

# MERGE-RNA: a physics-based model to predict RNA secondary structure ensembles with chemical probing

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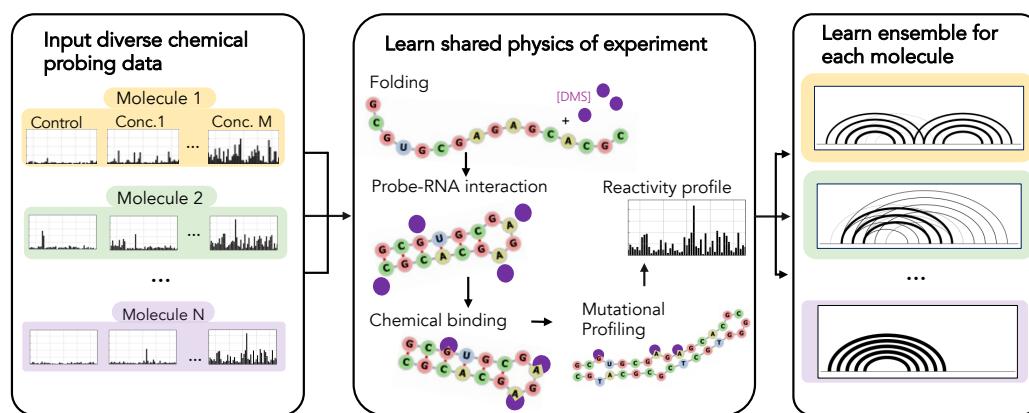
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## Abstract

The function of RNA molecules is deeply related to their secondary structure, which determines which nucleobases are accessible for pairing. Most RNA molecules however function through dynamic and heterogeneous structural ensembles. Chemical probing methods (e.g., DMS probing) rely on selective chemical modification of accessible RNA nucleotides to infer base-pairing status, yet the resulting nucleotide-resolution data represent ensemble averages over dynamic RNA conformations. We present MERGE-RNA, a unified, physics-based framework that explicitly models the full experimental pipeline, from the thermodynamics of probe binding to the mutational profiling readout. By integrating measurements across probe concentrations and replicates, our model learns a small set of transferable and interpretable parameters together with minimal sequence-specific soft constraints. This enables the prediction of secondary structure ensembles that best explain the data and the detection of suboptimal structures involved in dynamic processes. We validate MERGE-RNA on diverse RNAs, showing that it achieves strong structural accuracy while preserving essential conformational heterogeneity. In a designed RNA for which we report new DMS data, MERGE-RNA detects transient intermediate states associated with strand displacement, dynamics that remain invisible to traditional methods.

**Key words:** RNA secondary structure, Chemical probing, Ensemble prediction, DMS-MaP

## Graphical Abstract



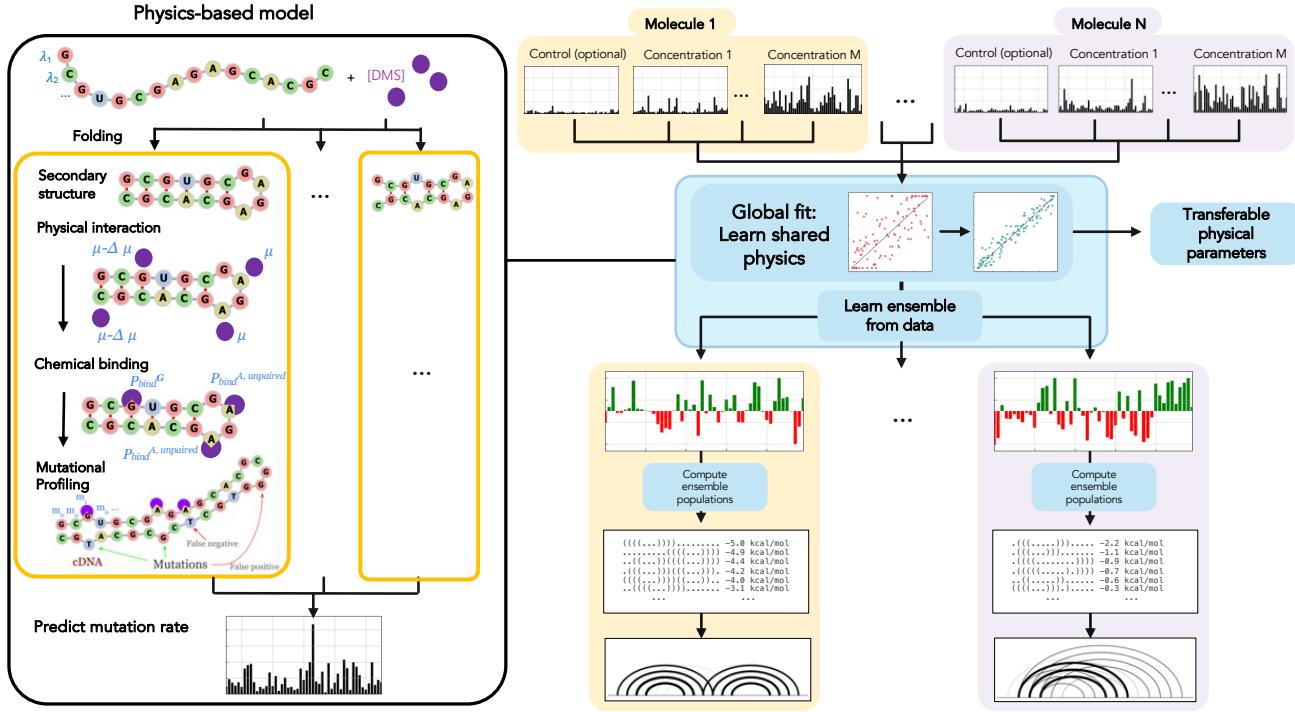


Fig. 1: Schematic overview of the method. Our approach builds a physical model—illustrated on the left side of the figure—that represents the entire pipeline of chemical probing experiments. This includes RNA folding into an ensemble of secondary structures, probe binding, adduct formation, and the final mutational profiling readout. The model is trained on chemical probing data from multiple RNA sequences, probe concentrations, and experimental replicates. From these data, it learns a set of physically meaningful parameters shared across all experiments ( $\mu$ ,  $\Delta\mu_{\text{pairing}}$ ,  $p_{\text{bind}}(A)$ ,  $p_{\text{bind}}(C)$ ,  $p_{\text{bind}}(G)$ ,  $p_{\text{bind}}(U)$ ,  $m_0$  and  $m_1$ , as defined in the main text). In addition, it estimates sequence-specific soft constraints  $\lambda_i$  (one per nucleotide position). Once trained, the model can predict the structural ensemble of each RNA at any probe concentration. Importantly, it can also extrapolate to zero probe concentration, providing the unperturbed structural ensemble.

## Introduction

RNA molecules play central roles in cellular processes, ranging from catalysis (ribozymes and ribosomes) [1, 2] to gene regulation (including riboswitches and microRNAs) [3]. This functional versatility arises from their ability to fold into specific secondary and tertiary structures [4]. Importantly, RNA molecules exist as thermodynamic ensembles at both the secondary [5] and tertiary [6] levels, with function arising from stable conformations, minority states, or dynamic equilibria. While tertiary structure modeling is computationally demanding [7], secondary structure thermodynamic models allow efficient computation of optimal structures [8] and base-pairing probabilities [9]. However, thermodynamic models face significant limitations from fundamental approximations: the nearest-neighbor approximation [10], the neglection of pseudoknots [8], and the hierarchical folding assumption [11] may make it difficult

to fully capture long-range interactions and context-dependent effects. To address these limitations, researchers have integrated co-evolutionary analysis or chemical probing data. Dimethyl sulfate (DMS) [12, 13] has become a powerful tool for structural analysis, providing single-nucleotide resolution data both *in vitro* and *in vivo*. DMS reacts preferentially with unpaired adenines and cytosines, with modifications detected as mutations during sequencing (mutational profiling, MaP) [13]. However, interpreting this data is challenging: measured reactivities report on structural ensembles rather than single conformations, and experimental processes introduce systematic biases from reverse transcription and sequencing artifacts. Current approaches typically convert measured reactivities into pseudo-free-energy restraints [14], but this can distort predicted ensembles when baseline models already agree with data [15], and the coefficients used to compute pseudo-free-energies are based on heuristics rather than physical principles. Despite widespread practice, focusing on a single

minimum free-energy (MFE) structure can be misleading as RNA molecules are known to adopt a very heterogeneous ensemble of conformations, of which the MFE structure is often only a minor component. In fact, ignoring structural variability and inter-nucleotide correlations (e.g., contact maps do not fully describe the ensemble) can pose problems both for interpreting RNA function and for properly modeling chemical probing experiments.

In this work we present MERGE-RNA (Multi-system Ensemble Refinement via Generalizable parameters Estimation), a unified physics-based model describes chemical probing experiments with physically meaningful parameters and predicts RNA secondary structure ensembles. Our approach combines a standard thermodynamic folding model with explicit probe-RNA interaction characterized by two key parameters: a chemical potential ( $\mu_r$ ) setting the probe concentration scale, and an energetic penalty ( $\Delta\mu_{\text{pairing}}$ ) for probe binding to paired nucleotides. We employ maximum entropy inference to determine sequence-specific soft constraints ( $\lambda_i$ ) representing minimal adjustments for model-data consistency, avoiding artificial constraints when baseline predictions already match data. The model explicitly accounts for mutational profiling artifacts, including false positives, false negatives, and position-specific biases. Crucially, our framework enables the simultaneous analysis of data from multiple RNA sequences, probe concentrations, and experimental replicates within a unified model, leveraging the shared physical parameters to improve robustness and extract transferable experimental knowledge.

In the next sections we outline the model and inference procedure, then validate transferability and accuracy on a group of well-characterized structured RNAs. We then apply the framework to the *cspA* 5' UTR (a known case of temperature-dependent rearrangement). Furthermore, we demonstrate accurate deconvolution of mixed states in synthetic bistable constructs, and finally leverage experimental data to reveal intermediate, suboptimal populations involved in dynamic processes (e.g., strand displacement) that are missed by traditional approaches.

## Methods

Our approach integrates experimental data from multiple RNA sequences, probe concentrations, and experimental replicates, and outputs the ensemble of secondary structures that are most compatible with the experimental data, as well as the physically meaningful parameters that describe the systems fitted.

### Physical Model

Our model follows the experimental pipeline of chemical probing experiments, as illustrated in Figure 1. The underlying physics remains consistent across experiments using the same reagent, probing time, and temperature, allowing us to simultaneously fit multiple experimental datasets within a unified framework. By maximizing the likelihood of the model to reproduce the observed mutation rates, our approach estimates physically meaningful parameters (detailed below) and identifies the ensemble of secondary structures most consistent with both the underlying physics and experimental evidence.

In this section we aim at introducing the physical model underlying our approach, while in the appendix A.1 we provide a more in depth description and rigorous discussion.

## RNA folding and chemical probing

### Folding

To model the RNA secondary structure ensemble, we utilize as baseline the ViennaRNA package [16]. The probability to observe a given secondary structure  $s$  at temperature  $T$  is defined as

$$\mathbb{P}(s) = \frac{\exp\left(-\frac{F(s)}{k_B T}\right)}{z} \quad (1)$$

where  $z$  is the partition function,  $k_B$  the Boltzmann constant and the baseline  $F(s) = F_0(s)$  is obtained as a sum of thermodynamic parameters. To improve predictions, we refine the ensemble to align with experimental data while minimally perturbing the baseline model. Using the principle of maximum entropy [17], we optimize sequence-specific parameters ( $\lambda_i$ ) that act as soft constraints for each nucleotide position [15]. These soft constraints modify the baseline ViennaRNA-predicted energy of pairing and remain consistent across experimental replicates and probe concentrations for a given RNA sequence. This results in a structure-dependent energy correction

$$\Delta F_{\text{opt}}(s) = \sum_{i \in \text{paired}(s)} \lambda_i \quad (2)$$

where  $\text{paired}(s)$  denotes the list of paired nucleobases in structure  $s$ .

### Probe-RNA interaction

We separately model the probe reactivity in two steps [18]: a reversible physical binding, described here, followed by an irreversible chemical reaction, as detailed in the next paragraph. At non-zero probe concentrations, we describe physical probe-RNA interactions through a thermodynamic binding model with two trainable parameters ( $\mu_r$  and  $\Delta\mu_{\text{pairing}}$ ). The reference chemical potential  $\mu_r$  captures the probability of an unpaired nucleotide to bind to a chemical probe at a reference DMS concentration  $[\text{DMS}]_r = 1\text{M}$ . The chemical potential is then corrected based on the actual probe concentration  $[\text{DMS}]$  as  $\mu = \mu_r + k_B T \ln\left(\frac{[\text{DMS}]}{[\text{DMS}]_r}\right)$ .  $\Delta\mu_{\text{pairing}}$  differentiates between paired and unpaired sites, so that the effective chemical potential for paired nucleotides becomes  $\mu' \equiv \mu - \Delta\mu_{\text{pairing}}$ . Since chemical probes typically exhibit reduced accessibility to base-paired regions,  $\Delta\mu_{\text{pairing}}$  is expected to be positive, though the model could be straightforwardly generalized to cases where this is not true. Incorporating a penalty term for base-paired sites results in a modified partition function. This modification is implemented by applying a concentration-dependent energetic contribution to paired bases  $\Delta F_{[\text{DMS}]}(s)$  (derivation is detailed in the appendix, Eq. 14).

Putting together all these terms, the corrected free energy of a given secondary structure  $s$  is

$$F(s) = \underbrace{F_0(s)}_{\text{baseline}} + \underbrace{\sum_{i \in \text{paired}(s)} \lambda_i}_{\text{site-specific corrections}} + \underbrace{\Delta F_{[\text{DMS}]}(s)}_{\text{concentration dependent perturbation}} \quad (3)$$

from which we can compute the population of observing each structure  $s$  via Equation 1.

### Chemical binding

Following the physical interaction between a chemical probe and RNA, adducts are formed with probabilities that depend

on both nucleobase identity and local RNA structure, under fixed experimental conditions (e.g., temperature and probing time). In the most general framework, this yields eight possible configurations, defined by the combination of nucleotide type (A, U, C, or G) and structural state (paired or unpaired). For DMS probing, due to the position of the potentially reactive nitrogens, adenine and cytosine show negligible reactivity when base-paired, while guanine and uracil retain reduced but structure-independent reactivity. Accordingly, the model is parameterized by four effective binding probabilities, corresponding to the reactive nucleotide-structure combinations:  $p_{\text{bind}}(\text{A, unpaired})$ ,  $p_{\text{bind}}(\text{C, unpaired})$ ,  $p_{\text{bind}}(\text{G})$ , and  $p_{\text{bind}}(\text{U})$ .

## Mutational Profiling and prediction of reactivity profiles

### Mutational profiling

Following chemical probing, modifications are typically detected through reverse transcription. In the following discussion, we focus on mutational profiling (MaP), though only minor modifications to the model would be required for compatibility with other experimental techniques, such as RT-stop. During reverse transcription, we account for the probability of producing false positive or false negative signals through the parameters  $m_0$  and  $m_1$ , as detailed in subsubsection A.1.1. Applying this framework to a given secondary structure  $s$ , we can compute the expected reactivity profile for that structure, then sum over all secondary structures in the ensemble to obtain the expected reactivity profile for the experiment.

### Handling systematic errors

We acknowledge that many sources of systematic error in the experimental pipeline cannot be fully captured by the model. For example, sequencing errors are known to introduce systematic biases in reactivity profiles [19], and reverse transcription can be affected by the presence of secondary structures [20]. To account for such effects, we make use of experimental controls (performed without reagent) to estimate position-dependent correction factors  $\epsilon_i$ , which effectively act as a baseline adjustment to the predicted mutation rates at each nucleotide position and are computed so that the prediction at zero concentration of DMS matches the control data. These factors capture systematic biases that are sequence-specific but independent of probe concentration. This allows the model to focus on the concentration-dependent signal in reactivity, which is most informative about RNA structure and probe interactions, while  $\epsilon_i$  absorbs background effects that remain constant across conditions. In other words, the sequence-dependent  $\epsilon_i$  parameters model the mutation rate in absence of reagents, whereas the sequence-dependent soft constraints  $\lambda_i$  affect the pairing population which, in turns, control how much the mutation rate increases when the reagent is added. As discussed in subsubsection A.1.1, when control experiments are available the parameter  $m_0$  is omitted, since background correction factors can be directly estimated from the control data. If controls are unavailable,  $m_0$  is instead fitted to provide a single background correction factor shared across the sequence.

## Parameter Optimization

Upon computation of the expected reactivity profile for a given sequence and probe concentration, we optimize the model parameters by minimizing a loss function that quantifies the discrepancy between predicted and observed reactivities. We

model per-position mutation counts as binomial: given read depth  $n_i$  and model-predicted mutation probability  $M_i$ , each read is an independent Bernoulli trial. Accordingly,

$$\mathbb{P}(\mathcal{M}_i^{\text{exp}} | n_i, M_i) = \text{Binomial}(\mathcal{M}_i^{\text{exp}}; n_i, M_i) \quad (4)$$

where  $\mathcal{M}_i^{\text{exp}}$  is the experimentally observed number of mutations at position  $i$ . As discussed in the appendix, the overall likelihood of the model across all nucleotide positions is the product of the individual position likelihoods. To facilitate optimization, we minimize the negative log-likelihood, yielding the following loss function:

$$\mathcal{L} = - \sum_i \ln \mathbb{P}(\mathcal{M}_i^{\text{exp}} | n_i, M_i). \quad (5)$$

The derivation and all definitions are expanded in Appendix A.

This optimization is implemented via a gradient-based algorithm that systematically adjusts model parameters to minimize the discrepancy between predicted and experimental reactivity profiles [21] as implemented in SciPy [22]. The parameter optimization is performed in two consecutive phases, as illustrated in Figure 1. In the first phase, we optimize the global physical parameters that are shared across all sequences and concentrations. These include: the reference chemical potential ( $\mu_r$ ), the binding penalty for structured regions ( $\Delta\mu_{\text{pairing}}$ ), the nucleotide-specific adduct formation probabilities ( $p_{\text{bind}}(\text{A, unpaired})$ ,  $p_{\text{bind}}(\text{C, unpaired})$ ,  $p_{\text{bind}}(\text{G})$ , and  $p_{\text{bind}}(\text{U})$ ), the false negative rate parameter ( $m_1$ ), and—when experimental controls are unavailable—the background correction factor ( $m_0$ ). This phase optimizes a total of 7 parameters (8 if no controls are available). In the second phase, we look to refine the secondary structure ensemble to find the one that best explains the experimental data. This is achieved by optimizing the sequence-specific soft constraints ( $\lambda_i$ ) for each nucleotide position, while keeping the global physical parameters fixed.

Following parameter optimization,  $\Delta F_{[\text{DMS}]}(s)$  is set to zero, extrapolating to zero probe concentration and thereby revealing the ensemble of secondary structures most consistent with the experimental data under native conditions.

## Experimental procedures

DMS probing of a synthetic RNA construct was performed as described in the appendix. DMS-treated RNA was column-purified and subjected to reverse transcription using MarathonRT [23, 24, 25, 26]. Sequencing reads were processed with an RNAFramework-based workflow [27, 28, 29].

## Results

### Transferability and accuracy of the physical model

In order to test our model's ability to generalize across different RNA systems, we evaluated our unified physics-based framework on a set of well-characterized RNA structures previously studied using DMS chemical probing [25]. This set spans a range of structural complexity, comprising five distinct systems: (i) bacterial RNase P (type A), (ii) hc16 ligase, (iii) *Tetrahymena* ribozyme, (iv) *V. cholerae* glycine riboswitch, and (v) HCV IRES. These sequences range from approximately 100 to 400 nucleotides in length. Reference structures for hc16 ligase, *Tetrahymena* ribozyme, and *V. cholerae* glycine riboswitch were obtained

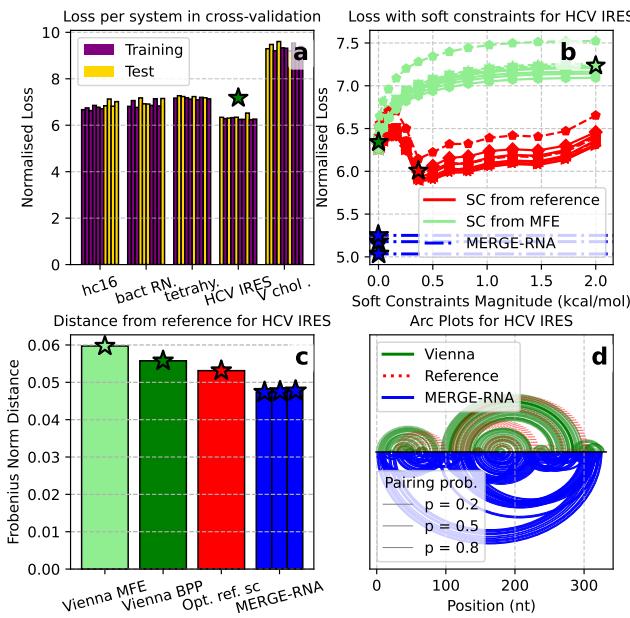


Fig. 2: (a) Cross-validation of physical parameters across five RNA systems. Each bar represents the per-datapoint loss obtained for the indicated system after training on three systems and testing on the remaining two. Yellow bars refer to results obtained when the corresponding system was excluded from the training set. (b) Normalized loss profiles for HCV IRES with soft constraints of varying magnitude applied. Red line: constraints from reference structure; light green line: constraints from minimum free energy (MFE) structure. Each curve represents an independent fit with physical parameters trained on different system triplets. The blue line corresponds to an improved fit achieved by optimizing the sequence-specific  $\lambda_i$  parameters with MERGE-RNA. Stars mark are reused across panels to indicate the same ensemble. (c) Frobenius distance between base pairing probability (bpp) matrices, quantifying structural similarity between the HCV IRES reference structure and: MFE structure, bpp of the original thermodynamic ensemble, bpp obtained from the minimum of the red curve in panel (b), and bpp from the fully optimized model. (d) Arc plot visualization comparing the secondary structures of HCV IRES: reference experimental structure (red), ensemble obtained from the thermodynamics model (green), and ensemble obtained from MERGE-RNA (blue).

from cryo-EM-guided structure determination [30], and secondary structure was annotated from the 3D structure with Barnaba [31]. HCV IRES reference structure was manually transcribed from Extended Data Fig. 6 of Ref. [25]. For bacterial RNase P (type A), although a crystal structure exists [32], sequence differences between the crystallized construct and the chemically probed sequence preclude its use as a reference. These reference structures are expected to be more accurate with respect to the predictions done using a simple thermodynamic model. However, since they represent single structures, they do not report on conformational heterogeneity.

Our first objective was to assess whether the physical parameters extracted from our model represent genuine physical properties of the experiment and are hence transferable to

different systems, rather than being overfitted on a specific case. We performed comprehensive cross-validation fits, training the physical parameters of the model (all the parameters except the  $\lambda_i$ ) on triplets of RNA systems and testing on the remaining pairs (Fig. 2a). Loss values (as defined in Equation 31) are then divided by the number of data points to enable direct comparison. The loss values obtained across all sets are consistent both in training and in test, demonstrating that our model generalises on multiple systems and successfully captures the underlying principles governing chemical probing experiments. Notably, physical parameters obtained through different minimizations might be different (see Table 1) but typically predict similar mutation profiles. One intuitive example of this is that by increasing the reference chemical potential ( $\mu_r$ ), the predicted mutation rate will—on average—increase, but this can be compensated by a decrease of binding probability  $p_{\text{bind}}$ . This under-determination of parameters can arise when the experiments do not cover a wide enough range of probe concentrations, but it does not affect the model’s ability to predict reactivity profiles within the range of conditions upon which it was trained.

### Importance of conformational heterogeneity in structured RNAs

Having established the generalizability of the physical parameters, we next used our framework to explore the balance between structural accuracy and ensemble heterogeneity. Using HCV IRES as a representative example (analogous results for other systems in Supplementary Figs. 6,7,8), we varied a uniform soft constraint (Fig. 2b): for a given scalar magnitude (x-axis), every nucleotide annotated as paired in the chosen reference structure (experimental or minimum-free-energy) was assigned that value and all other positions were set to zero, with no position-specific tuning. Increasing this magnitude progressively biases the ensemble toward the targeted structure, smoothly interpolating between the original thermodynamic ensemble and a single constrained structure while keeping the physical parameters fixed.

By interpolating between the original ensemble and its single most-likely structure (minimum-free-energy), we assess the role of conformational heterogeneity (light green line in Fig. 2b). In fact, favoring a single structure has the effect of progressively suppressing the population of alternative conformations that are likely present in solution and contribute to the observed mutation profiles. Interestingly, the loss function monotonically increases when the constraint is applied, degrading the agreement with experiment. On the other hand, incorporating knowledge from the reference experimental structure (red line in Fig. 2b) initially improves the model’s agreement with experimental data. However, even in this case, excessive constraint enforcement eventually degrades performance. This pattern indicates the existence of a “sweet spot” where the model partially incorporates reference structural information while preserving the conformational diversity necessary to explain the experimental measurements. Too strong a constraint toward any single structure—even the experimental reference—reduces the model’s ability to account for the heterogeneous structural ensemble that is captured in the experimental data. Overall, this analysis shows that a proper balance of information from the reference single experimental structure and ensemble diversity improves the capability of the model to reproduce chemical probing experiments.

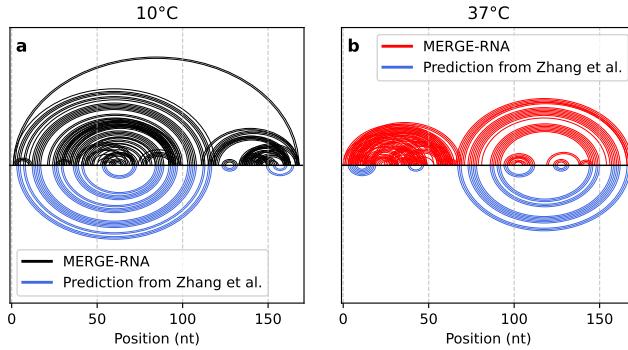


Fig. 3: Ensemble predictions for *cspA* 5' UTR at two different temperatures. (a,b) Arc plots corresponding to the DMS data collected at 10 and 37°C, respectively. Predictions from MERGE-RNA (red and black) are compared with predictions from Ref. [33] (blue).

#### Inference of optimal ensemble

Building on these insights, instead of borrowing from the reference structure, we inferred the best sequence-specific soft constraints ( $\lambda_i$  parameters) using our model. This resulted in substantially improved agreement with experimental data (blue line in Fig. 2b), as expected given that the experimental data is used to infer the soft constraints.

To validate our model independently, we computed the Frobenius distance between the reference structure and base-pairing probability matrices obtained by the baseline ViennaRNA MFE, Vienna BPP, and those optimally guided by the reference structure (Fig. 2c). Our model achieves comparable accuracy for some systems and clear improvements for others (Figs. 6,7,8). Remarkably, our inference performs strongly even against ensembles obtained using uniform soft constraints that are reference-informed (red star on the red curve in Fig. 2b) yet our model does this without knowledge of the underlying experimental structure. This apparent paradox arises because our model fits an array of constraints individually—allowing for greater flexibility, whereas enforcing the reference structure modifies the magnitude of all constraints simultaneously. A visualization of these improvements is illustrated through arc plot representations in Fig. 2d, which demonstrate that our model-derived ensemble (blue) captures the conformational diversity while recapitulating the reference structure (red) more accurately than the standard thermodynamic ensemble (green). Similar improvements were observed across all tested RNA systems, confirming the broad applicability of our approach.

In summary, with our model (1) the inferred physical parameters are generalisable across systems (Fig. 2a); (2) there is an optimal balance between structural accuracy and ensemble heterogeneity, and inference of sequence-specific corrections further improves agreement with experimental data (Fig. 2b); and (3) the resulting structural ensembles more accurately capture native conformations compared to standard thermodynamic approaches (Fig. 2c,d).

#### Application on a known case of functional structural rearrangement: *cspA* 5' UTR

We want to test our model's ability to capture complex structural rearrangements in relevant biological systems, so we consider two DMS mutational profiles for *cspA* 5' UTR recorded at 10 and 37°C [33]. This sequence is known to act as a thermoswitch [34], with different structures at the two reported temperatures. The two experiments have been recorded at different probing times (2 hours vs 10 minutes). Our approach has been designed to analyze homogeneous experiments and does not model the dependence of the observed mutation rate on the probing time. We hence normalize the dataset at 10°C to the same average mutation rate observed at 37°C. In addition, to avoid confusing the effect of the mutational profiles with the temperature-dependence of the thermodynamic model, we use the thermodynamic model set on the same intermediate temperature for every ensemble (23.5°C). Finally, we note that these experimental datasets lack a control mutation profile obtained in absence of DMS, making the separation of the physical signal from the systematic bias more difficult for our model. Given the mentioned limitations, we decided to perform a set of 6 independent fits, starting from different random initializations of the physical parameters. We report in Fig. 3 the ensemble predicted by the model that achieved the lowest loss, and the other fits are reported in Figs. 9-13 as Supplementary Information.

Figure 3(a,b) reports the ensembles predicted by MERGE-RNA, which are similar to the individual structures predicted in a previous work [33]. Interestingly, the ensembles that we predict at 37°C (10°C) are very similar to those predicted in ref. [33] in positions 70–170 (1–120), but differ in other regions. We note that our model reports a thermodynamic ensemble, where multiple competitive helices might appear with the proper population, where Ref. [33] reports a single minimum-free-energy structure.

Overall, this analysis demonstrates that our model can capture complex structural rearrangements in biological systems.

#### Deconvolution of mixed structural states on synthetic data

To test the model's ability to predict the correct secondary-structure populations in heterogeneous ensembles, we generated synthetic datasets for an in-house designed bistable RNA carrying two mutually exclusive hairpins. We deliberately designed this construct so that we could also assay the very same sequence experimentally (discussed in the next section); accordingly, we appended primer binding sites at the 3' and 5' ends. Concretely, the final construct has the architecture



where PBS1 and PBS2 are distinct primer-binding sites, A1 and A2 are identical copies of the same sequence, and B is complementary to A1/A2. In this design, A1 and A2 compete to pair with B, producing two mutually exclusive hairpins (helix 1: A1:B; helix 2: A2:B; as schematically visualised in Fig. 5c, see "baseline prediction"). L1 and L2 are short linkers designed to act as tetraloops for the hairpins.

While the original design targeted roughly equal populations of the two helices, the thermodynamic model allows us to artificially bias the ensemble to favor either helix. We then simulated mutation profiles for the two cases—when either helix is always formed—and combined these profiles in known proportions to create synthetic datasets with varying helix populations, ranging

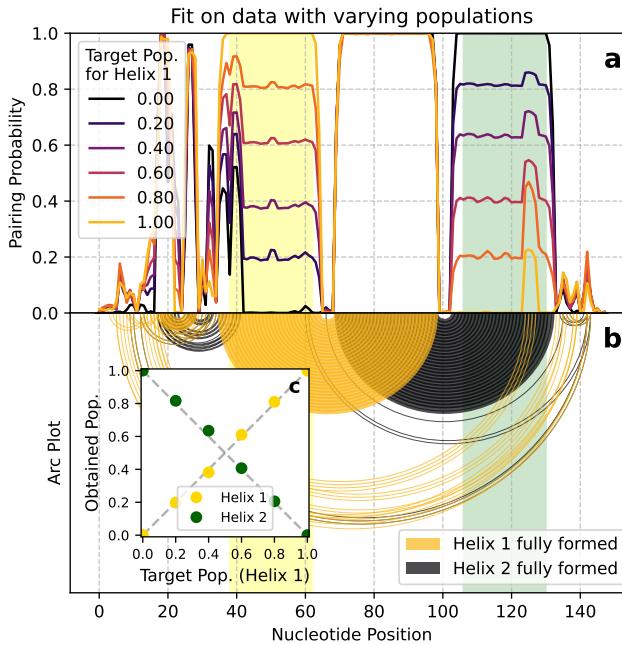


Fig. 4: Model accurately deconvolves mixed structural states from synthetic data. (a) Base-pairing probabilities inferred from fitting on synthetic data for a bistable RNA sequence. The data was generated from an RNA construct with two competing helices (helix 1 and helix 2), where the ground-truth population of helix 1 was systematically varied from 0 to 1 with steps of 0.2. The model’s predicted pairing probabilities are shown for each case. (b) Arc plots of the inferred structural ensemble for two limit cases: when helix 1 is dominant (gold) and when helix 2 is dominant (black). (c) The helix population inferred by the model is plotted against the ground-truth population. The inferred population is quantified as the median of the base-pairing probabilities over the respective helical regions, highlighted in panels (a) and (b).

from 0 to 1 in increments of 0.2. To generate realistic mutation profiles, we used the physical parameters inferred from our analysis on experimental data from biologically relevant RNAs (subsection ‘Transferability and accuracy of the physical model’ above).

We then trained our model on these synthetic datasets. Following the procedure established earlier, we first performed a joint fit of the physical parameters across all datasets, starting from random initializations. Subsequently, we optimized the soft constraints  $\lambda_i$  for each dataset individually. The results, presented in Fig. 4, show that the model accurately recovers the expected base-pairing probabilities for both helices across all synthetic datasets. The method is robust to the presence of additional base pairs formed by the random primers. This serves as a proof-of-concept for the model’s ability to deconvolve mixed structural states from chemical probing data across a wide range of populations (Fig. 4c).

#### A putatively bistable sequence exhibits a heterogeneous ensemble with strand displacement

To validate our model on experimental data, we collected DMS-MaP data on the putative bistable RNA construct described in

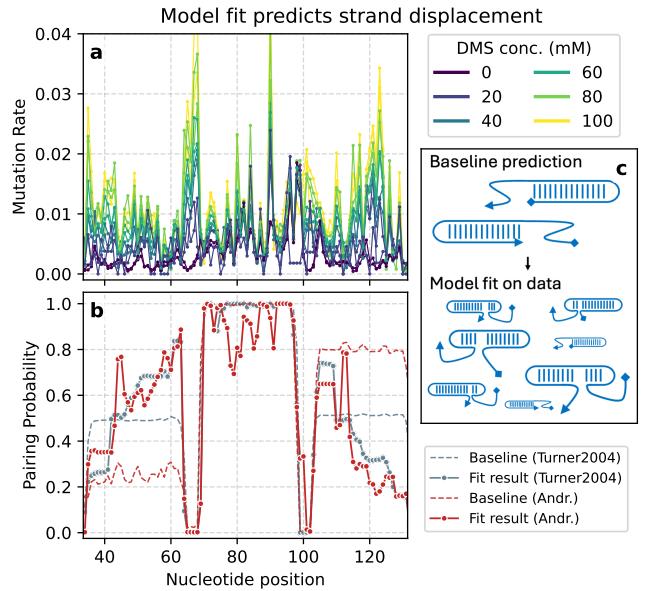


Fig. 5: Ensemble inference on experimental data for a putative bistable RNA exhibits evidence of strand displacement. (a) Per-nucleotide DMS-MaP mutation profiles across 0–100 mM (20 mM steps; two replicates each) for a putative bistable RNA. We leave out of the plot the pairing binding sites for better visualization. Two regions (A1 on the left and A2 on the right, as defined in (6)) are competing to pair with the central region (B). They show a slope in the reactivity profile that is compatible with a strand displacement mechanism. (b) Pairing probabilities obtained from the ensemble predicted by MERGE-RNA (solid lines) after fitting on experimental data, with Turner 2004 [35] (crimson) or Andronescu 2007 [36] (steel) thermodynamic models as baselines. MERGE-RNA is able to fix both the baseline predictions (dotted lines) and retrieve pairing probabilities consistent with the observed mutation rates. (c) Schematic depiction of the ensemble of structures as predicted by the baseline thermodynamic model (above) and MERGE-RNA fitted on the experimental data (below). While the thermodynamic model predicts only the two configurations with fully-formed helices, MERGE-RNA captures a wealth of intermediate states where strand displacement occurs.

the previous section. The data was collected at multiple DMS concentrations, 0 to 100 mM at steps of 20 mM, 2 replicas each. The obtained mutation profiles are visualised in Fig. 5a. We then trained our model on these experimental datasets, following the same procedure as in the previous section. Note that the sequence, as discussed earlier, was designed to adopt two mutually exclusive helices. We then applied our model to infer the structural ensembles from the data.

To challenge our model’s ability to recover the correct structural ensemble from an inaccurate thermodynamic baseline ( $F_0$  term in Equation 3), we performed the fitting procedure using two different baseline models. We first used the default Turner 2004 parameters [35], which were employed in the original sequence design. We then repeated the analysis with the Andronescu 2007 parameters [36], which predict a substantially different initial ensemble with populations of approximately 20% helix 1 and

80% helix 2, in stark contrast to the roughly 50-50 distribution predicted by the Turner 2004 parameters, as illustrated in Fig. 5b, dotted lines.

The results of our model fitting are presented in the same panel as solid lines. Remarkably, the two prediction of MERGE-RNA are consistent with each other, regardless of the initial thermodynamic parameter used, signaling robustness of the prediction with respect to the baseline parameters. In both cases, the model adjusts the ensemble of structure so that the resulting ensemble slightly favors helix 1, in accordance with the higher mutation rates observed in the experimental data (Fig. 5a).

Crucially, the model does more than just correct the populations of the two helices. It also predicts a gradient in base-pairing probabilities, where, within regions A1 and A2, nucleotides closer to the central domain (B) are more likely to be paired. This is a direct consequence of a corresponding slope in the observed reactivities (Fig. 5a). In fact, the model captures not only the two fully-formed helices but also intermediate states where the central domain is partially paired with both the competing strands, as schematically illustrated in Fig. 5c. This mechanism, known as strand displacement (or strand invasion) [37], has been observed in biological systems and is consistent with the observed mutation profile. In fact, regions nearest the central domain are expected to pair first during displacement, and, accordingly, the mutation profile shows reduced reactivity closer to the loops (Fig. 5a). The presence of intermediate states where a fraction of each of the two helices is formed is expected if the two loops are sufficiently stable. The loop co-occupancy analysis in Table 2 shows that baseline ensembles rarely populate states with both loops formed simultaneously (< 0.2%), whereas MERGE-RNA yields concurrent formation in 42–57% of structures.

This analysis underscores the importance of considering heterogeneous ensembles in RNA structure prediction, as they can capture dynamic processes such as strand displacement that are not accounted for in traditional Minimum Free Energy (MFE) approaches. Note that, since the potential strand displacement intermediates had a negligible population in the baseline thermodynamic model, this result would have very likely been missed by traditional approaches that enumerate and reweight only a limited set of suboptimal structures.

## Discussion

In this work, we have introduced MERGE-RNA, a physics-based framework for the analysis of chemical probing data that addresses fundamental limitations of conventional approaches. Our approach moves beyond the use of experimental data as simple pseudo-energetic restraints [14] and instead models the entire experimental pipeline, from the physical interaction of the probe with the RNA to the final mutational readout. This integrated approach allows for the determination of a set of transferable, physically meaningful parameters that describe the experimental pipeline, and ultimately enables the integration of multiple datasets for the inference of the unperturbed, native structural ensemble. As demonstrated by our successful deconvolution of mixed structural states (both on synthetic and experimental data), our method provides a powerful tool for quantitatively characterizing these populations. This capability is crucial for understanding the mechanisms of functional RNAs and for dissecting complex processes such as strand displacement and co-transcriptional folding, where the molecule transiently adopts a series of alternative conformations.

A central strength of our methodology is its ability to avoid over-constraining structural predictions. Standard approaches that convert reactivity data into pseudo-free energies tend to modify the energy landscape globally, often altering regions that are already correctly predicted. As previously noted, an ideal method should refrain from changing predictions that are already accurate and instead focus on introducing only the minimal adjustments needed to reconcile the model with the data, thereby highlighting where the baseline model is insufficient. Our framework achieves this by applying the maximum entropy principle to infer the minimal soft constraints ( $\lambda_i$ ) required to fit the data. This ensures that modifications arise only where the data provide strong evidence of a deviation from the initial model, refining the energy landscape locally while preserving the predictive power of the underlying thermodynamics. This manner of correcting the ensemble resembles the one introduced in Washietl et al [15] to analyze SHAPE data, although the derivation is different, and it improves in the capability of modeling the probe-RNA interactions directly.

The optimization of the structural ensemble is here done using the maximum entropy principle, which, among the ensembles with highest compatibility with experiment, choose the one that is as close as possible to the prior. The variability of the result was here limited by adding boundaries on the resulting soft constraints. Alternative approaches based on the use of entropy as a regularization term modulated by a chosen hyperparameter might be explored [38, 39].

Existing ensemble-deconvolution pipelines such as DREEM [40], DRACO [41], and DANCE-MaP [42] cluster sequencing reads with multiple co-occurring modifications and assign each cluster to a discrete structure. By capitalizing on per-read correlation patterns, these methods recover information about alternative conformations but at the cost of discarding the abundant reads that contain zero or single modifications and demanding deep coverage to resolve low-probability states. Our framework instead calibrates a physical model against the averaged mutational fractions, ensuring that every read (even negative ones) contributes signal even in sparse-modification regimes. The trade-off is the loss of explicit per-read co-variation, yet in principle the explicit reads can be integrated into our framework to fine-tune the populations of specific structures. We leave this as a possible future extension of the model. Moreover, read-level clustering can struggle to distinguish highly similar conformations and to represent continuous ensembles comprising many closely related intermediates (e.g., along strand-displacement pathways), which gives our approach an advantage. Consequently, our framework is well-suited not only to capture discrete alternative structures but also to quantify continuous ensembles that enable interpretation of dynamic RNA processes. Furthermore, alternative methods implicitly assume that each read originates from a single structure, which may not hold for RNAs undergoing rapid conformational dynamics and/or long probing timescales. Our framework instead models the ensemble-averaged behavior directly, naturally accommodating fast interconversions between states at equilibrium. Strikingly, even without using individual reads, the physics of the model enables (a) reconstructing correlations (e.g., the cooperative formation of two distant loops) and (b) measuring the population of minor substates (e.g., the strand-displaced intermediates).

A key feature of our calibrated framework is the ability to predict the structural ensemble at any probe concentration, and

most importantly, to extrapolate to zero probe concentration to obtain the native state. In the specific cases analyzed in this study, the dependence of the base-pairing probabilities on probe concentration was relatively modest, which introduces partial under-determination in the estimated parameters (e.g., increasing  $\mu$  can mimic decreasing  $p_{\text{bind}}$ ). This does not compromise robustness or transferability of the calibrated parameters (as shown above e.g. in Fig.2a) and can be mitigated by widening the concentration range. As a future direction, a concise Bayesian treatment (priors + posterior curvature) could quantify identifiability and provide credible intervals. As perspective, one may place weakly informative priors and consider  $\mathbb{P}(\theta \mid \text{data})$ ; Maximum a posteriori would remain a practical point estimate, while posterior would yield credible intervals and identifiability diagnostics. However, the model is fully equipped to handle systems where the probe induces significant non-linearities [18] rising from structural rearrangement, denaturation, or saturation. This behavior normally presents challenges in chemical probing experiments and suggests that one should work in the single-hit-kinetics regime [43], which is however difficult for large RNAs and effectively eliminates correlation information. The ability to explicitly model and remove these concentration-dependent perturbations is a significant step towards obtaining a more faithful representation of an RNA's native conformational landscape.

The explicit separation of intrinsic RNA thermodynamics (captured by the baseline model and the  $\lambda_i$  soft constraints) from the probe-specific interaction physics (captured by parameters such as  $\mu_r$  and  $\Delta\mu_{\text{pairing}}$ ) opens a natural avenue for future extensions. One could readily extend this framework to simultaneously analyze data from multiple, distinct chemical probes such as CMCT [44], SHAPE [45, 46, 47], hydroxyl radical probing [48], and ETS [49]. IPANEMAP [50] has shown that an improvement on performance is obtainable by integrating multi-probe measurements, but relies on empirical reactivity-to-energy conversions. In our framework, each probe would be described by its own set of physical parameters, but all datasets would be used to constrain and refine a single, shared set of  $\lambda_i$  parameters. As an example, SHAPE reagents could be modeled with the same formalism introduced here for DMS, but with different binding preferences (eight new  $p_{\text{bind}}$ , two for each nucleotide) and energetic penalties (new  $\mu_r$  and  $\Delta\mu_{\text{pairing}}$ ). This would yield a more robust and comprehensive determination of the structural ensemble, leveraging the orthogonal chemical information provided by different reagents and enabling the integration of multiple and more diverse datasets.

An additional extension concerns interactions with other biomolecules (e.g., RNA-protein binding) that can confound probing readouts. If reliable protein-RNA contacts are known on specific segments, those positions could be excluded from the loss to avoid bias from probe occlusion and simultaneously constrained to avoid internal pairing. Conversely, when interaction sites are unknown, the calibrated model could be used to localize discrepancies between predicted and observed mutational profiles, flagging sequence regions likely involved in protein or ligand interactions for follow-up experiments.

We acknowledge several important limitations of the current model. The underlying ViennaRNA framework does not account for the formation of pseudoknots, which are known to be critical for the function of many RNAs. Even in absence of pseudoknots, knowledge of the secondary structure alone might not be sufficient

to predict reactivities towards DMS probing, which might depend on 3D structural features [51]. Furthermore, while we have shown that the physical parameters are transferable across different RNA molecules under constant experimental conditions, their transferability between experiments conducted under varying conditions (e.g., different temperatures, buffers, or duration of exposition to the probe) is not guaranteed; therefore, we recommend fitting data from experiments conducted under similar conditions as a cohesive group. To achieve broader applicability, the model would need to be extended to explicitly describe the dependence of the physical parameters on these experimental variables.

## Data and code availability

The MERGE-RNA source code, together with analysis scripts, is available at <https://github.com/giusSacco/MERGE-RNA>.

## Competing interests

No competing interest is declared.

## Author contributions statement

Gi.S., Gu.S., and G.B. conceived and designed the study. Gi.S., Gu.S., and G.B. developed the theoretical modeling and statistical framework, interpreted the data and the results. Gi.S. implemented MERGE-RNA, led the primary data analysis, processed sequencing data, performed model benchmarking, and prepared the figures. G.B. performed exploratory analyses and contributed methodological input. J.L. and R.P.S. designed and performed the DMS-MaP experiments. G.B., Gu.S. and R.P.S. supervised the research. All authors wrote the manuscript, revised it critically, and approved the final version.

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## Supplementary information

### Physical Model

The physical model underlying our analysis of RNA chemical probing experiments is illustrated in the left panel of Figure 1 and will be detailed in this section. We derive and justify the expression for the free energy of a secondary structure  $s$  in the presence of a chemical probe (as reported in the main text Eq. 3) which enables the prediction of the ensemble of structures, and explain our methodology for producing a prediction of the reactivity profiles at varying probe concentrations, which serve as the theoretical basis for fitting experimental data.

### Secondary structure populations

The initial step in our analysis involves modeling the RNA folding into an ensemble of secondary structures, while also accounting for interactions with chemical probes. Our baseline is the prediction of the ViennaRNA package [16], specifically employing the `RNA.fold.compound.subopt` method to obtain an ensemble of suboptimal structures  $s$  with their associated free energies  $F_0(s)$ .

The maximum entropy formalism [17] provides a principled framework for integrating experimental data while making minimal adjustments to the baseline predictions from the classical thermodynamic model, ensuring that the resulting distribution of observables gets consistent with experimental measurements. Within this framework, the minimal perturbation to the baseline model that aligns with experimental data is achieved by introducing sequence-specific model parameters  $\lambda_i$  – one for each nucleotide position– that serve as Lagrange multipliers constraining the ensemble to match experimentally observed properties. These soft constraints can be effectively interpreted as positional corrections of pairing energy, allowing us to capture sequence-dependent effects that may not be fully accounted for in the standard thermodynamic model. This formalism allows to seamlessly integrate experimental data into the thermodynamic framework, and the corrections will be applied only where there are discrepancies between the model and the data, while leaving the areas where there is agreement unchanged. Importantly, the  $\lambda_i$  parameters are sequence-specific but remain consistent across all concentrations and experimental replicates for the same sequence.

The populations for each secondary structure  $s$  is determined by its free energy, which comprises three components: the baseline free energy  $E_0(s)$ , sequence-specific soft constraints  $\lambda_i$ , and the contribution from probe interactions  $\Delta F_{[\text{DMS}]}(s)$ :

$$F(s) = F_0(s) + \sum_{i \in \text{paired}(s)} \lambda_i + \Delta F_{[\text{DMS}]}(s) \quad (7)$$

The last term explicitly accounts for interactions between chemical probes and RNA structures, which modify the free energy in a concentration-dependent manner, whose explicit form is derived below. The concentration-dependent energy of the probe-RNA interaction is described by chemical potential  $\mu$ . Note that once a chemical potential for a reference probe concentration is known, the chemical potential for any other concentration can be derived from the ratio of the experimental concentrations as  $\mu = \mu_r + k_B T \ln \left( \frac{[\text{DMS}]}{[\text{DMS}]_r} \right)$ , where  $[\text{DMS}]$  is the concentration of the probe in the experiment and  $[\text{DMS}]_r$  and  $\mu_r$  are respectively the reference concentration and its chemical potential. Since for most chemical probes the interaction energy depends on local RNA structure (this is especially true for the most widely used

SHAPE and DMS), we include an additional parameter  $\Delta\mu_{\text{pairing}}$  that represents the energetic penalty for probe interactions with paired nucleotides, such that the effective binding energy for paired nucleotides becomes  $\mu' \equiv \mu - \Delta\mu_{\text{pairing}}$ . This formulation allows us to model the effect of varying probe concentrations with only two parameters ( $\mu_r$  and  $\Delta\mu_{\text{pairing}}$ ) and thus to combine data from multiple experiments into a single, coherent framework.

Importantly, in what follows we consider only the binding partition function, i.e., the contribution to the total partition function arising from independent probe-RNA binding degrees of freedom at fixed secondary structure  $s$ . Under the assumption of independent binding across sites, this factorizes as

$$Z_{\text{bind}}(s) = \prod_{i: s_i=0} z_u \prod_{i: s_i=1} z_p \quad (8)$$

$$= z_u^{N_u(s)} z_p^{N_p(s)} \quad (9)$$

with single-site partition functions  $z_u = 1 + e^{\beta\mu}$  for unpaired sites and  $z_p = 1 + e^{\beta\mu'}$  for paired sites, and where  $N_u(s)$  and  $N_p(s)$  denote the numbers of unpaired and paired nucleotides in structure  $s$ . The corresponding concentration-dependent contribution to the free energy (the last term in Equation 7) is then

$$\beta\Delta F_{[\text{DMS}]}(s) = -\ln(Z_{\text{bind}}(s)) \quad (10)$$

$$= -N_u(s) \ln z_u - N_p(s) \ln z_p \quad (11)$$

$$= -N_u(s) \ln(1 + e^{\beta\mu}) - N_p(s) \ln(1 + e^{\beta\mu'}) \quad (12)$$

$$= -(N - N_p(s)) \ln(1 + e^{\beta\mu}) - N_p(s) \ln(1 + e^{\beta\mu'}) \quad (13)$$

$$= -N \ln(1 + e^{\beta\mu}) - N_p(s) \ln \left( \frac{1 + e^{\beta\mu'}}{1 + e^{\beta\mu}} \right) \quad (14)$$

where  $N \equiv N_u(s) + N_p(s)$  is the sequence length. The first term is structure-independent and does not contribute to the populations in the experiment, so it can be safely ignored in our calculations. In practice, this formulation enables us to account for the probe influence on the secondary structure populations by adding  $-\ln \left( \frac{1 + e^{\beta\mu'}}{1 + e^{\beta\mu}} \right)$  as a soft constraint in ViennaRNA for each paired bases, allowing us to efficiently compute pairing probabilities within its computational framework. By substituting the expression for  $\Delta F_{[\text{DMS}]}(s)$  into Equation 7, we obtain the final expression for the free energy of a secondary structure

$$F(s) = \underbrace{F_0(s)}_{\text{baseline}} + \underbrace{\sum_{i \in \text{paired}(s)} \lambda_i}_{\text{site-specific corrections}} - \underbrace{k_B T N_p(s) \ln \left( \frac{1 + e^{\beta\mu'}}{1 + e^{\beta\mu}} \right)}_{\text{concentration dependent perturbation}} \quad (15)$$

### Physical Binding Probability

Once we have outlined how to compute the populations of secondary structures in the presence of a chemical probe, we can derive the expected reactivity profile through the modeling of the chemical probing experiment, beginning from the interaction between the probe and the RNA. The probability of physical binding between the probe and RNA follows a Boltzmann distribution:

$$\mathbb{P}(k_i = 1 | s_i = 0) = \frac{e^{\beta\mu}}{1 + e^{\beta\mu}} \quad (16)$$

$$\mathbb{P}(k_i = 1 | s_i = 1) = \frac{e^{\beta\mu'}}{1 + e^{\beta\mu'}} \quad (17)$$

where  $k_i \in \{0, 1\}$  denotes the physical binding state at position  $i$  (1 for probe-bound, 0 for unbound) and  $s_i \in \{0, 1\}$  represents the pairing state (1 for paired, 0 for unpaired).

We make two key assumptions in this model: first, that the probe physical interaction is independent of nucleotide identity, and second, that binding events occur independently at each site, with no interactions between probes. Note that the latter may not hold at high probe concentrations, where cooperative effects and non-linear increases in reactivity can occur, as investigated by [18] and [43].

### Probability of Chemical Modification

The probability of chemical modification at position  $i$  depends on both the physical binding event and the subsequent conversion from physical to chemical modification. We assume that when a probe physically interacts with nucleobase  $i$ , chemical modification occurs with a probability  $p_{\text{bind}}(n_i, s_i)$ , which depends only on the nucleotide identity  $n_i \in \{\text{A, U, G, C}\}$  and its pairing state  $s_i$ :

$$\mathbb{P}(c_i = 1 | s_i, n_i) = \mathbb{P}(c_i = 1 | k_i = 1, s_i, n_i) \mathbb{P}(k_i = 1 | s_i) \quad (18)$$

$$\equiv p_{\text{bind}}(n_i, s_i) \mathbb{P}(k_i = 1 | s_i) \quad (19)$$

where  $c_i \in \{0, 1\}$  represents the modification state (1 for chemically modified, 0 for unmodified). While this is the most general formulation and holds for widely used chemical probes such as SHAPE, we note that DMS can react only with unpaired A and C and in a structure independent manner with G and U. We can thus simplify the model and embed the physics of the probe in the model by setting

$$p_{\text{bind}}(\text{A}, 1) = 0$$

$$p_{\text{bind}}(\text{C}, 1) = 0$$

$$p_{\text{bind}}(\text{G}, 0) = p_{\text{bind}}(\text{G}, 1)$$

$$p_{\text{bind}}(\text{U}, 0) = p_{\text{bind}}(\text{U}, 1)$$

This leads to a total of four free parameters for the nucleotide-specific binding probabilities, one for each nucleotide type.

The overall probability of chemical modification at site  $i$ , denoted  $\mathbb{P}(c_i)$ , is obtained by averaging over the structural ensemble. Thanks to the assumption that binding and modification events are independent at each site, this average can be computed using the local pairing probabilities  $\mathbb{P}(s_i)$ , which are derived from the partition function without enumerating all possible secondary structures. The ensemble-averaged probability of chemical modification is thus given by:

$$\begin{aligned} \mathbb{P}(c_i) &= \langle \mathbb{P}(c_i | s, n_i) \rangle_s \\ &= \langle \mathbb{P}(c_i | s_i, n_i) \rangle_{s_i} \\ &= \sum_{s_i \in \{0, 1\}} \mathbb{P}(s_i) \mathbb{P}(c_i | s_i, n_i) \end{aligned} \quad (20)$$

### Expected Mutation Rate and Systematic Biases

In Mutation Profiling (MaP), chemical modifications induced by probes are detected as mutations in the cDNA during reverse transcription. To accurately model this process, we must establish the relationship between chemical modifications and the observed mutations while accounting for errors introduced during reverse transcription.

Although mutation probabilities could potentially depend on both sequence context and structural environment [20], we simplify our model by considering only the dependence on the chemical modification state  $c_i$ , treating other factors as systematic biases (which will be accounted for later in this section). We introduce two parameters  $m(c_i = 1)$  and  $m(c_i = 0)$  that govern respectively mutation probabilities for modified and unmodified nucleotides, according to the following definition:

$$\mathbb{P}(\text{mutation at position } i | c_i) \equiv 1 - e^{-(m(c_i) + \epsilon_i)} \quad (21)$$

In this formulation,  $m(c_i = 1)$  and  $m(c_i = 0)$  can be interpreted as sequence-independent probabilities governing respectively false negative and false positive mutations and will be fitted as model parameters. In the following and in the maintext we use the shorthand notation  $m_1 \equiv m(c_i = 1)$  and  $m_0 \equiv m(c_i = 0)$ . The position-specific  $\epsilon_i$  capture systematic biases common across all experimental replicates and probe concentrations for the same RNA sequence. These  $\epsilon_i$  values are calibrated to ensure that the model-predicted reactivity profile at zero probe concentration aligns with the control experiment, effectively serving as site-specific offsets common to all experimental conditions.

To compute the model prediction of the experimental reactivity profile, we average over all possible modification states, weighted by their probabilities:

$$M_i \equiv \langle M(c_i) \rangle_{c_i} \quad (22)$$

$$= \sum_{j \in \{0, 1\}} \mathbb{P}(c_i = j) M(c_i = j) \quad (23)$$

$$= \sum_{j \in \{0, 1\}} \mathbb{P}(c_i = j) (1 - e^{-(m(j) + \epsilon_i)}) \quad (24)$$

$$= 1 - e^{-\epsilon_i} \sum_{j \in \{0, 1\}} \mathbb{P}(c_i = j) e^{-m_j} \quad (25)$$

$$= 1 - e^{-\epsilon_i} [(1 - \mathbb{P}(c_i = 1)) e^{-m_0} + \mathbb{P}(c_i = 1) e^{-m_1}] \quad (26)$$

$$= 1 - e^{-\epsilon_i} [e^{-m_0} + \mathbb{P}(c_i = 1) (e^{-m_1} - e^{-m_0})] \quad (27)$$

This equation establishes a critical link between experimentally observed mutation rates and the underlying RNA structural ensemble through the chemical modification probabilities  $\mathbb{P}(c_i = 1)$ , which themselves depend on the pairing state probabilities  $\mathbb{P}(s_i)$  derived earlier.

Notably, the first term in the square brackets, containing  $m_0$ , produces a common background signal. When control experiments are available, this term can be incorporated into the systematic biases  $\epsilon_i$ ; otherwise, it must be fitted as a constant background term.

### Model Parameters Summary

Before moving on to how to obtain the optimal model parameters from experimental data, we summarize here all the parameters of our model that require fitting:

- **Physical parameters**, which describe the physics of the chemical probing experiment. These are shared across all RNA sequences and probe concentrations.
  - $\mu_r$ : quantifies the energy of probe-RNA interaction at a reference probe concentration, from which we derive chemical potentials for all other concentrations.

- $\Delta\mu_{\text{pairing}}$ : represents the energetic penalty for probe interaction with paired nucleobases.
- $p_{\text{bind}}(n_i, s_i)$ : is the probability of chemical modification given nucleotide identity  $n_i$  and pairing state  $s_i$ . These are four for DMS, one for each nucleotide type.
- $m_1$  and  $m_0$ : are parameters governing respectively false negative and false positive mutation probabilities during the cDNA transcription. If control experiments are not available,  $m_0$  gets absorbed into the systematic biases  $\epsilon_i$  and does not need to be fitted.
- **Soft constraints**, which are sequence-specific but consistent across all experimental conditions for a given RNA:
  - $\lambda_i$ : are site-specific soft constraints modifying the pairing energy of the baseline thermodynamic model.

Worth mentioning in this summary—although not fitted—are the  $\epsilon_i$ , which are calibrated from the control experiment and incorporate site-specific systematic errors shared across replicates and concentrations, and effectively serve as offsets to the predicted mutation profiles. They enable the decoupling of experimental biases from the thermodynamic and concentration-dependent effects, allowing the model to focus on physically relevant signal.

## Model Training

### Loss function

To fit the model parameters to experimental data, we define a loss function that quantifies the discrepancy between the expected reactivity profiles predicted by our model (as derived in the previous section) and the observed mutation rates from chemical probing experiments.

The binomial nature of the mutation counts, which arise from a series of independent Bernoulli trials (each read can either show a mutation or not), motivates the choice of a binomial likelihood for our loss function. Within this framework, the probability of observing  $\mathcal{M}_i$  mutations out of a coverage of  $n_i$  reads under predicted mutation rate  $M_i$  is given by:

$$\mathbb{P}(\mathcal{M}_i) = \text{Binomial}(\mathcal{M}_i, n_i, M_i) \quad (28)$$

$$= \binom{n_i}{\mathcal{M}_i} (1 - M_i)^{n_i - \mathcal{M}_i} \quad (29)$$

where  $i$  runs over all the data points, i.e. sequences, positions, concentrations and replicates. We thus define the loss function as the negative log-likelihood of observing the experimental mutation counts  $\mathcal{M}_i^{\text{exp}}$  given the model.

$$\mathcal{L} = \sum_i -\ln \mathbb{P}(\mathcal{M}_i^{\text{exp}} = \mathcal{M}_i) \quad (30)$$

$$= \sum_i -\ln \left[ \binom{n_i}{\mathcal{M}_i^{\text{exp}}} M_i^{\mathcal{M}_i^{\text{exp}}} (1 - M_i)^{n_i - \mathcal{M}_i^{\text{exp}}} \right] \quad (31)$$

### Fit of parameters

The model fitting process involves optimizing a set of parameters to best reproduce experimental data. We implemented the optimization procedure in a custom Python script utilizing the `scipy.optimize.minimize` function [22] with the L-BFGS-B algorithm [21]. This limited-memory quasi-Newton method is particularly well-suited for high-dimensional optimization problems, as it efficiently approximates the inverse Hessian

matrix while maintaining reasonable memory requirements. The algorithm's ability to handle bound constraints is especially valuable for ensuring physically meaningful parameter values throughout the optimization process. Specifically, we constrained the parameters to physically plausible ranges: the chemical potential at reference concentration,  $\mu_r$ , was bounded between -5 and 5 kcal/mol; the energetic penalty for pairing,  $\Delta\mu_{\text{pairing}}$ , was constrained to be non-negative, with an upper bound of 10 kcal/mol; the chemical modification probabilities,  $p_{\text{bind}}$ , were constrained to the range [0, 1], as is required for probabilities; the false positive mutation parameter,  $m_0$ , was kept small within [0, 0.1]; and the false negative parameter,  $m_1$ , was bounded within [0.1, 10].

The optimization is performed in a two-stage process to minimize the loss function. In the first stage, we fit the physical parameters that are common to all experiments, keeping the sequence-specific soft constraints ( $\lambda_i$ ) fixed. This global optimization starts from random initializations and runs until convergence. In the second stage, the optimized physical parameters are held constant while we fit the  $\lambda_i$  soft constraints independently for each RNA sequence. This hierarchical strategy allows us to first establish a robust physical model and then refine the secondary structure populations with sequence-specific adjustments. During the optimization, the  $\lambda_i$  parameters are constrained to the range [-1, 1] kcal/mol to ensure proper regularization and avoid overfitting.

## Experimental Procedures

### In vitro transcription (IVT)

A single-stranded full-length DNA oligonucleotide (5'-ATGGTCTGCTGGAGGTGGAATAGTTGTGAGTTGAAGTGGGGATTTTAATCCCCACTTCAACTCACAACATTCCAATTGGAAATAGTTGTGAGTTGAAGTGGGGATTGGCTGTGGCATAATG-3') was chemically synthesized (Integrated DNA Technologies). This full-length sequence was amplified by PCR using primers carrying a T7 promoter. The primers used were Full-T7-RV (5'-TAATACGACTCACTATAGGGCATTATGCCACAGCCAATCCCCCTTC-3') and Full-FW (5'-ATGGTCTGCTGGAGGTGGAATAGTTGTGAG-3').

PCR was performed in 50  $\mu\text{L}$  reactions containing the following final concentrations: GXL reaction buffer (1×, from a 5× stock; Takara Bio), each dNTP (0.2 mM), each primer (0.25  $\mu\text{M}$ ; Full-T7-RV and Full-FW), single-stranded full-length DNA template (1 nM), and PrimeSTAR GXL DNA Polymerase (0.025 U/ $\mu\text{L}$ ; Takara Bio). Thermal cycling conditions were as follows: initial denaturation at 98 °C for 2 min; 25 cycles of 98 °C for 20 s, 55 °C for 20 s, and 68 °C for 30 s; followed by a final extension at 68 °C for 5 min. PCR products were analyzed on a 2% agarose gel to verify the expected size, before purification using the NucleoSpin Gel and PCR Clean-up kit with NTC buffer (Macherey–Nagel), according to the manufacturer's instructions. The purified DNA was used as a template for T7 RNA polymerase-mediated in vitro transcription (IVT). IVT was performed in 50  $\mu\text{L}$  reactions containing the following final concentrations: T7 RNA Polymerase Reaction Buffer (1×, from a 10× stock; New England Biolabs), ATP, CTP, GTP and UTP (each 5 mM; Ribonucleotide Solution Mix, New England Biolabs), RNase Inhibitor, Murine (1 U/ $\mu\text{L}$ ; New England Biolabs), T7 RNA Polymerase (2.5 U/ $\mu\text{L}$ ; New England Biolabs), DNA template (25 nM), and inorganic pyrophosphatase (0.0005 U/ $\mu\text{L}$ ; Thermo Scientific), in nuclease-free water. Reactions were incubated at 37 °C for 2 h. Positive IVT

control reactions were set up identically, except that a previously validated DNA template was used. After IVT, residual DNA template was removed by DNase digestion. DNase digestion was carried out at 37 °C for 30 min in reactions containing the following final concentrations: DNase TURBO (0.1 U/µL) and DNase TURBO buffer (1×; Invitrogen). IVT products with and without DNase treatment were analyzed on a 2% agarose gel to assess the efficiency of DNA removal and the quality of the RNA transcripts.

### RNA refolding

DNase-treated IVT products were column-purified and used for RNA refolding. For the initial refolding step, RNA was diluted in ultrapure water to an RNA concentration of 26 nM, and refolding buffer was added to reach the following final concentrations: EDTA (0.5 mM), HEPES (200 mM, pH 7.5), NaCl (300 mM). The mixture was heated at 95 °C for 1.5 min to denature RNA secondary structures and then immediately placed on ice. While on ice, MgCl<sub>2</sub> was added to give a final concentration of 5 mM and an RNA concentration of 23.5 nM. Samples were then incubated at 37 °C for 30 min to allow RNA refolding. Refolded RNA was stored at -80 °C until further use.

### DMS probing

For dimethyl sulfate (DMS) probing, reactions were prepared with refolded RNA at a final concentration of 23.5 nM. DMS (Sigma-Aldrich) stock solutions were prepared in 100% ethanol such that, upon addition to the RNA samples, the final DMS concentrations were 20, 40, 60, 80, or 100 mM. β-mercaptoethanol (Sigma-Aldrich) was diluted in PBS such that, upon addition to the reaction, its final concentration was 1000 mM. For each DMS concentration, one negative control (receiving an equal volume of 100% ethanol instead of DMS) and two technical replicates with DMS were prepared. Refolded RNA samples were kept on ice, mixed thoroughly with the appropriate DMS working solution or with 100% ethanol for negative controls, and immediately transferred to an incubator pre-equilibrated to 37 °C for 7 min. Reactions were then placed back on ice, and β-mercaptoethanol working solution was added to each reaction and mixed thoroughly to reach its final concentration of 1000 mM and to quench DMS modification.

### Reverse transcription

DMS-treated RNA was column-purified and subjected to reverse transcription using MarathonRT [24, 25, 26]. Primer–RNA annealing reactions were prepared on ice with the following final concentrations: each dNTP (0.5 mM) and Full-FW primer (0.25 µM). Primer–RNA mixtures were incubated at 65 °C for 5 min and then rapidly cooled on ice. Reverse transcription was then performed in 40 µL reactions containing the primer–RNA annealing mixture and the following final concentrations: MarathonRT reaction buffer (1×; final composition 50 mM Tris–HCl, pH 8.3; 200 mM KCl; 20% (v/v) glycerol, prepared from a 3× stock containing 150 mM Tris–HCl, pH 8.3; 600 mM KCl; 60% (v/v) glycerol), MnCl<sub>2</sub> (1 mM), MarathonRT (1 U/µL; in-house purified, Addgene plasmid no. 109029 [23]; <http://n2t.net/addgene:109029>; RRID: Addgene\_109029; stored in 50 mM Tris–HCl, pH 8.3; 200 mM KCl; 20% (v/v) glycerol), RNase Inhibitor, Murine (1 U/µL; New England Biolabs), and DTT (5 mM). No–reverse transcriptase (no–RT) controls

were prepared in parallel with the same components and final concentrations, except that MarathonRT was omitted.

### PCR amplification

cDNA products were column-purified and subsequently amplified by PCR. PCR amplification was performed in 50 µL reactions containing the following final concentrations: GXL reaction buffer (1×, from a 5× stock; Takara Bio), each dNTP (0.2 mM), each primer (0.25 µM; Full-T7-RV and Full-FW), and PrimeSTAR GXL DNA Polymerase (0.025 U/µL; Takara Bio). An appropriate amount of cDNA was used as template. Thermal cycling conditions were: 98 °C for 2 min; 25 cycles of 98 °C for 20 s, 55 °C for 20 s, and 68 °C for 30 s; followed by a final extension at 68 °C for 5 min. PCR amplicons were analyzed on a 2% agarose gel and stained with ethidium bromide (EtBr) to assess amplification quality.

### Nanopore sequencing

PCR amplicons were column-purified, and DNA concentrations were determined using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific). After normalizing the molar concentration of each sample, a total of 1,600 fmol DNA was used in library preparation, with 5 µL taken from each sample. For each sample, dA-tailing and 5' phosphorylation were performed in 6 µL reactions containing 5 µL DNA, 0.7 µL NEBNext End-Repair Buffer (1× final concentration; New England Biolabs), and 0.3 µL NEBNext End-Repair Enzyme Mix (New England Biolabs). Reactions were incubated at 20 °C for 10 min, followed by 65 °C for 10 min. Barcoding ligation was performed using the SQK–NBD114–96 kit (Oxford Nanopore Technologies). For each sample, barcoding ligation was carried out in 5 µL reactions containing 1.5 µL end-repaired DNA, 1 µL barcode, and 2.5 µL NEB Blunt/TA Ligase Master Mix (New England Biolabs, added as supplied). Reactions were incubated at room temperature for 20 min, after which ligation was quenched by adding 1 µL EDTA (SQK–NBD114–96). Subsequently, 5 µL of each barcoded sample was pooled, and the resulting pool was purified with 1× AMPure XP beads (SQK–NBD114–96) and washed twice with Short Fragment Buffer (SFB; SQK–NBD114–96). The pooled barcoded DNA was eluted in 35 µL nuclease-free water. Adapter and motor protein ligation was performed in 56 µL reactions containing 35 µL pooled barcoded DNA, 11 µL NEBNext Quick Ligation Reaction Buffer (New England Biolabs, B6058S; 1× final concentration), 5 µL Native Adapter (NA; SQK–NBD114–96, Oxford Nanopore Technologies), and 5 µL high-concentration NEB T4 DNA Ligase (New England Biolabs, T2020M). Reactions were incubated at room temperature for 20 min. The library was then purified with 0.6× AMPure XP beads (SQK–NBD114–96) and washed twice with SFB, taking care to avoid drying of the beads during washing and prior to elution. The final library was loaded onto an R10.4.1 flow cell (FLO–PRO114M; Oxford Nanopore Technologies) and sequenced on a PromethION 2 Solo instrument (Oxford Nanopore Technologies) controlled by MinKNOW software (version 25.09.16). Basecalling was performed with Dorado v1.1.1 (Oxford Nanopore Technologies) in super-accuracy (“sup”) mode using the dna\_r10.4.1\_e8.2\_400bps\_sup@v5.2.0 model.

### Mapping and counting of mutations

Sequencing reads were processed with an RNAFramework-based [27] workflow using the functions `rf-map` and `rf-count`, following a similar pipeline to [41] with minor adaptations

for our constructs. For each library, we generated a Bowtie2 [28] index from the reference sequence using `bowtie2-build` and produced the companion FASTA index with `samtools faidx` [29]. Reads were aligned to the indexed reference with `rf-map -cq5 20 -cqo '--very-sensitive-local' -b2 -bi ../{ref_fasta}.index`, where `{ref_fasta}.index` are the files produced by `bowtie2-build`. The resulting BAM files were coordinate sorted and indexed (`samtools sort/index`) before quantification.

Mutation profiles were obtained with `rf-count` [27] using the options recommended by Ref.[41] (`-m -ds 75 -na -ni -md 3`). These parameters report mutational load rather than RT stops (`-m`), discard reads shorter than 75 nt (`-ds 75`), ignore ambiguous deletions (`-na`) and indels (`-ni`), and cap deletions at three nucleotides (`-md 3`). The full command sequence executed for each FASTQ file is summarized below:

```
# build indices of reference
bowtie2-build --quiet {ref_fasta} {ref_fasta}_index
samtools faidx {ref_fasta}
# alignment (see above)
rf-map \
  -cq5 20 \
  -cqo \
  -mp '--very-sensitive-local' \
  -b2 \
  -bi {ref_fasta}_index \
  -o rf_map_{output_suffix} \
  {fastq_file}

# sorting and indexing the BAM file
samtools sort {bam_file} -o {sorted_bam_file}
samtools index {sorted_bam_file}
# count mutations to obtain mutation profile and mutation map
rf-count \
  -f {ref_fasta} \
  -m \
  -ds 75 \
  -na \
  -ni \
  -md 3 \
  {sorted_bam_file}
```

For the reference structured RNAs (HCV IRES, bacterial RNase P, *V. cholerae* glycine riboswitch, *Tetrahymena* ribozyme, HC16), we instead adopted the filtering scheme described by the original work [25].

```
rf-count -r -p {num_threads} \
  -mf {primer_mask} \
  -f {reference_fasta} \
  --only-mut 'G>Y;A>B;C>D;T>V' \
  -m \
  -nd \
  -ni \
  -q {minq} \
  -eq 10
```

All downstream structural analyses reported in the main text rely on the coverage and mutation rates generated by this standardized workflow.

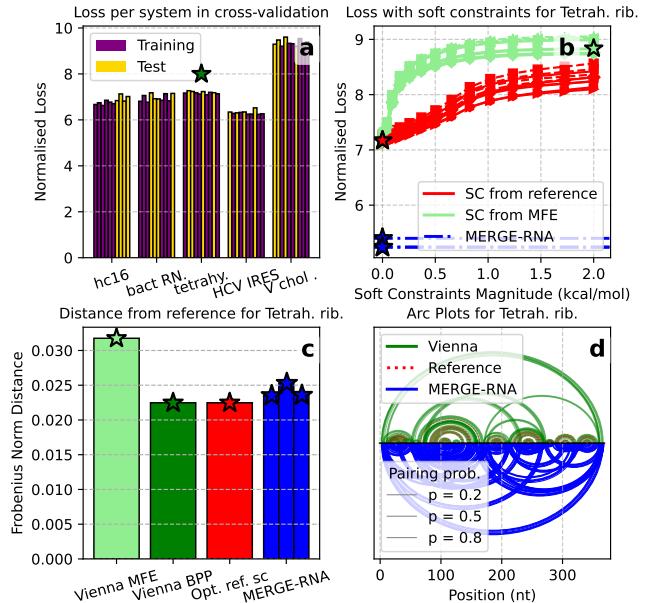


Fig. 6:

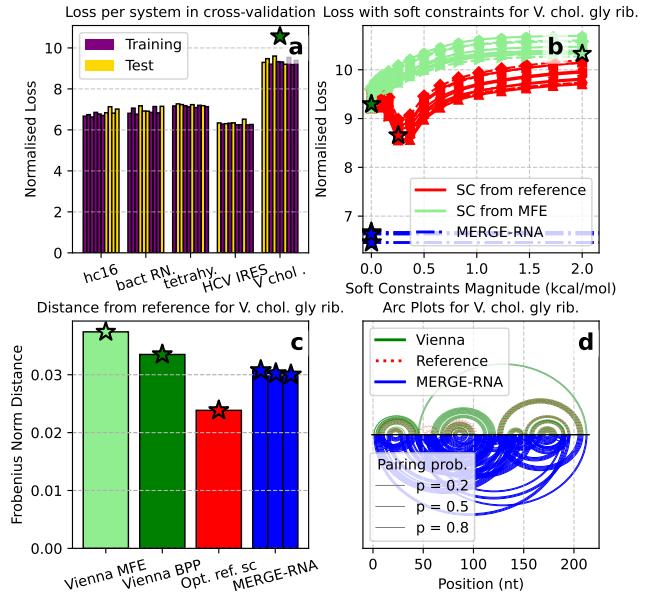


Fig. 7:

## Supplementary Information

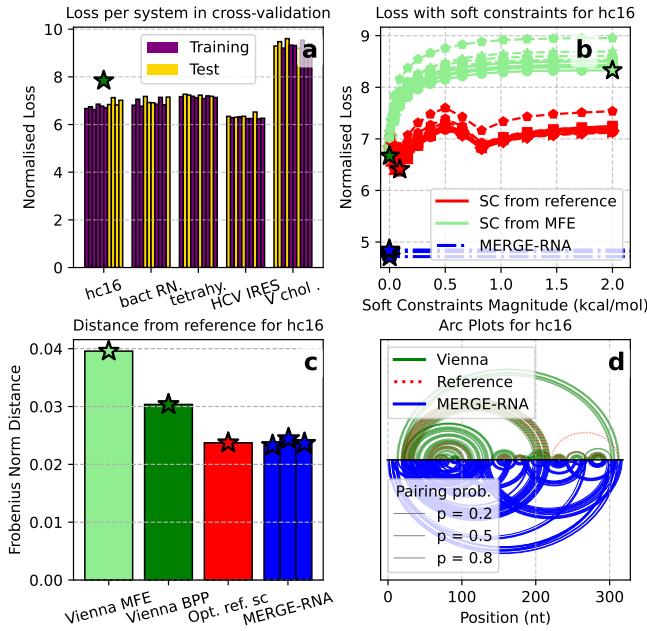


Fig. 8:

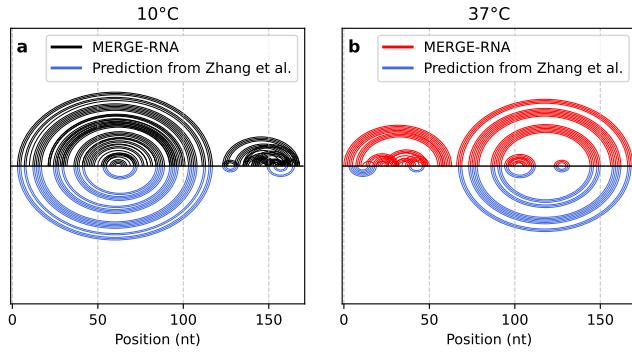


Fig. 9:

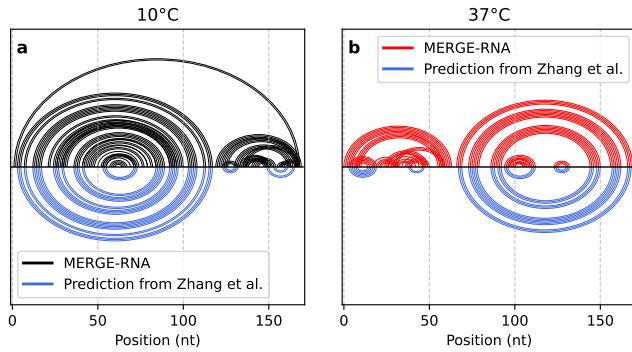


Fig. 10:

Training sets	$\mu_r$	$\Delta\mu_{\text{pairing}}$	$p_{\text{bind}}(A, \text{unpaired})$	$p_{\text{bind}}(C, \text{unpaired})$	$p_{\text{bind}}(G)$	$p_{\text{bind}}(\text{U})$	$m_1$
hc16 + HCV + V.chol.	-0.08	0.34	1.00	0.60	0.48	0.05	0.53
hc16 + RNaseP + HCV	-0.05	0.49	1.00	0.57	0.57	0.05	0.50
hc16 + RNaseP + Tetrah.	-0.02	0.15	0.89	0.56	0.28	0.02	0.56
hc16 + RNaseP + V.chol.	-0.36	0.03	1.00	0.66	0.31	0.04	1.05
hc16 + Tetrah. + HCV	-0.06	0.61	0.98	0.44	0.58	0.03	0.50
hc16 + Tetrah. + V.chol.	-0.07	0.40	0.91	0.64	0.40	0.03	0.53
RNaseP + HCV + V.chol.	0.08	0.18	0.99	0.58	0.35	0.05	0.39
RNaseP + Tetrah. + HCV	-0.08	0.24	0.97	0.51	0.35	0.04	0.53
RNaseP + Tetrah. + V.chol.	-0.05	0.79	0.91	0.61	0.41	0.07	0.54
Tetrah. + HCV + V.chol.	-0.09	0.50	1.00	0.49	0.57	0.05	0.45

Table 1. Physical parameters obtained by training on different triplets of RNA systems.

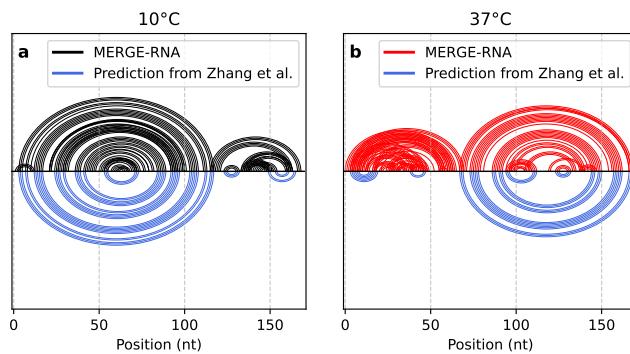


Fig. 11:

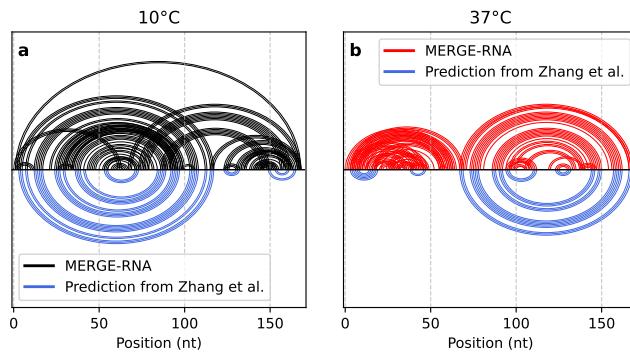


Fig. 12:

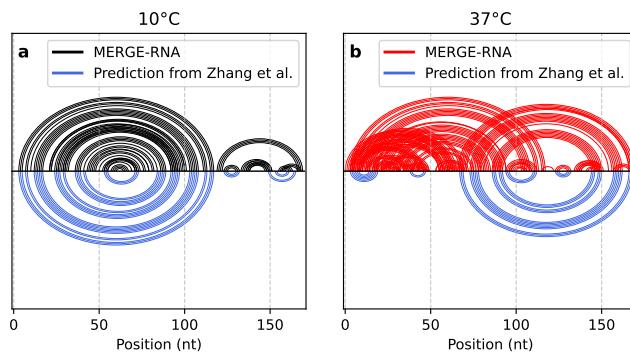


Fig. 13:

**Table 2.** Loop co-occupancy inferred from baseline thermodynamic ensembles and after MERGE-RNA refinement. Entries report the percentage of structures in which Loop1 and Loop2 are formed.

		Baseline		MERGE-RNA		
		Loop1		Loop1		
Turner	Loop2	0	1	Loop2	0	
		0	49%		27%	
	1	51%	0%	1	57%	
Andr.	Loop1		Loop1		Loop1	
	Loop2	0	1	Loop2	0	1
		0	19%		42%	0%
	1	81%	0%	1	16%	42%