spanned by the pseudoknot, 305-371 (Figure 3d, top). We considered a cluster and model to be “consistent” if the AUC-ROC was at least 0.90. The locally nested model (AS1 plus PS2 and PS3) was consistent with three clusters totaling 52% of the ensemble, while the extended model (AS1 plus all long-range stems) was consistent with one cluster (20%). No clusters were fully consistent with the pseudoknotted model, though the least-abundant cluster (7%) came close with an AUC-ROC of 0.88. The remaining cluster (21%) was not consistent with any model, suggesting that the ensemble contains structures beyond those in Figure 3c.

Adding an ASO targeting the 5' side of AS1 reduced the proportion of AS1-containing states (extended and locally nested) from 72% to 16% (Figure 3d, left; Supplementary Figure 4b). In their absence emerged clusters consistent with the pseudoknotted and truncated models, constituting 56% and 20% of the ensemble, respectively. Meanwhile, adding an ASO that blocked the part of LS2b that overlaps PS2 eliminated the extended state (which includes LS2b) and produced one cluster (13%) consistent with the pseudoknotted model (Figure 3d, right; Supplementary Figure 4c). Adding both ASOs simultaneously collapsed the ensemble into three clusters of which two (87%) were highly consistent with the pseudo-

knotted model (Figure 3d, bottom; Supplementary Figure 4d). Since blocking the PS2-overlapping portion of LS2b increased the proportion of clusters consistent (or nearly so) with the pseudoknotted model – both alone and combined with the anti-AS1 ASO – we conclude that the long-range stems do outcompete the pseudoknot.



**Figure 3: Refinement of the long-range structure model and competition with the frameshift pseudoknot.** (a) Refined model of the long-range stems (minimum free energy prediction based on cluster 1 in Figure 2c and d) including alternative stem 1 (AS1) [48]; the attenuator hairpin (AH) [50]; and long stems LS1, LS2a/b, LS3a/b, and LS4. Locations of pseudoknot stems PS1, PS2, and PS3 are also shown; as are the base pairs they overlap in AS1 and LS2b. (b) Rolling (window = 21 nt) Pearson correlation coefficient of DMS reactivities between each +ASO sample and a no-ASO control; base pairs targeted by each ASO are colored. (c) Models of possible structures for the FSE, by combining non-overlapping stems from (a). (d) Heatmaps comparing models in (c) to clusters of DMS reactivities over positions 305-371 via the area under the receiver operating characteristic curve (AUC-ROC). AUC-ROCs at least 0.90 are annotated. Cluster widths indicate proportions in the ensemble.

## Frameshift stimulating elements of multiple coronaviruses form long-range base pairs

We hypothesized that other coronaviruses would also feature long-range base pairs involving the FSE. To search for these structures, we performed SEARCH-MaP with FSE-targeted ASOs on 1,799 nt segments from eight coronaviral genomes.

As of December 2021, the NCBI Reference Sequence Database [51] contained 62 complete genomes of coronaviruses. To focus on those likely to have long-range base pairs involving the FSE, we predicted the likelihood that each base in a 2,000 nt section surrounding the FSE would pair with a base in the FSE (Supplementary Figure 5). Based on these predicted structures, we selected ten coronaviruses – at least one from each genus (Supplementary Figure 6a) – including SARS-CoV-2 as a positive control. Within the genus *Betacoronavirus*, we included all three SARS-related viruses – SARS coronaviruses 1 (NC\_004718.3) and 2 (NC\_045512.2) and bat coronavirus BM48-31 (NC\_014470.1) – because they clustered into their own structural outgroup. The other three strains of *Betacoronavirus* that we selected were MERS coronavirus (NC\_019843.3) with predicted base pairs at positions 510-530; and human coronavirus OC43 (NC\_006213.1) and murine hepatitis virus strain A59 (NC\_048217.1), both with a predicted upstream base pairs at positions 10-20. We selected two strains of *Alphacoronavirus*: transmissible gastroenteritis virus (NC\_038861.1) and bat coronavirus 1A (NC\_010437.1), predicted to have base pairs at positions 440-460 and 350-360, respectively. For avian infectious bronchitis virus strain Beaudette (NC\_001451.1) – a strain of *Gammacoronavirus* – the FSE was predicted to base-pair with positions 330-350; while common moorhen coronavirus HKU21 (NC\_016996.1) was the species of *Deltacoronavirus* with the most promising long-range base pairs.

We reasoned that if an FSE does interact with a distant RNA element, removing that element by truncating the RNA would change the structure of the FSE,

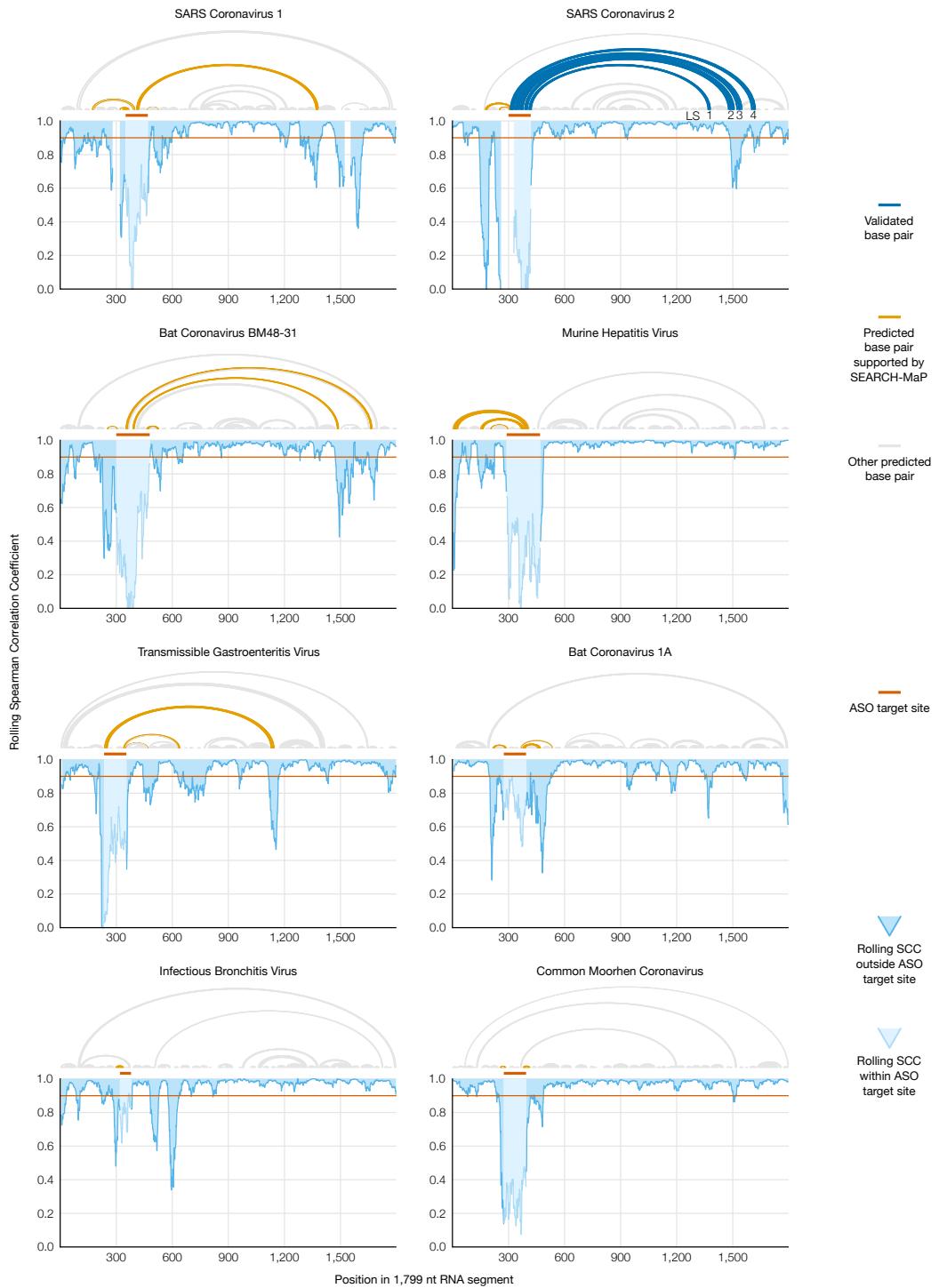
which we could detect with DMS-MaPseq [29]. For each of the ten coronaviruses that passed the computational screen, we *in vitro* transcribed and performed DMS-MaPseq on both a 239 nt segment comprising the FSE and minimal flanking sequences and a 1,799 nt segment encompassing the FSE and all sites with which it was predicted to interact. All coronaviruses except for human coronavirus OC43 and MERS coronavirus showed differences in their DMS reactivity profiles between the 239 nt and 1,799 nt segments (Supplementary Figure 6b), suggesting the FSE forms long-range base pairs.

To determine which RNA elements the FSE base-pairs with in each coronavirus, we performed SEARCH-MaP on the 1,799 nt RNA segment using DNA ASOs targeting the vicinity of the FSE (Figure 4, Supplementary Table 6). The rolling Spearman correlation coefficient (SCC) between the +ASO and no-ASO mutational profiles dipped below 0.9 at the ASO target site in every coronavirus segment, confirming the ASOs bound and altered the structure.

To confirm we could detect long-range base pairs, we compared the rolling SCC for the SARS-CoV-2 segment to our refined model of the FSE structure (Figure 4, blue). The SCC dipped below 0.9 at positions 1,483-1,560 and at 1,611-1,642, which coincide with stems LS2-LS3 (positions 1,476-1,550 within the 1,799 nt segment) and stem LS4 (positions 1,600-1,622). These dips were the two largest downstream of the FSE; although others (corresponding to no known base pairs) existed, they were barely below 0.9 and could have resulted from base pairing between these regions and other (non-FSE) regions. Near LS1 (positions 1,367-1,381), the SCC dipped only slightly to a minimum of 0.95, presumably because LS1 is the smallest (15 nt) and most isolated long-range stem. Therefore, this method was sensitive enough to detect all but the smallest long-range stem, and specific enough that the two largest dips corresponded to validated long-range stems.

We found similar long-range stems in SARS-CoV-1 and another SARS-related virus, bat coronavirus BM48-31. Both viruses showed dips in SCC at roughly the same positions as LS2-LS4 in SARS-CoV-2, indicating that they have homologous structures. SARS-CoV-1 also had a wide dip below 0.9 at positions 1,284-1,394,

corresponding to a homologous LS1. Thus, three SARS-related viruses share these long-range stems involving the FSE, hinting that these structures are functional.



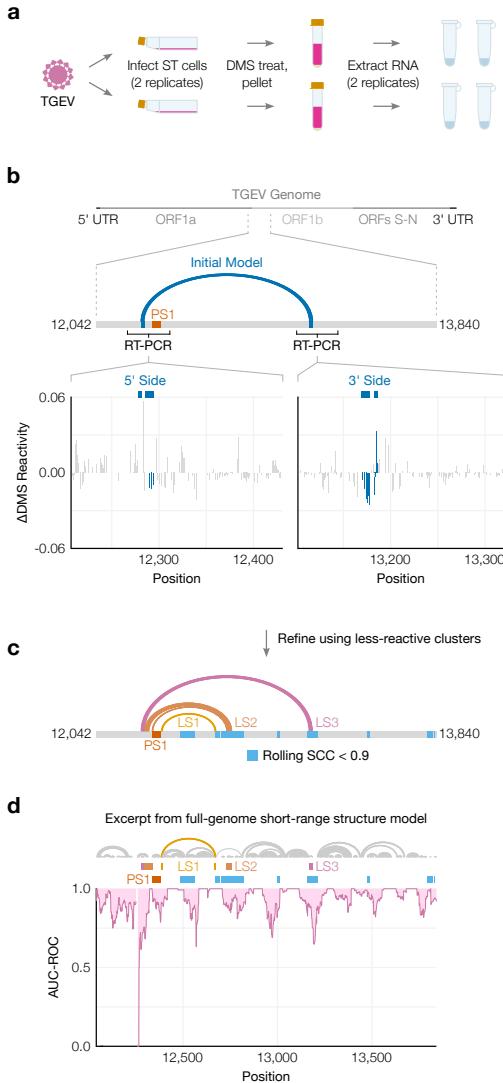
**Figure 4: Evidence for long-range RNA-RNA base pairs involving the FSE in four additional coronaviruses.** Rolling (window = 45 nt) Spearman correlation coefficient (SCC) of DMS reactivities between the +ASO and no-ASO samples for each 1,799 nt segment of a coronaviral genome. The target site of each ASO is highlighted on the SCC data and shown above each graph. Structures predicted with RNAstructure [52] using no-ASO ensemble average DMS reactivities as constraints [33] are drawn above each graph; base pairs connecting the ASO target site to an off-target position with SCC less than 0.9 are colored. For SARS-CoV-2, the refined model (Figure 3a) is also drawn, with LS1-LS4 labeled.

In every other species except common moorhen coronavirus, we found prominent dips in SCC at least 200 nt from the ASO target site. To model potential base pairing between these dip positions and the FSE, we used the Fold program from RNAstructure [52] with the no-ASO ensemble average DMS reactivities as constraints [33]. We surmised that using the DMS reactivities corresponding to the long-range base pairs formed would generally yield more accurate predictions of the long-range structure than would using the ensemble average DMS reactivities (a mixture of all structures). For instance, the prediction for SARS-CoV-2 based on the ensemble average included LS1 and LS2b but missed the other long-range stems. Although clustered data were unavailable in this case, we were still able to find long-range base pairs consistent with the SEARCH-MaP data for both murine hepatitis virus and transmissible gastroenteritis virus (Figure 4, orange). We conclude that long-range base pairing involving the FSE occurs more widely than in just SARS-CoV-2, including in the genus *Alphacoronavirus*.

## **Structure of the full TGEV genome in ST cells supports long-range base pairing involving the FSE**

Transmissible gastroenteritis virus (TGEV) is a strain of *Alphacoronavirus* 1 [53] that infects pigs and causes vomiting and diarrhea – almost always fatally in baby piglets [54]. Due to the impacts of TGEV on animal health and economics [54] and our evidence of a long-range stem, we sought to model the genomic secondary structures of live TGEV. We began by treating TGEV-infected ST cells with DMS (two biological replicates) and performing DMS-MaPseq [29] (two technical replicates per biological replicate) on the extracted RNA (Figure 5a). The DMS reactivities over the full genome were consistent between biological replicates ( $PCC = 0.97$ ), albeit not with the 1,799 nt segment *in vitro* ( $PCC = 0.82$ ), which showed that verifying the long-range stem in live TGEV would be necessary (Supplementary Figure 7).

To determine the structure ensembles, we performed RT-PCR on the extracted RNA using primers targeting both sides of the long-range stems. The DMS reactivities from RT-PCR were consistent with those over the full genome (Supplementary Figure). For each side of the long-range stem, we clustered the reads into two clusters (Figure 5b). These clusters had similar correlations with the +ASO sample and similar AUC-ROC scores (Supplementary Figure), making it more difficult to identify them for TGEV than for SARS-CoV-2 (Figure 2). Nevertheless, we realized that on each side, the bases that were predicted to interact had generally lower DMS reactivities in one cluster compared to the other cluster, and hypothesized that this cluster corresponded to the long-range stem formed (Figure 5b). On the 5' side, the less-reactive cluster constituted 52% of the ensemble; on the 3' side, 60%. To investigate, we refined the structure of the 1,799 nt segment using the DMS reactivities from both of these clusters. Consistent with our hypothesis, the minimum free energy (MFE) model included the long-range stem, which we hereafter call long stem 3 (LS3) (Figure 5c); predicting the structure using both more-reactive clusters did not produce LS3 (Supplementary Figure). The refined model also featured a prominent new stem connecting 20 nt upstream of the FSE with 400 nt downstream, which we call LS2. We suspect that LS2 exists because it coincides with a broad region perturbed by adding an ASO to the FSE in the 1,799 nt segment of TGEV (Figure 5c). Another stem spanning just under 300 nt, which we call LS1, was also predicted in the same location as in the 1,799 nt segment.



**Figure 5: Genomic secondary structure of live TGEV.** (a) Schematic of the experiment in which two biological replicates of ST cells were infected with TGEV, DMS-treated, and pelleted. Cell pellets were divided into two technical replicates prior to extraction of DMS-modified RNA. (b) Differences in DMS reactivities between the two clusters on each side of the long-range stem. Each bar represents one base. Bases are shaded dark blue if they pair in the initial model of the long-range stem (from Figure 4), shown above along with its location in the full genome. The locations of FSE pseudoknot stem 1 (PS1) and the regions amplified for clustering are also indicated. (c) Refined model of the long-range stem in TGEV based on the DMS reactivities of the less-reactive cluster from both sides. Long stems 1 (LS1), 2 (LS2), and 3 (LS3) are labeled. For comparison with the regions of the 1,799 nt segment perturbed by the ASO (Figure 4), positions after the FSE where the Spearman correlation coefficient (SCC) dipped below 0.9 are shaded light blue. (d) Rolling AUC-ROC (window = 45 nt) between the full-genome DMS reactivities and full-genome secondary structure modeled from the DMS reactivities (maximum 300 nt between paired bases). The structure model is drawn above the graph. Only positions 12,042-13,840 are shown here. For comparison, the locations of PS1, LS1, LS2, LS3, and dips in SCC after the FSE are also indicated.

We used the ensemble average DMS reactivities to produce one “ensemble average” model of the secondary structure of the full TGEV genome (Supplementary Figure). We restricted base pairs to a maximum distance of 300 nt to make the computation tractable and avoid over-predicting spurious long-range base pairs. To verify the model quality, we confirmed that the predicted structure of the first 520 nt included the highly conserved stem loops SL1, SL2, SL4, and SL5a/b/c in the 5' UTR [10] (Supplementary Figure 8a) and was consistent with the DMS reactivities (AUC-ROC = 0.94) (Supplementary Figure 8b).

The AUC-ROC was lower in many locations throughout the rest of the genome (Supplementary Figure), indicating that a single secondary structure consistent with the ensemble average DMS reactivities could not be found. We had noticed a similar phenomenon in SARS-CoV-2 – in particular, at the FSE [48]. Thus, we surmised that regions with low AUC-ROC scores likely form alternative structures or long-range base pairs – or both – that a single secondary structure model could not capture. Checking if this relationship also held for TGEV, we found a large dip in AUC-ROC just upstream of the FSE, centered on the 5' ends of LS2 and LS3, as well as smaller dips at the 3' ends of both stems (Figure 5d). In fact, at or near every location that SEARCH-MaP had evidenced to interact with the FSE – where the rolling SCC had dipped – the AUC-ROC also dipped. This finding supports the hypothesis that long-range base pairs and/or alternative structures are often the reason why predicted structures are not locally consistent with the DMS reactivities on which they were based.

## Discussion

In this work, we developed SEARCH-MaP and SEISMIC-RNA and applied them to detect structural ensembles involving long-range base pairs in SARS-CoV-2 and other coronaviruses. Previous studies have demonstrated that binding an ASO to one side of a long-range stem would perturb the chemical probing reactivities of the other side [55, 56, 57]. Here, we separated and identified the reactivities corresponding to long-range stems formed and unformed. This advance enables isolating the reactivities of the long-range stem formed – on not just one but both sides of the stem, linking corresponding alternative structures over distances much greater than the length of a read, which has not been possible in previous studies [30, 31]. Using the linked reactivities from both sides of a long-range stem, its secondary structure can be modeled more accurately than would be possible using the ensemble average reactivities, as we have done for SARS-CoV-2 (Figure 3) and TGEV (Figure 5).

SEISMIC-RNA builds upon our previous work, the DREEM algorithm [30]. Here, we have optimized the algorithm to run approximately 10-30 times faster and built an entirely new workflow around it for aligning reads, calling mutations, masking data, and outputting a variety of graphs. SEISMIC-RNA can process data from any mutational profiling experiment, including DMS-MaPseq [29] and SHAPE-MaP [28], not just SEARCH-MaP. The software is available from the Python Package Index ([pypi.org/project/seismic-rna](https://pypi.org/project/seismic-rna)) or GitHub ([github.com/rouskinlab/seismic-rna](https://github.com/rouskinlab/seismic-rna)) and can be used as a command line executable program (`seismic`) or via its Python application programming interface (`import seismicrna`).

We envision SEARCH-MaP and SEISMIC-RNA bridging the gap between broad and detailed investigations of RNA structure. Other methods such as proximity ligation [58, 59, 60, 61, 62] provide broad, transcriptome-wide information on RNA structure and could be used as a starting point to find structures of interest for deeper investigation with SEARCH-MaP/SEISMIC-RNA. Indeed, the first evidence

of the FSE-arch in SARS-CoV-2 came from such a study [49]. To investigate RNA structures in detail, M2-seq [35] and related methods [36] can pinpoint base pairs with up to single-nucleotide resolution and minimal need for structure prediction. However, base pairs are detectable only if the paired bases occur on the same sequencing read, which restricts their spans to at most the read length (typically 300 nt). Because the capabilities of M2-seq and SEARCH-MaP complement each other, they could be integrated: first SEARCH-MaP/SEISMIC-RNA to discover, quantify, and model long-range base pairs; then M2-seq for short-range base pairs. By providing the missing link – structure ensembles involving long-range base pairs – SEARCH-MaP and SEISMIC-RNA could combine broad and detailed views of RNA structure into one coherent model.

To understand structures of long RNA molecules, SEARCH-MaP and SEISMIC-RNA could also be used to validate predicted secondary structures and benchmark structure prediction algorithms. Algorithms that predict secondary structures achieve lower accuracies for longer sequences [26, 22], hence long-range base pairs in particular must be confirmed independently. We envision a workflow to determine the structure ensembles of an arbitrarily long RNA molecule that begins with DMS-MaPseq [29]. The DMS reactivities would be used [33] to predict two initial models of the structure: one with a limit to the base pair length (for short-range pairs), the other without (for long-range pairs). Sections of the RNA with potential long-range pairs would be flagged from the long-range model and from regions of the short-range model that disagreed with the DMS reactivities (as in Figure 5d). Then, SEARCH-MaP/SEISMIC-RNA could be used to validate, quantify, and refine the potential long-range base pairs; and other methods such as M2-seq [35] to do likewise for short-range base pairs. This integrated workflow could characterize the secondary structures of RNA molecules that have evaded existing methods (e.g. messenger RNAs [21]) as well provide much-needed benchmarks for secondary structure prediction algorithms [25].

In this study, we focused on the genomes of coronaviruses, specifically long-range base pairs involving the frameshift stimulating element (FSE). Long-range

base pairs implicated in frameshifting also occur in several plant viruses of the family *Tombusviridae* [63, 64, 65]. However, in *Tombusviridae* species, the frameshift pseudoknots themselves are made of long-range base pairs; in coronaviruses, the pseudoknots are local structures [43, 44, 45, 39] and (at least in SARS-CoV-2) compete with long-range base pairs. Consequently, the long-range base pairs are necessary for frameshifting in *Tombusviridae* species [63, 64, 65] but dispensable in coronaviruses: even the 80-90 nt core FSE of SARS-CoV-2 has stimulated 15-40% of ribosomes to frameshift in dual luciferase constructs [38, 66, 39, 67, 68, 48]. Surprisingly, frameshifting has appeared to be nearly twice as frequent (50-70%) in live SARS-CoV-2 [69, 70, 71]; whether this discrepancy is due to long-range base-pairing, methodological artifacts, or *trans* factors [72] is unknown [73].

If, how, and why the long-range base pairs affect frameshifting in coronaviruses are open questions. For *Tombusviridae*, one study [63] suggested that the long-range stem regulates viral RNA synthesis by negative feedback: without RNA polymerase, the long-range stem would form and stimulate frameshifting to produce polymerase, which would then unwind the long-range stem while replicating the genome. However, this mechanism seems implausible in coronaviruses, where RNA synthesis and translation occur in separate subcellular compartments (the double-membrane vesicles and the cytosol, respectively) [74]. Another study on *Tombusviridae* [65] hypothesized that after the ribosome has frameshifted, long-range stems destabilize the FSE so the ribosome can unwind it and continue translating. As the long-range base pairs in SARS-CoV-2 do compete with the pseudoknot, they might also have this role, which – for coronaviruses – could not be strictly necessary for frameshifting. One study [70] of translation in SARS-CoV-2 at different time points measured frameshifting around 20% at 4 hours post infection but 60-80% at 12-36 hours. This result is consistent with a previous hypothesis [75] that coronaviruses use frameshifting to time protein synthesis: first translating ORF1a to suppress the immune system, then translating ORF1b containing the RNA polymerase. We surmise the long-range base pairs would form in virions and persist when the virus released its genome into a host cell, where they

would initially suppress frameshifting. Once host protein synthesis had been inhibited and the double-membrane vesicles formed, a signal specific to the cytosol would disassemble the long-range base pairs so that frameshifting could occur efficiently and produce the replication machinery from ORF1b. The long-range base pairs would form in viral progeny but not in genomic RNA released into the cytosol for translation, so that more ORF1b could be translated. This possible role of long-range base pairs in the coronaviral life cycle could be tested by probing the RNA structure in subcellular compartments and virions, identifying cytosolic factors that could disassemble the long-range base pairs, and quantifying how they affect frameshifting in the context of a live coronavirus.

Future studies could also expand the scope of SEARCH-MaP and SEISMIC-RNA. While all SEARCH-MaP experiments in this study were performed *in vitro*, the method would likely also be feasible *in cellulo*: DMS-MaPseq can detect ASOs binding to RNAs within cells [76]. The main challenges would likely involve optimizing the ASO probes and transfection protocols to maximize the signal while minimizing unwanted side effects such as immunogenicity. SEARCH-MaP can screen an entire transcript (as in Figure 2), but scaling up to an entire transcriptome could prove challenging. One strategy for probing many RNAs simultaneously could involve adding a pool of ASOs – with no more than one ASO capable of binding each RNA – rather than one ASO at a time. In this manner, a similar number of samples would be needed to search all RNAs as would be needed for the longest RNA. Distinguishing direct from indirect base pairing is another area for development: if segment Q could base-pair with either P or R, then blocking P could perturb R (and vice versa) as a consequence of perturbing Q, even though P and R could not base-pair directly. A solution could be to first block Q with one ASO; then, if blocking P with another ASO caused no change in R (and vice versa), it would suggest that they could only interact indirectly (through Q).

We imagine that SEARCH-MaP and SEISMIC-RNA will make it practical to determine accurate secondary structure ensembles of entire messenger, long non-coding, and viral RNAs. Collected in a database of long RNA structures, these re-

sults would facilitate subsequent efforts to predict RNA structures and benchmark algorithms, culminating in a real “AlphaFold for RNA” [14] in the hands of every biologist.

# Methods

## Development of SEISMIC-RNA

SEISMIC-RNA was written in Python (currently compatible with version 3.10 or greater) using PyCharm Community Edition. Its dependencies include Python packages NumPy [77], Numba [78], Pandas [79, 80], and SciPy [81]; as well as Samtools [82], Cutadapt [83], Bowtie 2 [84], and RNAstructure [52].

## Confirmation of long-range base pairs in SARS-CoV-2 RNA

### RNA synthesis

A DNA template of the 2,924 nt segment of the SARS-CoV-2 genome, plus an upstream T7 promoter, was amplified from our previously constructed pmirGLO plasmid [48] with 250 nM primers TAATACGACTCACTATAGAATAATGAGCTTAGCCTGTTGCACTACG and TAAATTGCGGACATACTTATCGGCAATTTGTTACC (Thermo Fisher Scientific) using 2X CloneAmp HiFi PCR Premix (Takara Bio) in a 50 µl volume with initial denaturation at 98°C for 60 s; 35 cycles of 98°C for 10 s, 65°C for 10 s, and 72°C for 15 s; and final extension at 72°C for 60 s. The 50 µl PCR product was mixed with 10 µl of 6X Purple Loading Dye (New England Biolabs) and electrophoresed through a 1% agarose gel – 50 µl of 1X tris-acetate-EDTA buffer (Boston BioProducts), 0.5 g of SeaKem Agarose (Lonza), and 5 µl of 10,000X SYBR Safe (Invitrogen) – in 1X tris-acetate-EDTA buffer (Boston BioProducts) at 60 V for 60 min. The band at roughly 3 kb was excised and the DNA purified using a Zymoclean Gel DNA Recovery Kit (Zymo Research) according to the manufacturer's protocol; samples were eluted in 10 µl of nuclease-free water (Fisher Bioreagents) and measured with a NanoDrop (Thermo Fisher Scientific). To increase the DNA yield, the gel-extracted DNA was amplified by a second

round of PCR followed by gel extraction, using the same protocol as above. To remove contaminants after the second gel extraction, the DNA was further purified using a DNA Clean & Concentrator-5 kit (Zymo Research) according to the manufacturer's protocol; it was eluted in 10 µl of nuclease-free water (Fisher Bioreagents) and measured with a NanoDrop (Thermo Fisher Scientific).

RNA was transcribed using a MEGAscript T7 Transcription Kit (Invitrogen) according to the manufacturer's protocol. Specifically, 150 ng of DNA template from the previous step was diluted to 8 µl in nuclease-free water (Fisher Bioreagents), mixed with 2 µl of each of the four 10X NTP solutions followed by 2 µl of the 10X reaction buffer and 2 µl of the 10X enzyme mix, and then incubated at 37°C for 3 hr. The DNA template was then degraded by adding 1 µl of TURBO DNase (Invitrogen) and incubating at 37°C for 15 min. The RNA transcript was purified using an RNA Clean & Concentrator-5 kit (Zymo Research) according to the manufacturer's protocol; samples were eluted in 20 µl of nuclease-free water (Fisher Bioreagents) and measured with a NanoDrop (Thermo Fisher Scientific).

## DMS treatment

Antisense oligonucleotides (ASOs) were ordered from Integrated DNA Technologies in a 96-well PCR plate, each ASO already resuspended to 10 µM in 1X IDTE buffer (10 mM Tris, 0.1 mM EDTA). Each ASO pool (of up to 5 ASOs) was made by mixing 2.5 µl (25 pmol) of each constituent ASO (Supplementary Table 1); the total volume of each pool was adjusted to 12.5 µl by adding TE Buffer: 10 mM Tris (Invitrogen) with 0.1 mM EDTA (Invitrogen). Each 12.5 µl ASO pool was then mixed with 1 µl (425 ng, 453 fmol) of RNA (55X molar excess of each ASO) in a PCR tube. The tubes were heated to 95°C for 60 s to denature the RNA, then placed on ice for several minutes. The RNA was transferred to 1.5 ml tubes; to each, 35 µl of 1.4X refolding buffer comprising 400 mM sodium cacodylate pH 7.2 (Electron Microscopy Sciences) and 6 mM magnesium chloride (Invitrogen) was added, followed by incubation at 37°C for 25 min to allow the RNA to refold and bind the ASOs. No-ASO control 1 was handled in the same manner but with 12.5 µl of TE

Buffer in lieu of an ASO pool. For no-ASO control 2, 12.5  $\mu$ l of TE Buffer was added after placing on ice and before adding refolding buffer, to confirm that the timing of adding TE buffer would not alter the RNA structure.

For chemical probing, 1.5  $\mu$ l of neat DMS (Sigma-Aldrich) was added to each tube for a total volume of 50  $\mu$ l including 320 mM DMS, 280 mM sodium cacodylate, 4.2 mM magnesium chloride, and 9.1 nM RNA. DMS was initially mixed by swirling the pipette tip and then kept suspended by shaking at 500 rpm in a ThermoMixer C (Eppendorf) throughout the treatment at 37°C for 5 min. Reactions were quenched by adding 30  $\mu$ l neat beta-mercaptoethanol (Sigma-Aldrich) and mixing thoroughly by pipetting. Each sample of DMS-modified RNA was purified using an RNA Clean & Concentrator-5 kit (Zymo Research) according to the manufacturer's protocol; samples were eluted in 10  $\mu$ l of nuclease-free water (Fisher Bioreagents) and measured with a NanoDrop (Thermo Fisher Scientific).

ASOs were removed from each sample using TURBO DNase (Invitrogen) according to the manufacturer's protocol. Briefly, 4  $\mu$ l of each DMS-modified RNA was mixed with 4  $\mu$ l of nuclease-free water (Fisher Bioreagents), 1  $\mu$ l of 10X TURBO DNase Buffer, and 1  $\mu$ l of TURBO DNase in a PCR tube; and then incubated at 37°C for 30 min. To stop each reaction, 2  $\mu$ l of DNase Inactivation Reagent was mixed in and incubated at room temperature for 10 min, and mixed throughout by flicking several times. The DNase Inactivation Reagent was precipitated by spinning the tubes on a benchtop PCR tube centrifuge for 10 min, then transferring 4  $\mu$ l of each supernatant to a new tube.

## Sequencing library generation

For reverse transcription, each 4  $\mu$ l sample of DNased, DMS-modified RNA was mixed with 6  $\mu$ l of nuclease-free water (Fisher Bioreagents), 4  $\mu$ l of 5X First Strand Buffer (Invitrogen), 1  $\mu$ l of 10 mM dNTPs (Promega), 1  $\mu$ l of 100 mM dithiothreitol (Invitrogen), 1  $\mu$ l of RNaseOUT (Invitrogen), 1  $\mu$ l of TGIRT-III enzyme (InGex), 1  $\mu$ l of 10  $\mu$ M FSE reverse primer CTTCGTCCTTTCTTGGAAAGCGACA (Integrated DNA Technologies), and 1  $\mu$ l of 10  $\mu$ M section-specific reverse primer (Integrated DNA

Technologies, Supplementary Table 2), for a total volume of 20 µl. RNA was reverse transcribed at 57°C for 90 min, followed by inactivation at 85°C for 15 min. To remove the RNA template from the cDNA, 1 µl of Hybridase Thermostable RNase H (Lucigen) was added to each tube and incubated at 37°C for 20 min.

The cDNA products were amplified using the Advantage HF 2 PCR Kit (Takara Bio) according to the manufacturer's protocol. Specifically, 1 µl of unpurified cDNA was mixed with 8.25 µl of nuclease-free water (Fisher Bioreagents), 1.25 µl of 10X Advantage 2 PCR Buffer, 1.25 µl of 10X Advantage-HF 2 dNTP Mix, 0.25 µl of 50X Advantage-HF 2 Polymerase Mix, 0.25 µl of 10 mM forward primer (Integrated DNA Technologies, Supplementary Table 2), and 0.25 µl of 10 mM reverse primer (Integrated DNA Technologies, Supplementary Table 2), for a total volume of 12.5 µl. After an initial denaturation at 94°C for 60 s, 25 cycles of 94°C for 30 s, 60°C for 30 s, and 68°C for 60 s were run, followed by a final extension at 68°C for 60 s. For each cDNA, the PCR products were pooled (5 µl each) and then purified using a DNA Clean & Concentrator-5 kit (Zymo Research) according to the manufacturer's protocol; samples were eluted in 20 µl of nuclease-free water (Fisher Bioreagents) and measured with a NanoDrop (Thermo Fisher Scientific).

A 200 ng aliquot of each pool of PCR products was diluted in 10 mM Tris-HCl, pH 8 (Invitrogen) to a total of 50 µl. Aliquots were prepared for sequencing using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs) according to the manufacturer's protocol with the following modifications. During two-step size selection after adapter ligation, 27.5 µl and 12.5 µl of NEBNext Sample Purification Beads were used in the first and second steps, respectively, to select inserts of 280-290 bp. Indexing PCR was run at half volume (25 µl total volume) for 3 cycles. Each PCR product was mixed with 2.5 µl of 10X E-Gel Sample Loading Buffer (Invitrogen) and electrophoresed through a 2% E-Gel SizeSelect II Agarose Gel (Invitrogen) according to the manufacturer's protocol. Samples were extracted in 50 µl nuclease-free water (Fisher Bioreagents). DNA concentrations were measured using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific) according to the manufacturer's protocol. Samples were pooled and sequenced using an iSeq

100 Sequencing System (Illumina) with 2 x 150 bp paired-end reads according to the manufacturer's protocol.

## Data analysis

Sequencing reads (FASTQ files) were processed with SEISMIC-RNA versions 0.12 and 0.13 to compute mutation rates, clusters, correlations between samples, and secondary structure models. Commands for computing the effects of each ASO group (Figure 2b, Supplementary Figures 1 and 2) are in the script <https://github.com/rouskinlab/search-map/tree/main/Compute/sars2-2924/run-tile.sh>. Commands for finding alternative secondary structures and models (Figure 2c and d, Supplementary Figure 3a and b) are in the script <https://github.com/rouskinlab/search-map/tree/main/Compute/sars2-2924/run-deep.sh>. Because some samples contained amplicons that overlapped each other, sequence alignment map (SAM) files for these samples were filtered between SEISMIC-RNA's align and relate steps to select only reads with desired amplicons using a custom Python script (<https://github.com/rouskinlab/search-map/tree/main/Compute/sars2-2924/filter-deep.py>). For each cluster, the fraction of modeled structures containing long-range stems (Supplementary Figure 3c) was determined using a custom Python script ([https://github.com/rouskinlab/search-map/tree/main/Compute/sars2-2924/fraction\\_folded.py](https://github.com/rouskinlab/search-map/tree/main/Compute/sars2-2924/fraction_folded.py)).

## Verification of the refined model of long-range base pairs and mutually exclusive base pairs in SARS-CoV-2

### RNA synthesis

A 1,799 nt segment of the SARS-CoV-2 genome, beginning 290 nt upstream of and ending 1,502 nt downstream of the conserved 7 nt slippery site (TTTAAAC), was ordered from Twist Bioscience as a gene fragment flanked by the standard 5' and 3' adapters

CAATCCGCCCTCACTACAACCG and CTACTCTGGCGTCGATGAGGGA, respectively. A DNA template for *in vitro* transcription, including a T7 promoter, was amplified from the 1,799 bp construct with 250 nM primers TAATACGACTCACTATAGGTACTGGTCAGGCAATAACAGTTACAC and GACCCCATTATTAAATGGAAAACCAGCTG (Integrated DNA Technologies) using 2X CloneAmp HiFi PCR Premix (Takara Bio) in a 20 µl volume with initial denaturation at 98°C for 30 s; 30 cycles of 98°C for 10 s, 65°C for 10 s, and 72°C for 10 s; and final extension at 72°C for 60 s. The PCR product was purified using a DNA Clean & Concentrator-5 kit (Zymo Research) according to the manufacturer's protocol; it was eluted in 18 µl of 10 mM Tris-HCl pH 8 (Invitrogen) and measured with a NanoDrop One (Thermo Fisher Scientific). RNA was transcribed using a HiScribe T7 High Yield RNA Synthesis Kit (New England Biolabs) according to the manufacturer's protocol. Specifically, 100 ng of DNA template from the previous step was diluted to 8 µl in nuclease-free water (Fisher Bioreagents), mixed with 2 µl of each of the four 10X (100 mM) NTP solutions followed by 2 µl of the 10X reaction buffer and 2 µl of the 10X T7 RNA polymerase mix, and then incubated at 37°C for 11 hr. The DNA template was then degraded by adding 1 µl of TURBO DNase (Invitrogen) and incubating at 37°C for 30 min. The RNA transcript was purified using an RNA Clean & Concentrator-25 kit (Zymo Research) according to the manufacturer's protocol; samples were eluted in 50 µl of nuclease-free water (Fisher Bioreagents) and measured with a NanoDrop One (Thermo Fisher Scientific).

## DMS treatment

For each antisense oligonucleotide (ASO) treatment, 1 pmol (580 ng) of RNA was diluted to 8 µl in nuclease-free water (Fisher Bioreagents) and mixed with 100 pmol (1 µl at 100 µM) each of zero, one, or two ASOs (Integrated DNA Technologies, Supplementary Table 3); the total volume was adjusted to 10 µl by adding nuclease-free water (Fisher Bioreagents). In a PCR tube, the RNA was heated to 95°C for 60 s to denature it, then placed on ice for 5-10 min. Meanwhile, 1.15X refolding

buffer was assembled from 75  $\mu$ l of 400 mM sodium cacodylate pH 7.2 (Electron Microscopy Sciences), 0.6  $\mu$ l of 1 M magnesium chloride (Invitrogen), and 11.5  $\mu$ l nuclease-free water (Fisher Bioreagents). If no ASO would be added during refolding, then an additional 1  $\mu$ l of nuclease-free water (Fisher Bioreagents) was added. The refolding buffer was pre-warmed to 37°C in a 1.5 ml tube. The denatured, chilled RNA was pipetted into the pre-warmed refolding buffer and incubated at 37°C for 15-20 min to refold the RNA. If an ASO would be added during refolding, then 1  $\mu$ l (100 pmol) of ASO was added. The RNA was incubated for another 15 min to allow any newly added ASOs to bind.

For chemical probing, 1.9  $\mu$ l of neat DMS (Sigma-Aldrich) was added for a total volume of 100  $\mu$ l including 200 mM DMS, 300 mM sodium cacodylate, 6 mM magnesium chloride, and 10 nM RNA. DMS was initially mixed by swirling the pipette tip and then kept suspended by shaking at 500 rpm in a ThermoMixer C (Eppendorf) throughout the treatment at 37°C for 5 min. The reaction was quenched by adding 20  $\mu$ l neat beta-mercaptoethanol (Sigma-Aldrich) and mixing thoroughly by pipetting. DMS-modified RNA was purified using an RNA Clean & Concentrator-5 kit (Zymo Research) according to the manufacturer's protocol; RNA was eluted in 15  $\mu$ l of nuclease-free water (Fisher Bioreagents) and measured with a NanoDrop One (Thermo Fisher Scientific).

## Sequencing library generation

Before reverse transcription, 1  $\mu$ l of DMS-modified RNA was mixed with 7  $\mu$ l nuclease-free water (Fisher Bioreagents), 1  $\mu$ l of 10 mM dNTPs (Promega), and 1  $\mu$ l of 10  $\mu$ M FSE reverse primer CTTCGTCCTTTCTTGGAAAGCGACA (Integrated DNA Technologies) in a PCR tube. The tube was heated at 65°C for 5 min to denature the RNA and anneal the primer, then placed on ice for 5 min. Meanwhile, 2X reverse transcription mix was assembled from 5  $\mu$ l of nuclease-free water (Fisher Bioreagents), 4  $\mu$ l of 5X Induro RT Reaction Buffer (New England Biolabs), and 1  $\mu$ l of Induro Reverse Transcriptase (New England Biolabs). The 10  $\mu$ l of denatured nu-

cleic acids was mixed with 10 µl of 2X reverse transcription mix. RNA was reverse transcribed at 57°C for 30 min, followed by inactivation at 95°C for 1 min.

The FSE section was amplified by mixing 1 µl of unpurified cDNA with 7 µl of nuclease-free water (Fisher Bioreagents), 10 µl of Q5 High-Fidelity 2X Master Mix (New England Biolabs), 1 µl of 10 µM FSE forward primer CCCTGTGGGTTTA-CACTTAAAAAC (Integrated DNA Technologies), and 1 µl of 10 µM FSE reverse primer CTTCGTCCCTTCTTGGAAAGCGACA (Integrated DNA Technologies); initially denaturing at 98°C for 30 s; 30 cycles of 98°C for 10 s, 65°C for 20 s, and 72°C for 20 s; and finally extending at 72°C for 120 s. Amplification was confirmed by electrophoresing 1 µl of the PCR product through an E-Gel EX Agarose Gel, 2% (Invitrogen) and checking for a 283 bp band. The PCR product was purified using a DNA Clean & Concentrator-5 kit (Zymo Research) according to the manufacturer's protocol; it was eluted in 20 µl of 10 mM Tris-HCl pH 8 (Invitrogen) and measured with a NanoDrop One (Thermo Fisher Scientific).

A 50-100 ng aliquot of the purified FSE amplicon was diluted in 10 mM Tris-HCl pH 8 (Invitrogen) to a total of 25 µl. A sequencing library was generated using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs) according to the manufacturer's protocol with the following modifications. To conserve reagents, all steps were performed at half of the volume specified in the protocol, including reactions, bead cleanups, and washes. During two-step size selection after adapter ligation, 14 µl and 7 µl of SPRIselect Beads (Beckman Coulter) were used in the first and second steps, respectively, to select inserts of 283 bp. Also to conserve reagents, indexing PCR was run not only at half volume (25 µl) but also with each primer at 400 nM (instead of 1000 nM specified in the protocol) for 4 cycles. After indexing, PCR products were pooled in pairs; each pool (50 µl) was mixed with 5 µl of 10X E-Gel Sample Loading Buffer (Invitrogen), and 25 µl was electrophoresed through a 2% E-Gel SizeSelect II Agarose Gel (Invitrogen) according to the manufacturer's protocol. Samples were extracted in 50 µl nuclease-free water (Fisher Bioreagents). DNA concentrations were measured using a Qubit 4 Fluorometer (Thermo Fisher Scientific) according to the manufacturer's protocol.

Samples were pooled and sequenced using a NextSeq 1000 Sequencing System (Illumina) with 2 x 150 bp paired-end reads according to the manufacturer's protocol.

## Data analysis

Sequencing reads (FASTQ files) were processed with SEISMIC-RNA versions 0.11 and 0.12 to compute mutation rates, clusters, and correlations between samples using the commands in the shell script <https://github.com/rouskinlab/search-map/tree/main/Compute/sars2-1799/run.sh>. Heatmaps of the reproducibility of clustering between replicates (Supplementary Figure 4) were generated using the Python script <https://github.com/rouskinlab/search-map/tree/main/Compute/sars2-1799/compare-clusters.py>. After the two replicates were confirmed to give similar clusters, they were pooled for subsequent analyses. Secondary structures with rolling correlations (Figure 3b) were drawn using the Python script <https://github.com/rouskinlab/search-map/tree/main/Compute/sars2-1799/draw-structure.py>. Alternative structure models (Figure 3c) were selected and created with the help of the Python scripts <https://github.com/rouskinlab/search-map/tree/main/Compute/sars2-1799/choose-model-parts.py> and <https://github.com/rouskinlab/search-map/tree/main/Compute/sars2-1799/make-models.py>. Heatmaps of areas under the curve (Figure 3d) were generated using the Python script <https://github.com/rouskinlab/search-map/tree/main/Compute/sars2-1799/atlas-plot.py>.

## Investigation of long-range base pairing in multiple coronaviruses

### Computational screen of long-range base pairing in coronaviruses

All coronaviruses with reference genomes in the NCBI Reference Sequence Database [51] were searched for using the following query:

```
refseq[filter] AND ("Alphacoronavirus" [Organism] OR  
"Betacoronavirus" [Organism] OR  
"Gammacoronavirus" [Organism] OR  
"Deltacoronavirus" [Organism])
```

The reference sequences were downloaded in FASTA format ([https://github.com/rouskinlab/search-map/tree/main/Compute/covs-screen/cov\\_refseq.fasta](https://github.com/rouskinlab/search-map/tree/main/Compute/covs-screen/cov_refseq.fasta)) and the complete records in Feature Table format ([https://github.com/rouskinlab/search-map/tree/main/Compute/covs-screen/cov\\_features.txt](https://github.com/rouskinlab/search-map/tree/main/Compute/covs-screen/cov_features.txt)).

The location of the frameshift stimulating element (FSE) in each genome was estimated from the feature table, and the nearest instance of UUUAAAC was used as the slippery site, using a custom Python script ([https://github.com/rouskinlab/search-map/tree/main/Compute/covs-screen/extract\\_long\\_fse.py](https://github.com/rouskinlab/search-map/tree/main/Compute/covs-screen/extract_long_fse.py)). The 2,000 nt segment beginning 100 nt upstream of and ending 1,893 nt downstream of the slippery site was used for predicting long-range interactions involving the FSE. Genomes with ambiguous nucleotides (e.g. N) in this segment were discarded. For each coronavirus genome, up to 100 secondary structure models of the 2,000 nt segment were generated using Fold version 6.3 from RNAstructure [52] with -M 100 and otherwise default parameters, using a custom Python script ([https://github.com/rouskinlab/search-map/tree/main/Compute/covs-screen/fold\\_long\\_fse.py](https://github.com/rouskinlab/search-map/tree/main/Compute/covs-screen/fold_long_fse.py)).

Then, for each position, the fraction of models for the coronavirus in which the base at the position paired with any other base between positions 101 (the first base of the slippery sequence) and 250 was calculated using a custom Python script ([https://github.com/rouskinlab/search-map/tree/main/Compute/covs-screen/analyze\\_interactions.py](https://github.com/rouskinlab/search-map/tree/main/Compute/covs-screen/analyze_interactions.py)). The coronaviruses were clustered by their fraction vectors using the unweighted pair group method with arithmetic mean (UPGMA) and a euclidean distance metric, implemented in Seaborn version 0.11 [85] and SciPy version 1.7 [81]. The resulting hierarchically-clustered heatmap (Supplementary Figure 5) was examined manually to select

coronaviruses based on the prominence of potential long-range interactions with the FSE (relatively large fractions far from positions 101-250).

## RNA synthesis

For each selected coronavirus, a 1,799 nt segment beginning 290 nt upstream of and ending 1,502 nt downstream of the conserved 7 nt slippery site (TTTAAAC) was ordered from Twist Bioscience as a gene fragment flanked by the standard 5' and 3' adapters CAATCCGCCCTCACTACAACCG and CTACTCTGGCGTCGAT-GAGGGA, respectively. Gene fragments were resuspended to 10 ng/ $\mu$ l in 10 mM Tris-HCl pH 8 (Invitrogen). Each DNA template for *in vitro* transcription of 1,799 nt RNA segments, including a T7 promoter, was amplified from 0.5  $\mu$ l (5 ng) of a gene fragment with 250 nM of each primer TAATACGACTCACTATAGGCAATCCGCC-CTCACTACAACCG and TCCCTCATCGACGCCAGAGTAG using 2X CloneAmp HiFi PCR Premix (Takara Bio) in a 20  $\mu$ l volume with initial denaturation at 98°C for 30 s; 30 cycles of 98°C for 10 s, X°C (see Supplementary Table 4) for 10 s, and 72°C for 15 s; and final extension at 72°C for 60 s. DNA templates for *in vitro* transcription of 239 nt RNA segments were amplified using the same procedure but with the forward primers with T7 promoters (F+T7) and reverse primers (R) in Supplementary Table 5.

For experiments in which the RNAs were transcribed as a pool of all coronaviruses, all PCR products of the same length (i.e. 1,799 nt or 239 nt) were pooled, then purified. Otherwise, PCR products were purified individually. Purification was performed using a DNA Clean & Concentrator-5 kit (Zymo Research) according to the manufacturer's protocol; concentrations were measured with a NanoDrop (Thermo Fisher Scientific).

RNA was transcribed using a HiScribe T7 High Yield RNA Synthesis Kit (New England Biolabs) according to the manufacturer's protocol but at one-quarter volume (5  $\mu$ l). Specifically, 50 ng of DNA template from the previous step was diluted to 1.75  $\mu$ l in nuclease-free water (Fisher Bioreagents), mixed with 0.5  $\mu$ l of each of the four 10X (100 mM) NTP solutions followed by 0.5  $\mu$ l of the 10X reaction buffer

and 0.5 µl of the 10X T7 RNA polymerase mix, supplemented with 0.25 µl RNase-OUT (Invitrogen), and then incubated at 37°C for 16 hr. DNA templates were then degraded by adding 0.5 µl of TURBO DNase (Invitrogen) and incubating at 37°C for 30 min. RNA transcripts were purified using an RNA Clean & Concentrator-5 kit (Zymo Research) according to the manufacturer's protocol; RNA was eluted in 50 µl of nuclease-free water (Fisher Bioreagents) and measured with a NanoDrop (Thermo Fisher Scientific).

## DMS treatment

Refolding buffer was assembled from 750 µl of 400 mM sodium cacodylate pH 7.2 (Electron Microscopy Sciences), 6 µl of 1 M magnesium chloride (Invitrogen), and 244 µl of nuclease-free water (Fisher Bioreagents), then pre-warmed to 37°C. Antisense oligonucleotides (ASOs) in Supplementary Table 6 were ordered from Integrated DNA Technologies. Each ASO was resuspended to 100 µM in low-EDTA TE buffer: 10 mM Tris pH 7.4 with 0.1 mM EDTA (Integrated DNA Technologies). For each coronavirus, 5 µl of each corresponding ASO (Supplementary Table 6) was pooled; the pool of ASOs was diluted with low-EDTA TE buffer to a final volume of 100 µl, bringing each ASO to 5 µM.

If the RNAs had already been pooled, then 300 ng of pooled RNA was diluted in 2.5 µl of nuclease-free water (Fisher Bioreagents) in a PCR tube. The tube was heated to 95°C for 1 min to denature the RNA, then chilled on ice for 3 min. The denatured, chilled RNA (2.5 µl) was added to 95 µl of pre-warmed refolding buffer and incubated at 37°C for 20 min to refold the RNA.

If the RNAs had not been pooled, then for each coronavirus, 1 pmol of RNA for that coronavirus was mixed with 10 µl of either low-EDTA TE buffer (for probing without ASOs) or the ASO pool for that coronavirus (for probing with ASOs) in a PCR tube. Each tube was heated to 95°C for 1 min to denature the RNA, then chilled on ice for 3 min. Each denatured, chilled RNA was added to pre-warmed refolding buffer for a total volume of 100 µl and incubated at 37°C for 20 min

to refold the RNA (possibly with ASOs). Subsequently, equimolar amounts of all refolded RNAs were combined into one 97 µl pool in a 1.5 ml tube.

Neat DMS (Sigma-Aldrich) was added to a total volume of 100 µl (2.5 µl of DMS for RNAs transcribed as pools, 3 µl of DMS for RNAs pooled after transcription), mixed by pipetting up and down and swirling the tip, and shaken at 800 rpm and 37°C in a ThermoMixer C (Eppendorf) for 5 min. To quench the reaction, 60 µl of neat beta-mercaptoethanol (Sigma-Aldrich) was added and mixed thoroughly by pipetting. DMS-modified RNA was purified using an RNA Clean & Concentrator-5 kit (Zymo Research) according to the manufacturer's protocol; RNA was eluted in 16 µl of nuclease-free water (Fisher Bioreagents) and measured with a NanoDrop (Thermo Fisher Scientific).

For samples containing ASOs, 5 µl of TURBO DNase Buffer (Invitrogen) and 1 µl of TURBO DNase Enzyme (Invitrogen) were added along with nuclease-free water (Fisher Bioreagents) to a total volume of 50 µl. Samples were incubated at 37°C for 30 min to degrade the ASOs. Then, the RNA was purified with an RNA Clean & Concentrator-5 kit (Zymo Research) according to the manufacturer's protocol; RNA was eluted in 16 µl of nuclease-free water (Fisher Bioreagents) and measured with a NanoDrop (Thermo Fisher Scientific).

## **Sequencing library generation**

Sequencing libraries were generated from 100 ng of DMS-modified RNA using the xGen Broad-Range RNA Library Preparation Kit (Integrated DNA Technologies) according to the manufacturer's protocol, with the following modifications. During fragmentation, 8 µl of RNA was combined with 1 µl of Reagent F1, 4 µl of Reagent F3, and 2 µl of Reagent F2. For reverse transcription, 1 µl of Enzyme R1, 2 µl of TGIRT-III enzyme (InGex), and 1 µl of 100 mM dithiothreitol (Invitrogen) was added to the fragmented RNA (instead of the reaction mix), then incubated at room temperature for 30 minutes before adding 2 µl of Reagent F2. Reverse transcription was stopped by adding 1 µl of 4 M sodium hydroxide (Fluka), heating to 95°C for 3 min, and chilling at 4°C. The pH was neutralized with 1µl of 4 M hydrochloric

acid ([SUPPLIER]). Instead of a bead cleanup after the final PCR, unpurified PCR products were mixed with 4 µl of 6X DNA loading dye (Invitrogen) and run alongside 4 µl of 1 Kb Plus DNA Ladder (Invitrogen) on an 8% Tris-borate-EDTA (TBE) gel (Invitrogen) in 1X TBE buffer (Invitrogen) at 180 V for 55 min. The gel was stained with SYBR Gold (Invitrogen). The section between 250 and 500 bp was excised and placed in a 0.5 ml tube with a hole punctured in the bottom by an 18-gauge needle (BD Biosciences). The 0.5 ml tube was nested inside a 1.5 ml tube and centrifuged at [SPEED] for 1 min to crush the gel slice into the latter. The crushed gel pieces were suspended in 500 µl of 300 mM sodium chloride (Boston Bioproducts) and shaken in a ThermoMixer C (Eppendorf) at 1,500 rpm while incubating at 70°C for 20 min. The entire slurry was then centrifuged at [SPEED] through a 0.22 µm Costar Spin-X filter column to remove the gel pieces. The filtrate was mixed with 600 µl isopropanol (Sigma-Aldrich) and 3 µl GlycoBlue Coprecipitant (Invitrogen), vortexed briefly, and stored at -20°C overnight. DNA was then pelleted by centrifugation at [SPEED] for 45 min in an Eppendorf 5430R benchtop centrifuge cooled to 4°C. The supernatant was aspirated, and the pellet was washed with 1 ml of ice-cold 70% ethanol (Sigma-Aldrich). The pellet was re-suspended in 15 µl nuclease-free water (Fisher Bioreagents) and quantified using the 1X dsDNA High Sensitivity Assay Kit for the Qubit 3.0 Fluorometer (Thermo Fisher Scientific) according to the manufacturer's protocol. Samples were pooled and sequenced using an iSeq 100 Sequencing System (Illumina) with 2 x 150 bp paired-end reads according to the manufacturer's protocol.

## Data analysis

Sequencing reads (FASTQ files) were processed with SEISMIC-RNA versions 0.11 and 0.12 to compute mutation rates, correlations between samples, and secondary structure models using the commands in the shell script <https://github.com/rouskinlab/search-map/tree/main/Compute/covs-1799/run.sh>. For the 239 nt and 1,799 nt RNAs that had been pooled during *in vitro* transcription, the two replicates for each coronavirus for each length were confirmed to give similar results,

then merged before comparing the 239 nt and 1,799 nt RNAs to each other. For the comparison of RNAs with and without ASOs, the no-ASO samples that had been transcribed individually were confirmed to give similar results to those transcribed as a pool; then, all no-ASO samples were pooled before comparing to samples with ASOs. For each coronavirus, the DMS reactivities of the combined no-ASO samples were used to model up to 20 secondary structures of the 1,799 nt segment using Fold from RNAstructure v6.3 [52]. Structure models were checked manually for correspondence with the rolling correlation between the +ASO and no-ASO conditions; the minimum free energy structure was chosen for every coronavirus except for transmissible gastroenteritis virus, in which the first sub-optimal structure – but not the minimum free energy structure – contained long-range base pairs supported by the rolling correlation. Rolling correlations between +ASO and no-ASO conditions superimposed on secondary structure models (Figure 4) were graphed using the Python script [https://github.com/rouskinlab/search-map/tree/main/Compute/util/pairs\\_vs\\_correl.py](https://github.com/rouskinlab/search-map/tree/main/Compute/util/pairs_vs_correl.py).

## DMS-MaPseq of transmissible gastroenteritis virus in ST cells

### Amplicons of the frameshift stimulating element and long-range interaction element

1  $\mu$ l of rRNA-depleted RNA was mixed with 2.5  $\mu$ l of nuclease-free water (Fisher Bioreagents), 0.5  $\mu$ l of 10 mM dNTPs (Promega), 0.5  $\mu$ l of 10  $\mu$ M primer ACAATTCTGCTTAAGGAATTACCAATACACGCAA (Integrated DNA Technologies), and 0.5  $\mu$ l of 10  $\mu$ M primer CTATACCAAGTTGTTGAAATGGTAACCTGCAGTAACA (Integrated DNA Technologies) in a PCR tube; denatured at 65°C for 5 min; and chilled on ice. Meanwhile, 2.5  $\mu$ l of nuclease-free water (Fisher Bioreagents), 2  $\mu$ l of 5X Induro reaction buffer (New England Biolabs), and 0.5  $\mu$ l of Induro RT (New England Biolabs) were mixed, then added to the denatured RNA. Reverse

transcription proceeded at 57°C for 30 min, followed by inactivation at 95°C for 1 min. 1 µl of unpurified RT product was amplified in 10 µl using Q5 High-Fidelity 2X Master Mix (New England Biolabs) with 1 µM of each primer, either GCCGCTACAAAGGTAAGTCGTGCAAATACCAACT and ACAATTCTCGTCTTAAGGAATTACCAATACACGCAA or GTGAAAAGTGACATCTATGGTTCTGATTATAAGCAGTA and CTATACCAAGTTGTTGAAATGGTAACCTGCAGTAACA (Integrated DNA Technologies); initially denaturing at 98°C for 30 s; 30 cycles of 98°C for 5 s, 69°C for 20 s, and 72°C for 15 s; and finally extending at 72°C for 120 s. Amplification was confirmed by electrophoresing 1 µl of each PCR product. PCR products for both pairs of primers were pooled and then purified using a DNA Clean & Concentrator-5 kit (Zymo Research) according to the manufacturer's protocol, eluted in 18 µl of 10 mM Tris-HCl pH 8 (Invitrogen), and measured with a NanoDrop (Thermo Fisher Scientific).

175-225 µl of DNA was prepared for sequencing using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs) according to the manufacturer's protocol with the following modifications. All steps were performed at half of the volume specified in the protocol, including reactions, bead cleanups, and washes. During size selection after adapter ligation, 14 µl and 7 µl of SPRIselect Beads (Beckman Coulter) were used in the first and second steps, respectively, to select inserts of 295 bp. Indexing PCR was run with 400 nM of each primer for 4 cycles. 420 bp inserts were selected using a 2% E-Gel SizeSelect II Agarose Gel (Invitrogen) according to the manufacturer's protocol. DNA concentrations were measured using a Qubit 4 Fluorometer (Thermo Fisher Scientific) according to the manufacturer's protocol. Samples were pooled and sequenced using a NextSeq 1000 Sequencing System (Illumina) with 2 x 150 bp paired-end reads according to the manufacturer's protocol.

## Data analysis

The genomic sequence of this TGEV strain was determined using the script <https://github.com/rouskinlab/search-map/tree/main/Compute/tgev-virus/consensus.sh>: reads from the untreated sample were aligned to the TGEV reference genome (NC\_038861.1) using Bowtie 2 [84] and the consensus sequence was determined using Samtools [82]. All reads were processed with SEISMIC-RNA version 0.15 to compute mutation rates, correlations between samples, and secondary structure models using the commands in the shell script <https://github.com/rouskinlab/search-map/tree/main/Compute/tgev-virus/run.sh>. Positions in the untreated sample with mutation rates greater than 1% were masked. Replicates were checked for reproducibility and pooled for clustering and structure modeling. A model of short-range base pairs (maximum distance 300 nt) in the TGEV genome was generated from the DMS reactivities using Fold-smp from RNAstructure [52] in five overlapping 10 kb segments, which were merged using the script <https://github.com/rouskinlab/search-map/tree/main/Compute/tgev-virus/assemble-tgev-ss.py>. Rolling area under the curve superimposed on secondary structure models (Figure 5d) was graphed using the script <https://github.com/rouskinlab/search-map/tree/main/Compute/tgev-virus/make-figure-6d.py>.

## Data Availability

All sequencing data generated in this study have been deposited into the NCBI Short Read Archive under accession code PRJNA1103196.

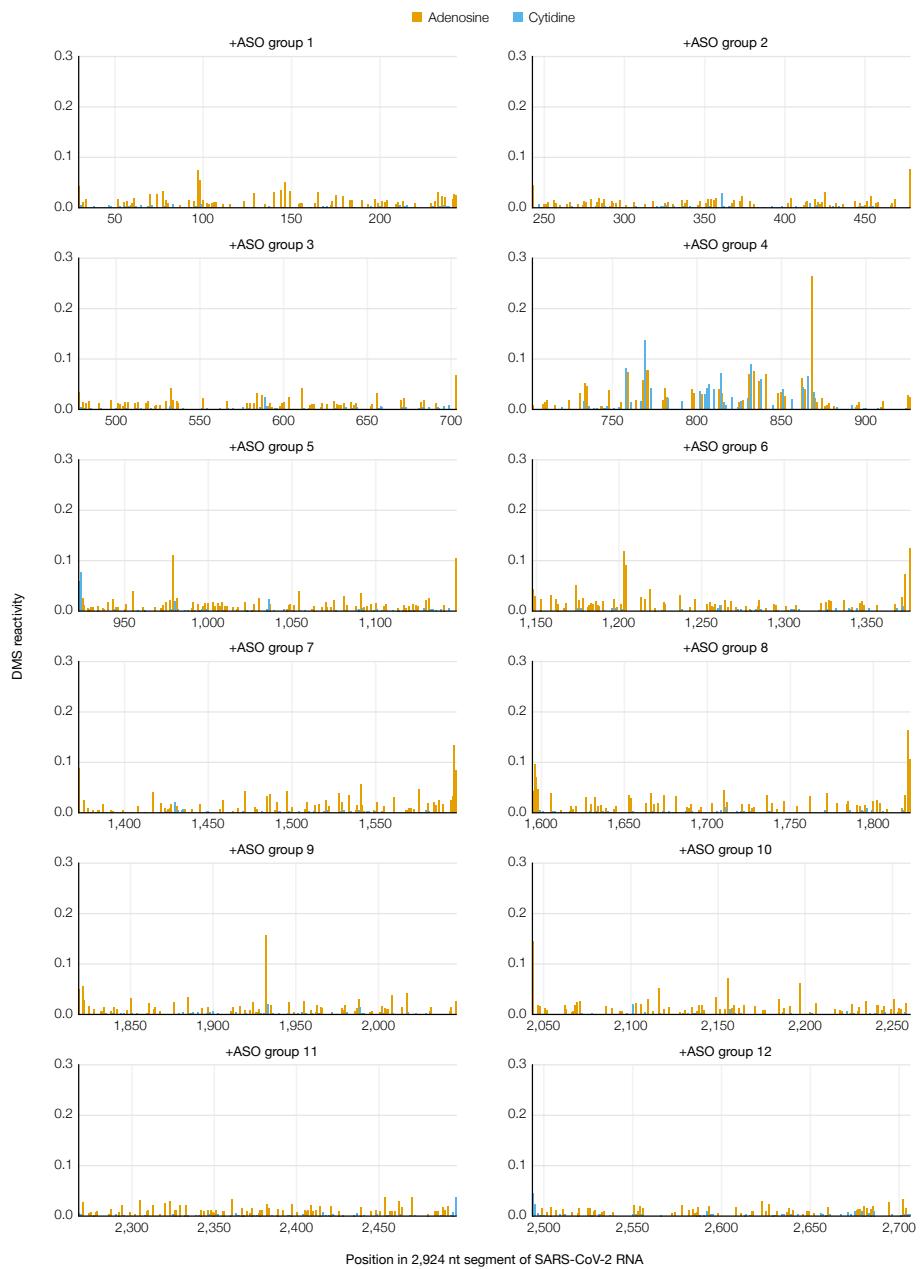
## Code Availability

Documentation for SEISMIC-RNA, including instructions for installation, is hosted on GitHub Pages: <https://rouskinlab.github.io/seismic-rna>. Source code for SEISMIC-RNA is available from GitHub: <https://github.com/rouskinlab/seismic-rna>.

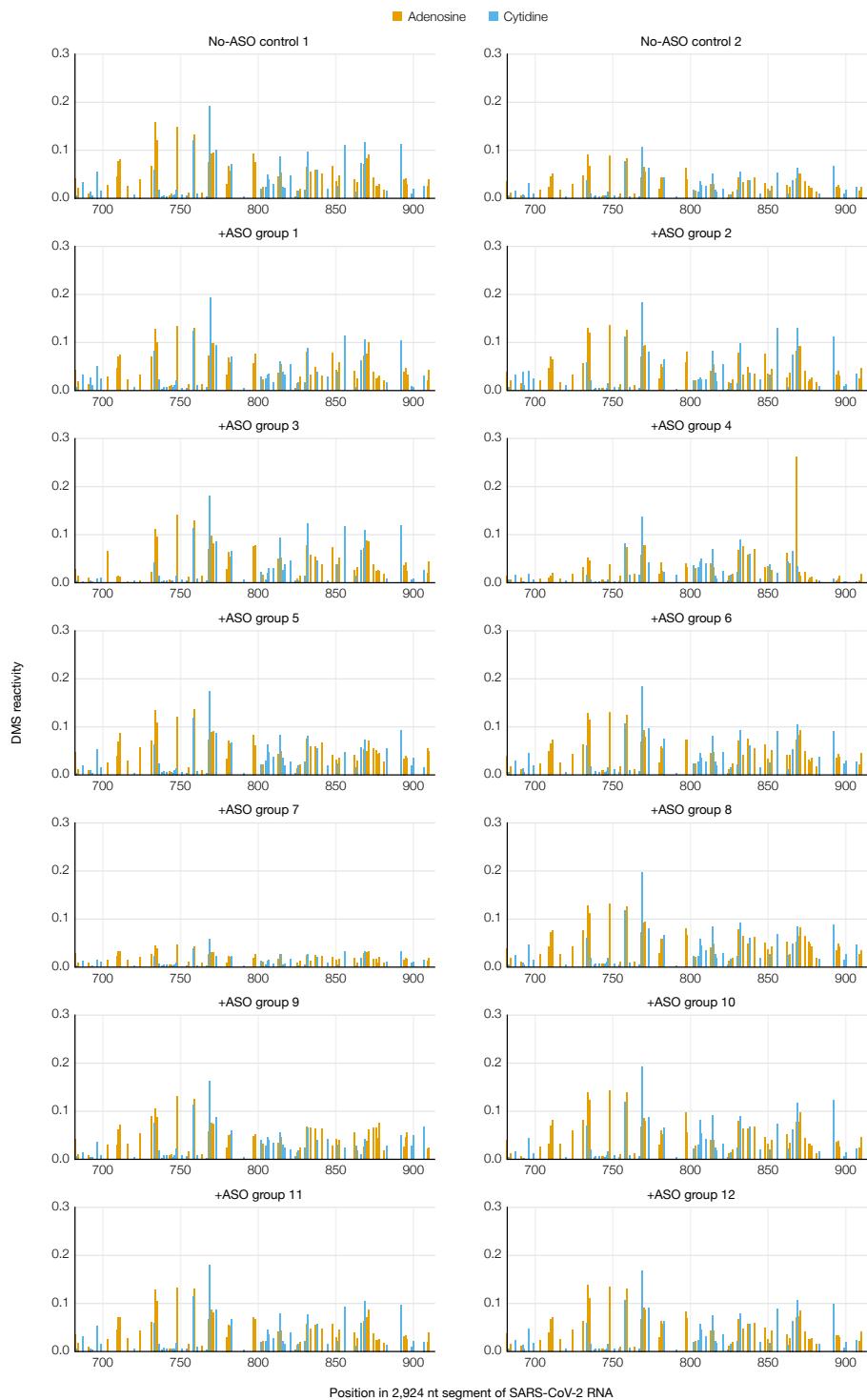
Shell scripts for running SEISMIC-RNA, auxiliary scripts for data analysis, supplementary files, and LaTeX source code for this manuscript are also available from GitHub: <https://github.com/rouskinlab/search-map>. The Methods section explains how each script was used.

# Supplementary Information

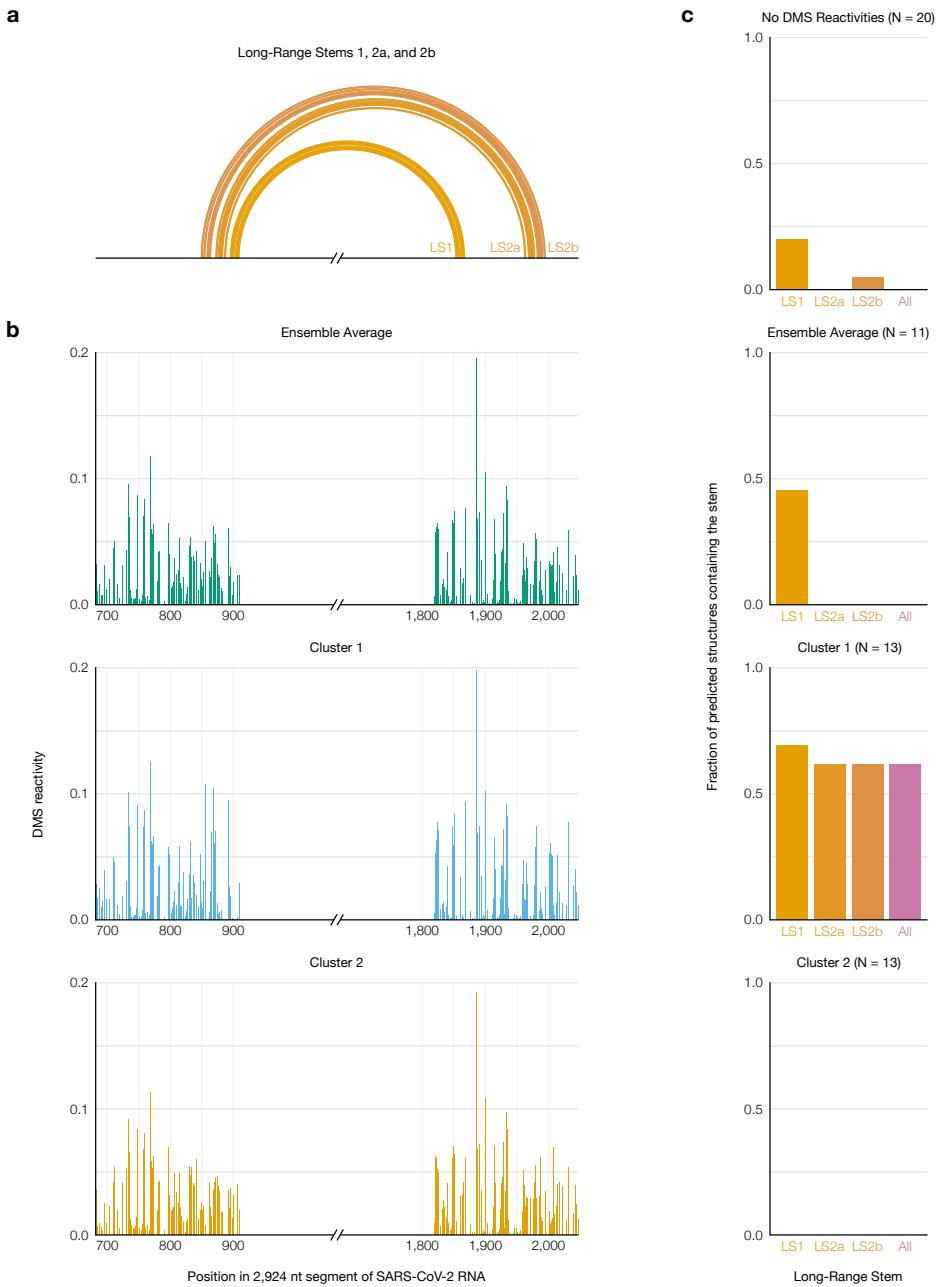
## Supplementary Figures



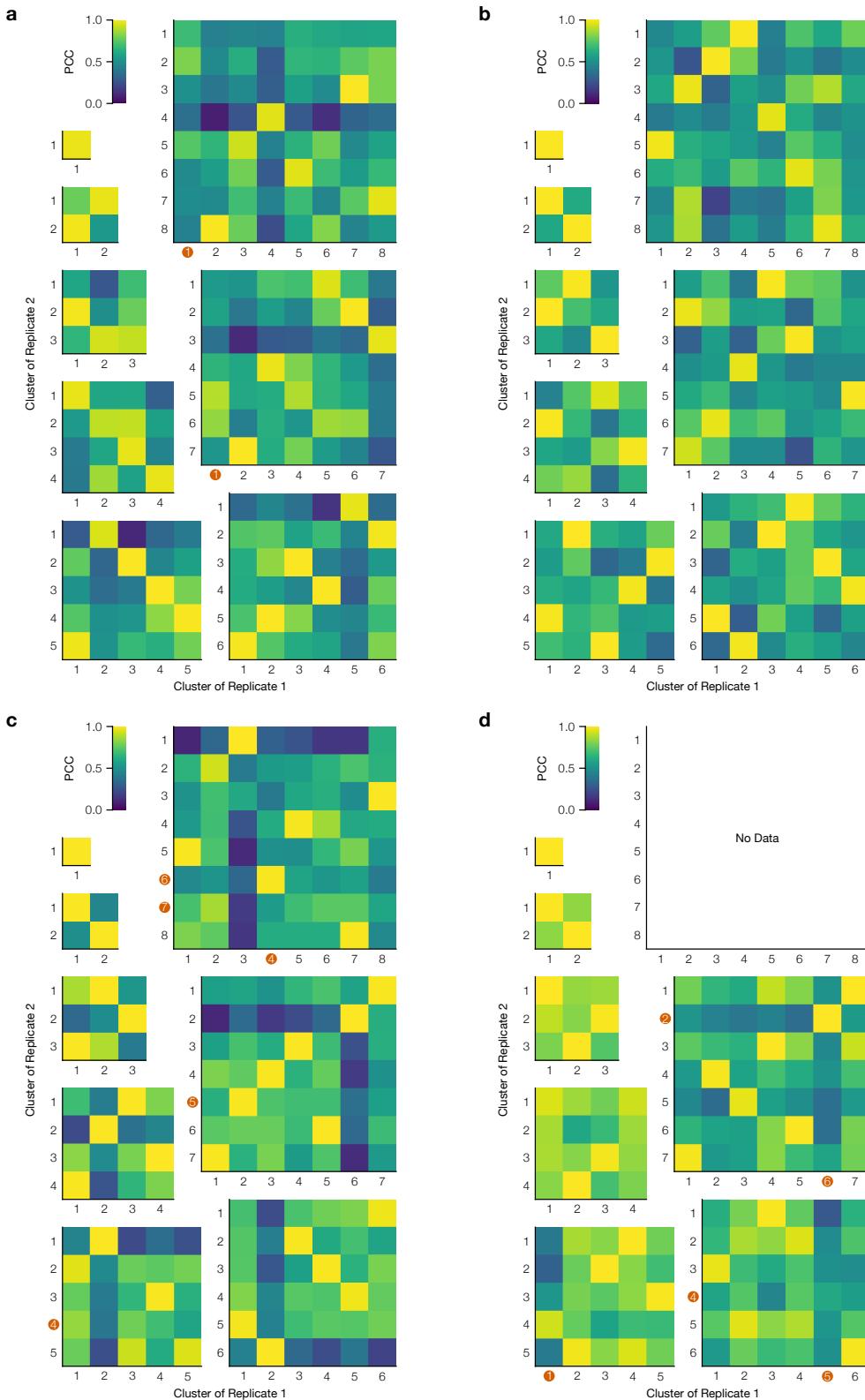
Supplementary Figure 1: Mutational profile of each ASO target section upon adding the corresponding group of ASOs to the 2,924 nt segment of SARS-CoV-2 genomic RNA. Positions are colored based on the RNA sequence.



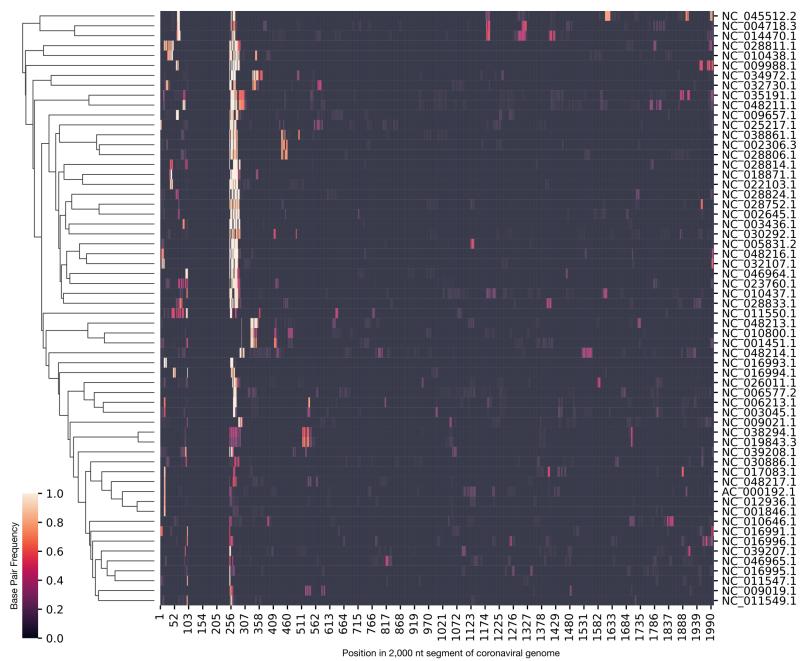
**Supplementary Figure 2: Mutational profiles of the FSE section upon adding each group of ASOs to the 2,924 nt segment of SARS-CoV-2 genomic RNA. Positions are colored based on the RNA sequence.**



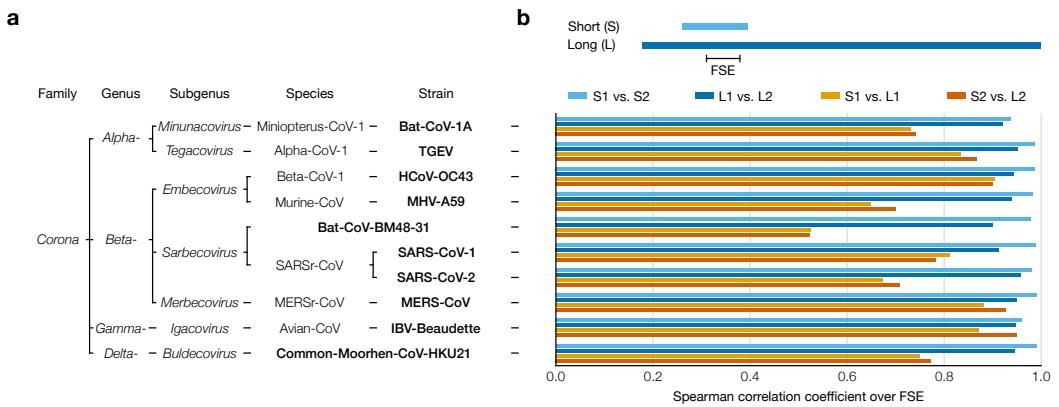
**Supplementary Figure 3: Improved prediction of long-range stems in SARS-CoV-2 using clustered DMS reactivities.** (a) Model of the two inner stems of the FSE-arch [49], denoted long stems (LS) 1 and 2a/b. (b) Mutational profiles of the ensemble average and of clusters 1 and 2 on both sides of the FSE-arch. (c) For each mutational profile (as well as a purely thermodynamic prediction with no DMS reactivities), the fraction of predicted structures in which each long stem was predicted perfectly (i.e. all base pairs were present). The numbers of predicted structures (N) are indicated.



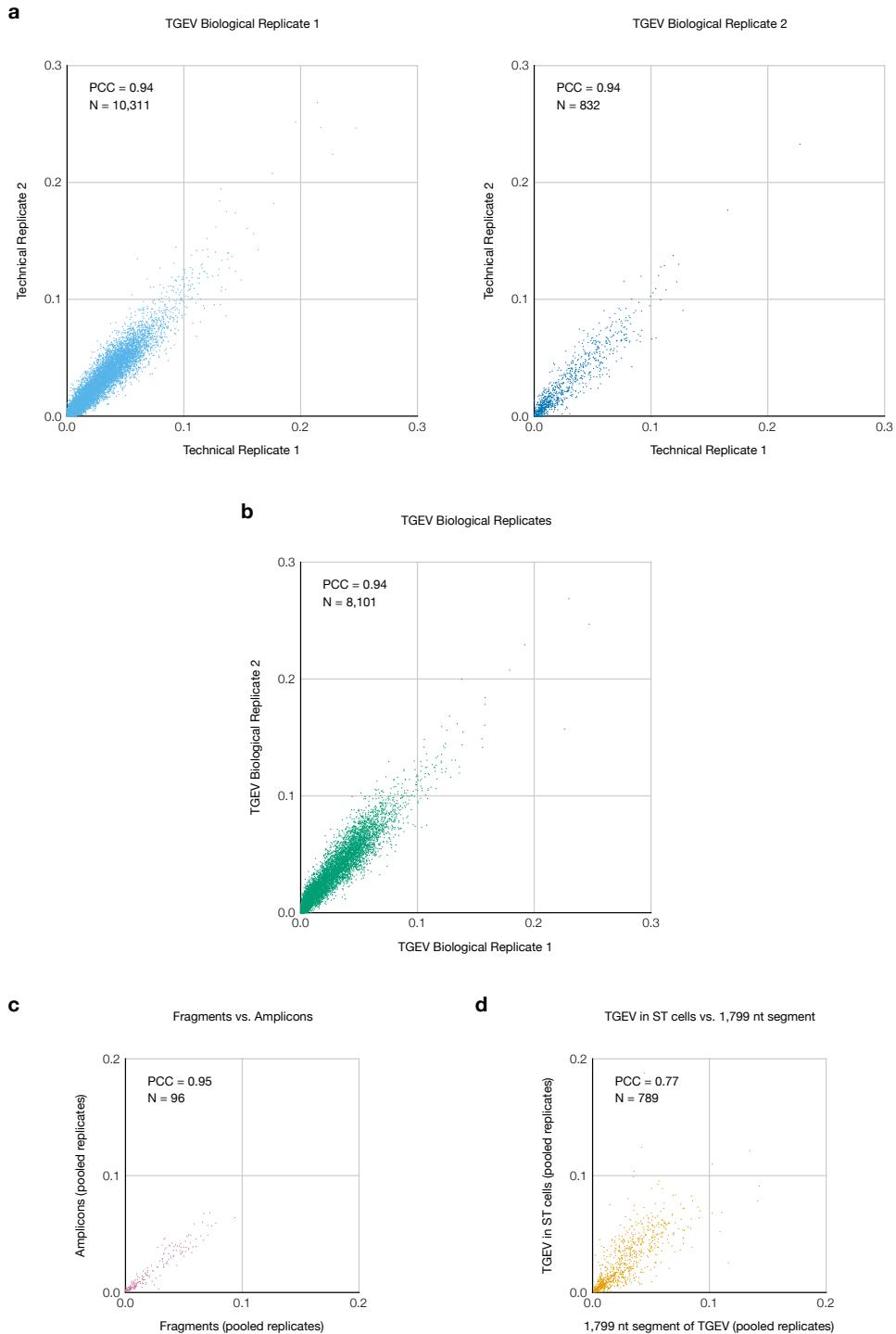
**Supplementary Figure 4: Reproducibility of clustering the SARS-CoV-2 FSE after adding ASOs.** (a) Heatmaps of the Pearson correlation coefficient (PCC) between each pair of clusters from two replicates of the 1,799 nt segment of SARS-CoV-2. Each heatmap corresponds to one order (i.e. number of clusters). Clusters are marked with red circles if at least one DMS reactivity exceeded 0.3. (b) Same as (a) plus Anti-AS1 ASO. (c) Same as (a) plus Anti-PS2-overlap ASO. (d) Same as (a) plus Anti-AS1 and Anti-PS2-overlap ASOs.



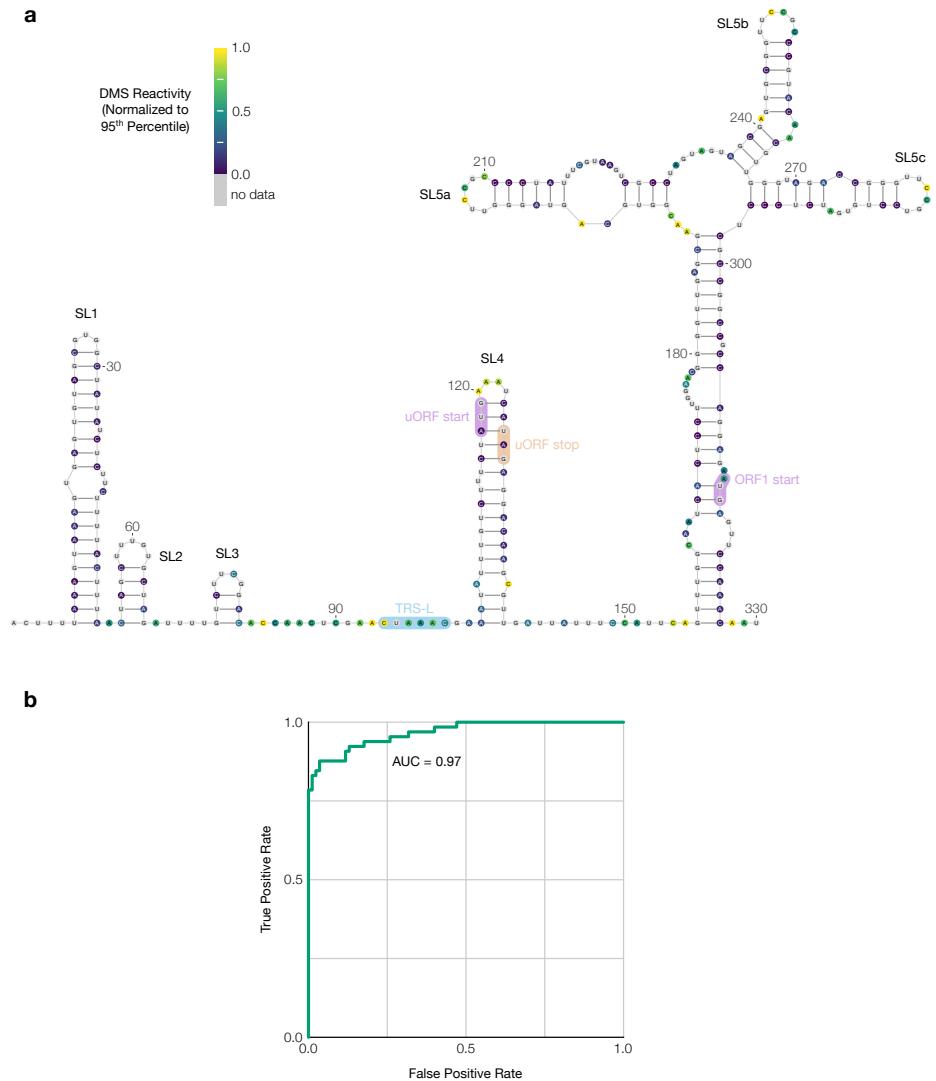
**Supplementary Figure 5: Computational screen of long-range base pairing near the FSE in 60 coronaviruses.** For each 2,000 nt segment of each coronaviral genome, the fraction of predicted structures in which each position outside the range 101-250 base-paired with any position in the range 101-250 is indicated. Genomes are clustered by their base-pairing frequencies. For each genome, the accession number for NCBI [51] is indicated.



**Supplementary Figure 6: Experimental screen of long-range base pairing near the FSE in 10 coronaviruses.** (a) Taxonomy of the ten coronavirus species/strains in this screen; the lowest-level group for each virus is bolded. Bat-CoV-1A: bat coronavirus 1A (NC\_010437.1), TGEV: transmissible gastroenteritis virus (NC\_038861.1), HCoV-OC43: human coronavirus OC43 (NC\_006213.1), MHV-A59: murine hepatitis virus strain A59 (NC\_048217.1), Bat-CoV-BM48-31: bat coronavirus BM48-31 (NC\_014470.1), SARS-CoV-1: severe acute respiratory syndrome coronavirus 1 (NC\_004718.3), SARS-CoV-2: severe acute respiratory syndrome coronavirus 2 (NC\_045512.2), MERS-CoV: Middle East respiratory syndrome coronavirus (NC\_019843.3), IBV-Beaudette: avian infectious bronchitis virus strain Beaudette (NC\_001451.1), Common-Moorhen-CoV-HKU21: common moorhen coronavirus HKU21 (NC\_016996.1). (b) Spearman correlation coefficients of DMS reactivities over the FSE between replicates 1 and 2 of short (239 nt) and long (1,799 nt) segments of each coronaviral genome.



**Supplementary Figure 7: Replicates of TGEV in ST cells and comparison to the 1,799 nt segment.** (a) Scatter plots comparing the DMS reactivities of the two technical replicates for each biological replicate of TGEV in ST cells. Each point represents one base in the sequence. The number of points (N) and Pearson correlation coefficient (PCC) are indicated for each plot. (b) Scatter plot comparing the DMS reactivities of the two biological replicates (each biological replicate comprises the reads for both of its technical replicates pooled together). (c) Scatter plot comparing the DMS reactivities of TGEV in ST cells using random fragmentation and amplicons of the FSE (the reads for both biological replicates pooled together). (d) Scatter plot comparing the DMS reactivities of TGEV in ST cells (the reads for both biological replicates pooled together) and for the 1,799 nt segment *in vitro*.



**Supplementary Figure 8: Secondary structure of the TGEV 5' UTR.** (a) Model of the secondary structure of the first 330 nt of the TGEV genome, based on DMS reactivities in infected ST cells normalized to the 95<sup>th</sup> percentile. Bases are colored by DMS reactivity. The model includes the conserved stem loops SL1, SL2, SL3, SL4, SL5a, SL5b, and SL5c [10]. The leader transcription regulatory sequence (TRS-L) [86], upstream open reading frame (uORF) [87], and start codon of ORF1 are also labeled. The model was drawn using VARNA [88]. (b) Receiver operating characteristic curve showing agreement between the DMS reactivities and the secondary structure model; the area under the curve (AUC) is indicated.

# Supplementary Methods

## Correcting observer bias due to drop-out of reads

Let  $N$  reads from  $K$  clusters align to a reference sequence of length  $L$ . Let the proportion of reads whose 5' and 3' ends align, respectively, to coordinates  $a$  and  $b$  ( $1 \leq a \leq b \leq L$ ) be  $\eta_{ab}$  (assuming these proportions are equal for all clusters).

Let the mutation rate of base  $j$  ( $1 \leq j \leq L$ ) in cluster  $k$  ( $1 \leq k \leq K$ ) be  $\mu_{jk}$ . Let the proportion of cluster  $k$  in the ensemble be  $\pi_k$ . To express these quantities as probabilities, let  $C_k$  be the event that a read comes from cluster  $k$ ; let  $E_{ab}$  be the event that a read aligns with 5' and 3' coordinates  $a$  and  $b$ , respectively; let  $S_j$  be the event that a read contains position  $j$  (i.e. its alignment coordinates  $a$  and  $b$  satisfy  $1 \leq a \leq j \leq b \leq L$ ); let  $M_j$  be the event that a read has a mutation at position  $j$ ; and let  $G_g$  be the event that a read has no two mutations separated by fewer than  $g$  non-mutated bases.

### Deriving mutation rates of reads with no two mutations too close

In terms of these events, the total mutation rates ( $\mu_{jk}$ ) are  $P(M_j|S_jC_k)$ , i.e. the probability that a read would have a mutation at position  $j$  given that it contained position  $j$  and came from cluster  $k$ ; and the observable mutation rates ( $m_{jk}$ ) are  $P(M_j|S_jC_kG_g)$ , i.e. the probability that a read would have a mutation at position  $j$  given that it contained position  $j$ , came from cluster  $k$ , and had no two mutations closer than  $g$  bases. Using these definitions and Bayes' theorem yields a probabilistic formula for  $m_{jk}$ :

$$m_{jk} = P(M_j|S_jC_kG_g) = P(M_j|S_jC_k) \frac{P(G_g|S_jM_jC_k)}{P(G_g|S_jC_k)} = \mu_{jk} \frac{P(G_g|S_jM_jC_k)}{P(G_g|S_jC_k)}$$

The term  $P(G_g|S_jC_k)$  is the probability that a read would have no two mutations closer than  $g$  bases given that it contained position  $j$  and came from cluster  $k$ . It can be computed using  $P(G_g|E_{ab}C_k)$  (abbreviated  $d_{abk}$ ): the probability that a

read would contain no two mutations closer than  $g$  bases given that its 5' and 3' coordinates are  $a$  and  $b$ , respectively ( $1 \leq a \leq b \leq L$ ), and that it came from cluster  $k$ . If position  $b$  were mutated (probability  $\mu_{bk}$ ), then the read would contain no two mutations closer than  $g$  bases if and only if none of the  $g$  bases preceding  $b$  (i.e. positions  $b-g$  to  $b-1$ , inclusive) were mutated (probability  $\prod_{j'=\max(b-g,a)}^{b-1} (1-\mu_{j'k})$ , abbreviated  $w_{\max(b-g,a),b-1,k}$ ) and two no mutations between positions  $a$  and  $b-(g+1)$ , inclusive, were too close (probability  $d_{a,\max(b-(g+1),a),k}$ ). If position  $b$  were not mutated (probability  $1 - \mu_{bk}$ ), then the read would contain no two mutations closer than  $g$  bases if and only if no mutations between positions  $a$  and  $b-1$ , inclusive, were too close (probability  $d_{a,\max(b-1,a),k}$ ). These two possibilities generate a recurrence relation:

$$d_{abk} = \mu_{bk} w_{\max(b-g,a),b-1,k} d_{a,\max(b-(g+1),a),k} + (1 - \mu_{bk}) d_{a,\max(b-1,a),k}$$

The base case is  $d_{abk} = 1$  when  $a = b$  because such a read would contain one position and thus be guaranteed to have no two mutations too close. Then,  $P(G_g|S_j C_k)$  is the average of  $d_{abk}$  over every read that contains position  $j$ , weighted by the proportions  $\eta_{ab}$ :

$$P(G_g|S_j C_k) = \frac{\sum_{a=1}^j \sum_{b=j}^L \eta_{ab} d_{abk}}{\sum_{a=1}^j \sum_{b=j}^L \eta_{ab}}$$

The term  $P(G_g|M_j E_{ab} C_k)$  is the probability that a read would have no two mutations too close given that it contained a mutation at position  $j$  and came from cluster  $k$ . It can be computed using  $P(G_g|M_j E_{ab} C_k)$  (abbreviated  $f_{abjk}$ ): the probability that a read would contain no two mutations too close given that position  $j$  is mutated ( $1 \leq a \leq j \leq b \leq L$ ), that its 5' and 3' coordinates are  $a$  and  $b$  (respectively), and that it came from cluster  $k$ . Because position  $j$  is mutated, having no two mutations too close requires that none of the  $g$  bases on both sides of position  $j$  be mutated. The probability that none of the preceding  $g$  positions ( $j-g$  to  $j-1$ ) is mutated is  $w_{\max(j-g,a),j-1,k}$ , while that of the following  $g$  positions ( $j+1$  to  $j+g$ ) is  $w_{j+1,\min(j+g,b),k}$ . Upstream of the  $g$  bases flanking position  $j$  (i.e. positions  $a$  to  $j-(g+1)$ ), the probability that no two mutations are too close is  $d_{a,\max(j-(g+1),a),k}$ ;

downstream (i.e. positions  $j + (g + 1)$  to  $b$ ), the probability is  $d_{\min(j+(g+1), b), b, k}$ . Since mutations in these four sections are independent, the probability that the read contains no two mutations too close is the product:

$$f_{abjk} = d_{a, \max(j-(g+1), a), k} w_{\max(j-g, a), j-1, k} w_{j+1, \min(j+g, b), k} d_{\min(j+(g+1), b), b, k}$$

Then,  $P(G_g | S_j M_j C_k)$  is the average of  $f_{abjk}$  over every read that contains position  $j$ , weighted by the proportions  $\eta_{ab}$ .

$$P(G_g | S_j M_j C_k) = \frac{\sum_{a=1}^j \sum_{b=j}^L \eta_{ab} f_{abjk}}{\sum_{a=1}^j \sum_{b=j}^L \eta_{ab}}$$

Combining the above results yields an explicit formula for  $m_{jk}$ :

$$m_{jk} = \mu_{jk} \frac{\sum_{a=1}^j \sum_{b=j}^L \eta_{ab} f_{abjk}}{\sum_{a=1}^j \sum_{b=j}^L \eta_{ab} d_{abk}}$$

## Deriving end coordinate proportions of reads with no two mutations too close

The total proportions ( $\eta_{ab}$ ) of reads aligned to 5' and 3' coordinates  $a$  and  $b$ , respectively, are  $P(E_{ab})$ ; and the proportions of reads with no two mutations too close that align with coordinates  $a$  and  $b$  ( $e_{abk}$ ) are  $P(E_{ab} | G_g C_k)$ . Note that, while reads are assumed to come from the same distribution of coordinates ( $\eta_{ab}$ ) regardless of their cluster  $k$ , the observable distribution of coordinates ( $e_{abk}$ ) varies by cluster because  $P(G_g C_k)$  depends on  $k$ . Using these definitions and Bayes' theorem yields a probabilistic formula for  $e_{abk}$ :

$$e_{abk} = P(E_{ab} | G_g C_k) = P(G_g | E_{ab} C_k) \frac{P(E_{ab} | C_k)}{P(G_g | C_k)} = d_{abk} \frac{\eta_{ab}}{P(G_g | C_k)}$$

The term  $P(G_g | C_k)$  is the probability that a read would have no two mutations too close given that it came from cluster  $k$ . It can be computed as an average of  $P(G_g | E_{ab} C_k)$  (i.e.  $d_{abk}$ ) over all coordinates  $a$  and  $b$  (such that  $1 \leq a \leq b \leq L$ ),

weighted by the proportion of each coordinate,  $P(E_{ab})$  (i.e.  $\eta_{ab}$ ):

$$P(G_g|C_k) = \frac{\sum_{a=1}^L \sum_{b=a}^L \eta_{ab} d_{abk}}{\sum_{a=1}^L \sum_{b=a}^L \eta_{ab}} = \sum_{a=1}^L \sum_{b=a}^L \eta_{ab} d_{abk}$$

This expression is already normalized because  $\sum_{a=1}^L \sum_{b=a}^L \eta_{ab} = 1$ , by definition.

Combining the above results yields an explicit formula for  $e_{abk}$ :

$$e_{abk} = \frac{\eta_{ab} d_{abk}}{\sum_{a'=1}^L \sum_{b'=a'}^L \eta_{a'b'} d_{a'b'k}}$$

## Deriving cluster proportions of reads with no two mutations too close

The proportion of total reads in cluster  $k$  is  $\pi_k = P(C_k)$ . The proportion among only reads with no two mutations closer than  $g$  bases is

$$p_k = P(C_k|G_g) = P(G_g|C_k) \frac{P(C_k)}{P(G_g)} = \pi_k \frac{\sum_{a=1}^L \sum_{b=a}^L \eta_{ab} d_{abk}}{P(G_g)}$$

The term  $P(G_g)$  is the probability that a read from any cluster would have no two mutations closer than  $g$  bases and can be solved for by leveraging that the cluster proportions ( $p_k$ ) must sum to 1:

$$1 = \sum_{k=1}^K p_k = \sum_{k=1}^K \pi_k \frac{\sum_{a=1}^L \sum_{b=a}^L \eta_{ab} d_{abk}}{P(G_g)} = \frac{1}{P(G_g)} \sum_{k=1}^K \pi_k \sum_{a=1}^L \sum_{b=a}^L \eta_{ab} d_{abk}$$

$$P(G_g) = \sum_{k=1}^K \pi_k \sum_{a=1}^L \sum_{b=a}^L \eta_{ab} d_{abk}$$

The result is an explicit formula for  $p_k$ :

$$p_k = \frac{\pi_k \sum_{a=1}^L \sum_{b=a}^L \eta_{ab} d_{abk}}{\sum_{k'=1}^K \pi_{k'} \sum_{a=1}^L \sum_{b=a}^L \eta_{ab} d_{abk'}}$$

## Solving total mutation rates and cluster and coordinate proportions

The observed mutation rates ( $m_{jk}$ ), end coordinate proportions ( $e_{abk}$ ), and cluster proportions ( $p_k$ ) can be calculated as weighted averages over the  $N$  reads with no

two mutations too close:

$$m_{jk} = \frac{\sum_{i=1}^N z_{ik} x_{ij}}{\sum_{i=1}^N z_{ik}}$$

$$e_{abk} = \frac{\sum_{i=1}^N z_{ik} y_{abi}}{\sum_{i=1}^N z_{ik}}$$

$$p_k = \frac{\sum_{i=1}^N z_{ik}}{N}$$

where  $x_{ij}$  is 1 if read  $i$  has a mutation at position  $j$ , otherwise 0;  $y_{abi}$  is 1 if read  $i$  aligns to coordinates  $a$  and  $b$ , otherwise 0; and  $z_{ik}$  is the probability that read  $i$  came from cluster  $k$ .

The original parameters  $\mu_{jk}$ ,  $\eta_{abk}$ , and  $\pi_k$  can be solved by setting the two formula each for  $m_{jk}$ ,  $e_{abk}$ , and  $p_k$  equal to each other, creating a system of equations:

$$\mu_{jk} \frac{\sum_{a=1}^j \sum_{b=j}^L \eta_{ab} f_{abjk}}{\sum_{a=1}^j \sum_{b=j}^L \eta_{ab} d_{abk}} = m_{jk} = \frac{\sum_{i=1}^N z_{ik} x_{ij}}{\sum_{i=1}^N z_{ik}}$$

$$\eta_{ab} \frac{d_{abk}}{\sum_{a'=1}^L \sum_{b'=a'}^L \eta_{a'b'} d_{a'b'k}} = e_{ab} = \frac{\sum_{i=1}^N z_{ik} y_{abi}}{\sum_{i=1}^N z_{ik}}$$

$$\pi_k \frac{\sum_{a=1}^L \sum_{b=a}^L \eta_{ab} d_{abk}}{\sum_{k'=1}^K \pi_{k'} \sum_{a=1}^L \sum_{b=a}^L \eta_{ab} d_{abk'}} = p_k = \frac{\sum_{i=1}^N z_{ik}}{N}$$

Solving this entire system at once has proven computationally impractical for all but extremely short sequences. A more feasible approach is to first solve for  $\mu_{jk}$  given an initial guess for  $\eta_{ab}$ , next solve for  $\eta_{ab}$  given the updated  $\mu_{jk}$ , then solve for  $\pi_k$  given the updated  $\mu_{jk}$  and  $\eta_{ab}$ , and iterate until all three sets of parameters converge.

Even assuming every  $\eta_{ab}$  is a constant, these equations are still too complex to solve for  $\mu_{jk}$  analytically because  $d_{abk}$  and  $f_{abjk}$  also depend on  $\mu_{jk}$  (as well as on other  $\mu$  variables). Thus, every  $\mu_{jk}$  is solved for numerically by rearranging each equation to

$$\mu_{jk} \frac{\sum_{a=1}^j \sum_{b=j}^L \eta_{ab} f_{abjk}}{\sum_{a=1}^j \sum_{b=j}^L \eta_{ab} d_{abk}} - m_{jk} = 0$$

and applying the Netwon-Krylov method [89] implemented in SciPy [81].

Once every  $\mu_{jk}$  has been solved for, every  $\eta_{ab}$  can be updated. Because  $d_{abk}$  does not depend on  $\eta_{ab}$  (except indirectly through the  $\mu_{jk}$  parameters, which are

now assumed to be constants), each equation can be rearranged to

$$\eta_{ab} = \frac{e_{ab}}{d_{abk}} \sum_{a'=1}^L \sum_{b'=a'}^L \eta_{a'b'} d_{a'b'k}$$

Leveraging that  $\sum_{a=1}^L \sum_{b=a}^L \eta_{ab} = 1$ , by definition, leads to

$$\sum_{a=1}^L \sum_{b=a}^L \frac{e_{ab}}{d_{abk}} \sum_{a'=1}^L \sum_{b'=a'}^L \eta_{a'b'} d_{a'b'k} = 1$$

$$\sum_{a'=1}^L \sum_{b'=a'}^L \eta_{a'b'} d_{a'b'k} = \frac{1}{\sum_{a=1}^L \sum_{b=a}^L \frac{e_{ab}}{d_{abk}}}$$

and finally a closed-form expression for each  $\eta_{ab}$  given  $\mu_{jk}$  (and hence  $d_{abk}$ ) and  $e_{abk}$ :

$$\eta_{ab} = \frac{\frac{e_{ab}}{d_{abk}}}{\sum_{a'=1}^L \sum_{b'=a'}^L \frac{e_{a'b'}}{d_{a'b'k}}}$$

This equation should theoretically yield the same value of  $\eta_{ab}$  for every  $k$ . In practice, the values will differ due to inexactness in floating-point arithmetic. Thus, the consensus value of  $\eta_{ab}$  is taken to be the average  $\eta_{ab}$  over every  $k$ , weighted by  $\pi_k$ :

$$\eta_{ab} = \sum_{k=1}^K \pi_k \frac{\frac{e_{ab}}{d_{abk}}}{\sum_{a'=1}^L \sum_{b'=a'}^L \frac{e_{a'b'}}{d_{a'b'k}}}$$

With updated values of  $\mu_{jk}$  and  $\eta_{ab}$ ,  $\pi_k$  can also be solved. The above equations can be rearranged to

$$\pi_k = p_k \frac{\sum_{k'=1}^K \pi_{k'} \sum_{a=1}^L \sum_{b=a}^L \eta_{ab} d_{abk'}}{\sum_{a=1}^L \sum_{b=a}^L \eta_{ab} d_{abk}}$$

Given that  $\sum_{k=1}^K \pi_k = 1$ , by definition:

$$\sum_{k=1}^K p_k \frac{\sum_{k'=1}^K \pi_{k'} \sum_{a=1}^L \sum_{b=a}^L \eta_{ab} d_{abk'}}{\sum_{a=1}^L \sum_{b=a}^L \eta_{ab} d_{abk}} = 1$$

$$\sum_{k'=1}^K \pi_{k'} \sum_{a=1}^L \sum_{b=a}^L \eta_{ab} d_{abk'} = \frac{1}{\sum_{k=1}^K \frac{p_k}{\sum_{a=1}^L \sum_{b=a}^L \eta_{ab} d_{abk}}}$$

which leads to a closed-form expression for each  $\pi_k$  given  $\mu_{jk}$  (and hence  $d_{abk}$ ),  $\eta_{ab}$ , and  $p_k$ :

$$\pi_k = \frac{\sum_{a=1}^L \sum_{b=a}^L \eta_{ab} d_{abk}}{\sum_{k'=1}^K \sum_{a=1}^L \sum_{b=a}^L \eta_{ab} d_{abk'}}$$

## Clustering reads with the expectation-maximization algorithm

Let  $N$  reads from  $K$  clusters align to a reference sequence of length  $L$ . Let the proportion of reads whose 5' and 3' ends align, respectively, to coordinates  $a$  and  $b$  ( $1 \leq a \leq b \leq L$ ) be  $\eta_{ab}$  (assuming these proportions are equal for all clusters). Let the mutation rate of base  $j$  ( $1 \leq j \leq L$ ) in cluster  $k$  ( $1 \leq k \leq K$ ) be  $\mu_{jk}$ . Let the proportion of cluster  $k$  in the ensemble be  $\pi_k$ .

### Maximization step

The maximization step updates the parameters ( $\mu_{jk}$ ,  $\eta_{ab}$ , and  $\pi_k$ ) using the current cluster memberships ( $z_{ik}$ ). The observed estimates of the parameters  $m_{jk}$ ,  $e_{ab}$ , and  $p_k$  are first computed; then, the underlying parameters  $\mu_{jk}$ ,  $\eta_{ab}$ , and  $\pi_k$  are solved for as described in 6.1.4.

### Expectation step

The expectation step updates the cluster memberships ( $z_{ik}$ ) and the likelihood function ( $L$ ) using the current parameters ( $\mu_{jk}$ ,  $\eta_{ab}$ , and  $\pi_k$ ). Each cluster membership is defined as the probability that read  $i$  came from cluster  $k$  given its 5'/3' end coordinates ( $E_{ab}$ ) and mutations ( $M$ ) and given that no two mutations are too close ( $G_g$ ):  $z_{ik} = P(C_k | E_{ab} M G_g)$ . The likelihood of the model ( $L$ ) is the product of the marginal probability ( $L_i$ ) of observing each read  $i$  from any cluster:  $L_i = P(E_{ab} M | G_g)$ . Both  $L_i$  and  $z_{ik}$  can be expressed in terms of the joint probability ( $L_{ik} = P(E_{ab} M C_k | G_g)$ )

of observing each read  $i$  from each cluster  $k$ :

$$L_i = P(E_{ab}M|G_g) = \sum_{k=1}^K P(E_{ab}MC_k|G_g) = \sum_{k=1}^K L_{ik}$$

$$z_{ik} = P(C_k|E_{ab}MG_g) = \frac{P(E_{ab}MC_kG_g)}{P(E_{ab}MG_g)} = \frac{P(E_{ab}MC_k|G_g)}{P(E_{ab}M|G_g)} = \frac{L_{ik}}{L_i}$$

To derive a formula for  $L_{ik}$ , it can be factored into three parts using the chain rule for probability:

$$L_{ik} = P(E_{ab}MC_k|G_g) = \frac{P(E_{ab}MC_kG_g)}{P(G_g)} = P(M|E_{ab}C_kG_g)P(E_{ab}|C_kG_g)P(C_k|G_g)$$

The first part – the probability that a read would have the specific mutations  $x_{ij}$  given that its 5'/3' end coordinates are  $a$  and  $b$  (respectively), it comes from cluster  $k$ , and no two mutations are too close – is the product over every position  $j$  from  $a$  to  $b$  of the probability of a mutation ( $\mu_{jk}$ ) if read  $i$  is mutated at position  $j$  ( $x_{ij} = 1$ ), otherwise ( $x_{ij} = 0$ ) the probability of no mutation ( $1 - \mu_{jk}$ ), normalized by the probability that no two mutations would be too close ( $d_{abk}$ ):

$$P(M|E_{ab}C_kG_g) = \frac{1}{d_{abk}} \prod_{j=a}^b \mu_{jk}^{x_{ij}} (1 - \mu_{jk})^{(1-x_{ij})}$$

The second part,  $P(E_{ab}|C_kG_g) = e_{abk}$ , can be calculated from the parameters  $\mu_{jk}$ ,  $\eta_{ab}$ , and  $\pi_k$ , as explained in 6.1.2. Likewise, the third part,  $P(C_k|G_g) = p_k$ , can also be calculated from the parameters, as explained in 6.1.3. Combining all parts yields a formula for  $L_{ik}$  in terms of the parameters  $\mu_{jk}$ ,  $\eta_{ab}$ , and  $\pi_k$  and of their derived values  $d_{abk}$ ,  $e_{abk}$ , and  $p_k$ :

$$L_{ik} = p_k \frac{e_{abk}}{d_{abk}} \prod_{j=a}^b \mu_{jk}^{x_{ij}} (1 - \mu_{jk})^{(1-x_{ij})}$$

The formula for the total likelihood of the model and its parameters follows:

$$L(\mu, \eta, \pi) = \prod_{i=1}^N L_i = \prod_{i=1}^N \sum_{k=1}^K p_k \frac{e_{abk}}{d_{abk}} \prod_{j=a}^b \mu_{jk}^{x_{ij}} (1 - \mu_{jk})^{(1-x_{ij})}$$

# Supplementary Tables

Table 1: Sequences of the antisense oligonucleotides (ASOs) targeting the 2,924 nt segment of SARS-CoV-2 RNA.

Group	ASO	Sequence
1	1	GGCAGCACAAAGACATCTGTCGTAGTGCAACAGGACTAAGC- TCATTATT
	2	TGTAGTAAGCTAACGCATTGTCATCAGTGCAAGCAGTTGT- GTAGTACC
	3	TGTAAATCGGATAACAGTGCAAGTACAAACCTACCTCCCTT- TGTTGTGT
	4	GATAGTACCAGTTCCATCACTCTTAGGGAATCTAGCCCATT- TCAAATCC
	5	CTTAGGTGTCTGTAACAAACCTACAAGGTGGTCCAGT- TCTGTATA
2	1	ATACCTCTATTAGGTTTTAACCTTAATAAAAGTATAAA- TACTTCACTTAGGAC
	2	CACTTCTGTTGCATTACCAGCTTGAGACGTACTGTGGCAG- CTAAACTACCAAGTACC
	3	AAGCTTAGCAGCATCTACAGCAAAAGCACAGAAAGATAA- TACAGTTGAATTGGCAGG
	4	CACAACATCTAACACAATTAGTGATTGGTTGTCCCCACT- AGCTAGATAATCTTG
3	1	GATCCATATTGGCTCCGGTGTAACTGTTATTGCCTGACCA- GTACCAGTGTGTGA
	2	ATGATCTATGTGGCAACGGCAGTACAGACAACACGATGCA- CCACCAAAGGATTCTT

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Table 1: Sequences of the antisense oligonucleotides (ASOs) targeting the 2,924 nt segment of SARS-CoV-2 RNA. (Continued)

		3	GTTGTAGGTATTTGTACATACTTACCTTTAAGTCACAAAAT-
			CCTTTAGGATTGG
		4	CCGCAGACGGTACAGACTGTGTTTAAGTGTAAAACCCAC-
			AGGGTCATTAGCACAA
4	1		CTGAAGCATGGGTTCGCGGAGTTGATCACAACACAGCCA-
			TAACCTTCCACATA
	2		AAGACGGGCTGCACTTACACCGCAAACCCGTTAAAAACG-
			ATTGTGCATCAGCTGA
	3		TAGATGTAAAAGCCCTGTATACGACATCAGTACTAGTGCC-
			TGTGCCGCACGGTGT
	4		GGAAGCGACAACAATTAGTTTAGGAATTAGCAAAACCA-
			GCTACTTTATCATTG
5	1		TGTCTCTTAACACAAAGTAAGAATCAATTAAATTGTCATCT-
			TCGTCCTTTCTT
	2		GACAATCCTTAAGTAAATTATAAATTGTTCTTCATGTTGGT-
			AGTTAGAGAAAGTG
	3		GGTACCATGTCACCGTCTATTCTAAACTAAAGAAGTCATG-
			TTTAGCAACAGCTG
	4		AAGCATAGACGAGGTCTGCCATTGTGTATTTAGTAAGACGT-
			TGACGTGATATATGT
6	1		TGTATGTGACAAGTATTTCTTTAATGTGTACAATTACCTT-
			CATCAAAATGCCTTA
	2		GGTTTCTACAAAATCATACCAGTCCTTTATTGAAATAAT-
			CATCATCACACAAT

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Table 1: Sequences of the antisense oligonucleotides (ASOs) targeting the 2,924 nt segment of SARS-CoV-2 RNA. (Continued)

	3	TTAACAAAGCTTGGCGTACACGTTCACCTAAGTTGGCGTAT-
		ACGCGTAATATATCTG
	4	ATGTCAGTACACCAACAATACCAGCATTGCGATGGCATCA-
		CAGAATTGTACTGTTT
7	1	GTTTGTATGAAATCACCGAAATCATACCAGTTACCATTGAG-
		ATCTTGATTATCTA
	2	TAGGCATTAACAATGAATAATAAGAATCTACAACAGGAACT-
		CCACTACCTGGCGTG
	3	GTAAAGTCAGTGTCAACATGTGACTCTGCAGTTAAAGCCCT-
		GGTCAAGGTTAATA
	4	TTAACCTCTCTCCGTGAAGTCATATTTAACAAATCCCCT-
		TAATGTAAGGCTTT
8	1	AACACAATTGGGTGGTATGTCTGATCCAATATTTAAAAT-
		AACGGTCAAAGAGTT
	2	GAGAATAAAACATTAAAGTTGCACAATGCAGAATGCATCT-
		GTCATCCAACAGTT
	3	CATCAACAAATATTTCTCACTAGTGGTCCAAAAC TTGTA-
		GGTGGGAACACTGTA
	4	ATGTACAACACCTAGCTCTGAAGTGGTATCCAGTTGAAA-
		CTACAAATGGAACAC
9	1	TACACAAGTAATT CCTTAAA ACTAAGTCTAGAGCTATGTAA-
		GTTTACATCCTGATT
	2	TGC GTTTATCTAGTAATAGATTACCAGAAGCAGCGTGCATA-
		GCAGGGTCAGCAGCA

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Table 1: Sequences of the antisense oligonucleotides (ASOs) targeting the 2,924 nt segment of SARS-CoV-2 RNA. (Continued)

		3 TTTGACAGTTGAAAAGCAACATTGTTAGTAAGTGCAGCTA-
		CTGAAAAGCACGTAG
		4 CTTAAAGAAACCCTTAGACACAGCAAAGTCATAGAAGTCTT-
		TGTTAAAATTACCGGG
10	1	CAGCATTACCACCATCCTGAGCAAAGAAGAAGTGTAAATTCA-
		ACAGAACCTCCTTC
	2	CTGATATCACACATTGTTGGTAGATTATAACGATAGTAGTC-
		ATAATCGCTGATAG
	3	ACCATCGTAACAATCAAAGTACTTATCAACAACTCAACTA-
		CAAATAGTAGTTGT
	4	AACCAGCTGATTGTCTAGGTTGTTGACGATGACTTGGTTA-
		GCATTAATACAGCC
11	1	CCTCATAACTCATTGAATCATAATAAAGTCTAGCCTTACCC-
		CATTATTAAATGGAA
	2	ATTTGAGTTAGTAGGGATGACATTACGTTTGATATGC-
		GAAAAGTGCATCTTGAT
	3	GAGACACCAGCTACGGTGCAGCTCTATTCTTGCACTAAT-
		GGCATACTTAAGATTC
	4	GGCTATTGATTCAATAATTTGATGAAACTGTCTATTGGT-
		CATAGTACTACAGATA
12	1	CAACCACCATAGAATTGCTTCCAATTACTACAGTAGC-
		TCCTCTAGTGGC
	2	CCATAAGGTGAGGGTTTCTACATCACTATAAACAGTTTT-
		AACATGTTGTGC

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Table 1: Sequences of the antisense oligonucleotides (ASOs) targeting the 2,924 nt segment of SARS-CoV-2 RNA. (Continued)

3 CATAATTCTAAGCATGTTAGGCATGGCTCTATCACATTAG-  
GATAATCCCAAC

4 ACGGTGTGACAAGCTACAACACAGTTATGTTGCGAGCA-  
AGAACAAAGTGAGGC

13 1 ACACATGACCATTCACTCAATACTTGAGCACACTCATTAG-  
CTAATCTATAGAA

2 AGTTGTGGCATCTCCTGATGAGGTTCCACCTGGTTAACAT-  
ATAGTGAACCGCC

3 ATTAACATTGGCCGTGACAGCTTGACAAATGTTAAAAACAC-  
TATTAGCATAAGC

4 TAAATTGCGGACATACTTATCGGCAATTGTTACCATCAG-  
TAGATAAAAGTGC

Table 2: Sequences of the forward (F) and reverse (R) primers for amplifying the target site of each ASO group in the 2,924 nt segment of SARS-CoV-2 RNA.

Group	Primer	Sequence
1	F	AATAATGAGCTTAGTCCTGTTGCACTAGC
	R	AGGTTGTTAACCTTAATAAAGTATAAAACTTCACTTT- AGG
2	F	ACCTTGTAGGTTGTTACAGACACACCTAA
	R	TTGCCTGACCAGTACCACTAGTGTGTG
3	F	GGACAACCAATCACTAATTGTGTTAAGATGTTG
	R	TCACAACATACAGCCATAACCTTCCACA
4	F	CTTAAAAACACAGTCTGTACCGTCTGC
	R	GTAAGAACATTAAATTGTCATCTCGTCCTTTC
5	F	TGCTAAATTCTAAAAACTAATTGTTGTCGCTT
	R	ATGTGTCACAATTACCTTCATCAAAATGCCT
6	F	CAATGGCAGACCTCGTCTATGC
	R	GAAATCATACCAGTTACCATTGAGATCTTGATTATC
7	F	CGAAATGCTGGTATTGTTGGTGTACTGAC
	R	GTCTGATCCAATATTAAAATAACGGTCAAAGAG
8	F	TGTTAAAATATGACTTCACGGAAGAGAGGTT
	R	AAGTCTAGAGCTATGTAAGTTACATCCTGA
9	F	CCACTTCAGAGAGCTAGGTGTTGTAC
	R	CAAAGAAGAAGTGTAAATTCAACAGAACTTCCT
10	F	TGACTTGCTGTCTAACGGTTCTTAA
	R	CATAATAAAGTCTAGCCTACCCATTATTAAATGG
11	F	CGTCAACAAACCTAGACAAATCAGCTGG

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Table 2: Sequences of the forward (F) and reverse (R) primers for amplifying the target site of each ASO group in the 2,924 nt segment of SARS-CoV-2 RNA. (Continued)

	R	TTCCAATTACTACAGTAGCTCCTCTAGTG
12	F	GACCAATAGACAGTTCATCAAAATTATTGAAATCAATA-
	G	
	R	ATACTTGAGCACACTCATTAGCTAATCTATAG
13	F	ACAACGTGTTGTAGCTTGTACACC
	R	TAAATTGCGGACATACTTATCGGCAATTTG

**Table 3: Sequences of the antisense oligonucleotides (ASOs) targeting the 1,799 nt segment of SARS-CoV-2 genomic RNA.** A plus sign (+) indicates that the following nucleotide is locked nucleic acid (LNA).

ASO	Sequence
Anti-LS1	GTAATTC+CTTAAAA+CTAAG
Anti-LS2a	TGAAA+AGCAA+CATTGTT
Anti-LS2b	TA+CCGGGTTTGACAG
Anti-LS3b	A+CCCTTAGACACAGCA
Anti-AS1	TGGGTTCGCG+GAGTTG
Anti-PS2-overlap	GT+TAAAATTA+CCG+GG

Table 4: PCR primer annealing temperatures for coronavirus gene fragments.

Coronavirus	Annealing Temperature (°C)
Bat Coronavirus 1A	55
Bat Coronavirus BM48-31	60
Common Moorhen Coronavirus	55
Human Coronavirus OC43	55
Infectious Bronchitis Virus	60
MERS Coronavirus	60
Murine Hepatitis Virus	60
SARS Coronavirus 1	60
SARS Coronavirus 2	55
Transmissible Gastroenteritis Virus	55

**Table 5: Sequences of the forward (F), forward with T7 promoter (F+T7), and reverse (R) primers for amplifying the 239 nt segment of each 1,799 nt segment of coronaviral RNAs.**

Coronavirus	Primer	Sequence
Bat Coronavirus 1A	F	GGACCCTATA CGGTTTGCT-TGAAAA
	F+T7	TAATACGACTCACTATAGGAC-CCTATACGGTTTGCTTGAA-AA
	R	TTTTACAATAAAGAAAGCATICATGCTT
Bat Coronavirus BM48-31	F	GGGTTTATTCTTAGAACAC-AGTCTG
	F+T7	TAATACGACTCACTATAGGTTTTATTCTTAGAACACAGTC-TG
	R	GGAGTCTAATAAGTTGCCCTCTTCATC
Common Moorhen Coronavirus	F	GGATAAAGATAAGGAACCTG-TTTCTTT
	F+T7	TAATACGACTCACTATAGGATAAAGATAAGGAACCTGTTCTT
	R	ACTATTAGGTATTGGCAAATT-AATGCG
Human Coronavirus OC43	F	GGCTGTGTCTTATGTTTGAC-ACATGA

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**Table 5: Sequences of the forward (F), forward with T7 promoter (F+T7), and reverse (R) primers for amplifying the 239 nt segment of each 1,799 nt segment of coronaviral RNAs. (Continued)**

	F+T7	TAATACGACTCACTATAAGGCT-
		GTGTCTTATGTTTGACACAT-
		GA
	R	ATCTAATTATCACCGTTCTC-
		ATCAAC
Infectious Bronchitis Virus	F	GGTTTGCAGTGTGTTGCCAGTG-
		TTGGAT
	F+T7	TAATACGACTCACTATAAGGTT-
		TGCACTGTTGCCAGTGTGG-
		AT
	R	CTCAAGATTCCATCTTCAGT-
		ATCGCG
MERS Coronavirus	F	GGGATTGGTTGTCAAATAC-
		CCCCTG
	F+T7	TAATACGACTCACTATAAGGGA-
		TTTGTTGTCAAATACCCCT-
		G
	R	ATGATGCCCTGGTCATCTAA-
		TTCTAC
Murine Hepatitis Virus	F	GGCTGTGTCATATGTGTTGAC-
		GCATGA
	F+T7	TAATACGACTCACTATAAGGCT-
		GTGTCATATGTGTTGACGCAT-
		GA

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**Table 5: Sequences of the forward (F), forward with T7 promoter (F+T7), and reverse (R) primers for amplifying the 239 nt segment of each 1,799 nt segment of coronaviral RNAs. (Continued)**

	R	ATCCAAC TTGTTGCCGTCCCTC- ATCTAC
SARS Coronavirus 1	F	GGGTTTTACACTTAGAAACAC- AGTCTG
	F+T7	TAATACGACTCACTATAAGGT- TTTACACTTAGAAACACAGTC- TG
	R	AGAGTCTAATAAATTGCCTTC- CTCATC
SARS Coronavirus 2	F	GGGTTTTACACTAAAAACAC- AGTCTG
	F+T7	TAATACGACTCACTATAAGGT- TTTACACTAAAAACACAGTC- TG
	R	AGAACATTAAATTGTCATC- TTCGTC
Transmissible Gastroenteritis Virus	F	GGCAATT CGGTTCTGTATTGA- AAATGA
	F+T7	TAATACGACTCACTATAAGGCA- ATT CGGTTCTGTATTGAAAAT- GA
	R	TTTGACAATGTAGTAGGCATC- ATGTTT

Table 6: Sequences of the antisense oligonucleotides (ASOs) targeting the 1,799 nt segments of coronaviral RNAs.

Coronavirus	ASO	Sequence
Bat Coronavirus 1A	1	CAGGGCTCTAGTCGAGCTGCAC-TAGAGCCCCTGCTCGTTAAA-TAACGCCTGATCAACAG
	2	GCAACTTCTTATTGTAAATATC-AAAGGCGCGTACAACATGCTCC-GGTTCAGTACCATTA
Bat Coronavirus BM48-31	1	GACATCAGTGCTTGTGCCTGTG-CCGCACGGTGTAAAGACGGGCC-GCACTTACACCGCAAAC
	2	TTTAGGAACTTGCAAAACCA-GCAACTTCTCATTATAAAATATC-AAAAGCCCTGTAAAC
	3	AAAATAGGAGTCTAATAAGTTG-CCCTCTTCATCAACTCCTGGAA-ACGGCAACAATTGT
Common Moorhen Coronavirus	1	TGGGGTTCTAGACGGGCATCAC-TAGAACCCCTTACTCGTTAAAT-AAGCTGTATTTGCA
	2	GTTATATTATTATGTACATGAAA-CGCCCTTTACAATATCCGGCT-GAGTGCCAGACTGT
Infectious Bronchitis Virus	1	ACATCAAAGGCTCGCTTACAA-CATCAGGATCACATCCACTAGC-AAGGGTATCAGCCGA

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**Table 6: Sequences of the antisense oligonucleotides (ASOs) targeting the 1,799 nt segments of coronaviral RNAs. (Continued)**

Murine Hepatitis Virus	1 AAGCCACTGGCACAGGGTACAA- GACGGGCATTTACACTTGTACC- CCGAATCCGTTAAAA  2 CCAATGCCAGCTCGATTAGCAT- TACAAATGTCAAATGCCCTTAA- TTGAACATCAGTGTCC  3 AACTTGTGCCGTCTCATCTAC- ACGCTGGAAGCGGCAGCAATTCA- ACTTTATAATACAAA
SARS Coronavirus 1	1 TTTGAAAACCAGCAACTTTTC- GTTGTAAATATCAAAAGCCCTG- TAGACGACATCAGTA  2 TCTAATAAATTGCCCTCCTCATC- CTTCTCCTGGAAGCGACAGCAA- TTAGTTTTAGGAAC
SARS Coronavirus 2	1 GACATCAGTACTAGTGCCTGTG- CCGCACGGTGTAAGACGGGCT- GCACTTACACCGCAAAC  2 TTTTAGGAATTAGCAAAACCA- GCTACTTTATCATTGTAGATGTC- AAAAGCCCTGTATAC
Transmissible Gastroenteritis Virus	1 TAAATAACTTGATCAACAGTA- AAACTCTGCATAGAAGTACGAT- CGCACATGCAACCATT

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**Table 6: Sequences of the antisense oligonucleotides (ASOs) targeting the 1,799 nt segments of coronaviral RNAs. (Continued)**

2 GGTCTGGATCAGTACCATTGCA-  
GGGTTCTAGTCGAGCTGCACTA-  
GAACCCCGCACTCGTT

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