PKProbDesign:
RNA inverse folding including pseudoknots by optimizing thermodynamic folding probability

Takumi Otagaki, Junichi Iwakiri, Goro Terai, Kiyoshi Asai, Kengo Sato

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New Results

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Institute of SCIENCE TOKYO

This is my second time Benasque!

I really enjoyed my previous Benasque.








Professor Asai cooked lunch for us every day!
I was spoiled - just like last time in Benasque.

RNA inverse folding

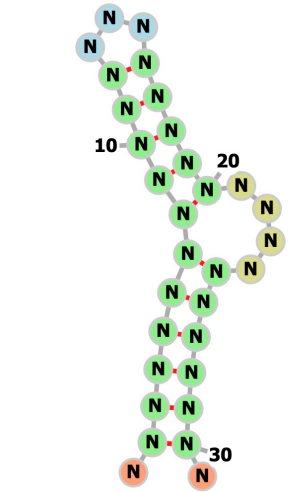
- Inverse Folding problem is
  to find the sequence q^* which folds the target structure σ^t.

Sequence q^*

??????????????

Folds into

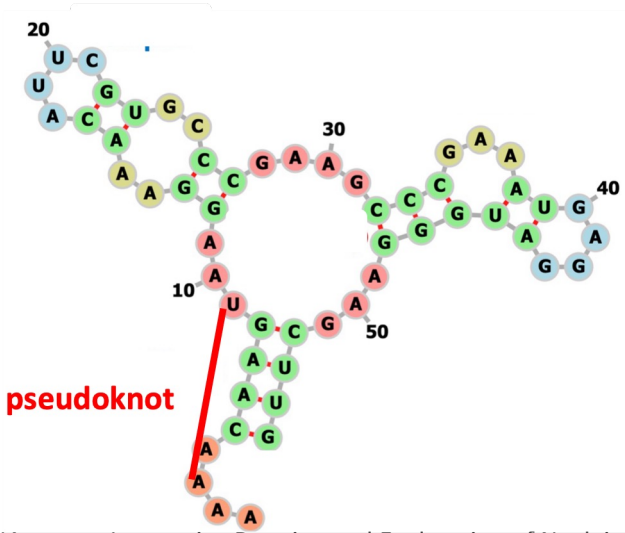
Target Structure σ^t

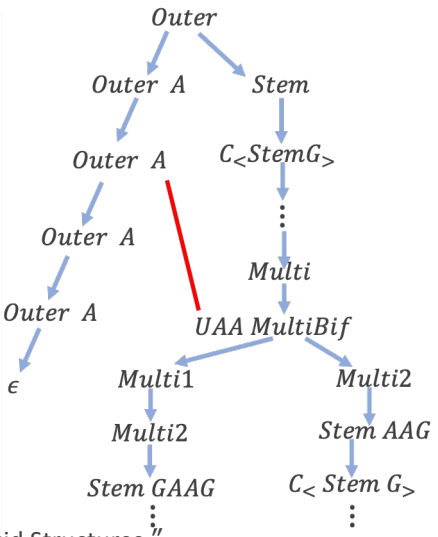


.((((((((((...))))))....))))))

RNA inverse folding with pseudoknots

- **Pseudoknots are important motifs** in functional and viral RNAs and often contribute to tertiary folding.
- They **violate the nested structure** assumed by standard secondary-structure models, making them **computationally challenging**.





Pseudoknots break the tree.

Johnson, Philip Z., and Anne E. Simon. 2023. "RNAcanvas: Interactive Drawing and Exploration of Nucleic Acid Structures." *Nucleic Acids Research* 51 (W1): W501–8.

Minimum Free Energy (MFE) based inverse folding

Find **the sequence s whose MFE structure is σ^t**.

$$s^* \in \{s \mid \text{MFE\_structure}(s) = \sigma^t\}$$

$$\text{MFE\_stucrure(s)} = \text{argmin}_\sigma \, \text{Energy}(\sigma, s)$$

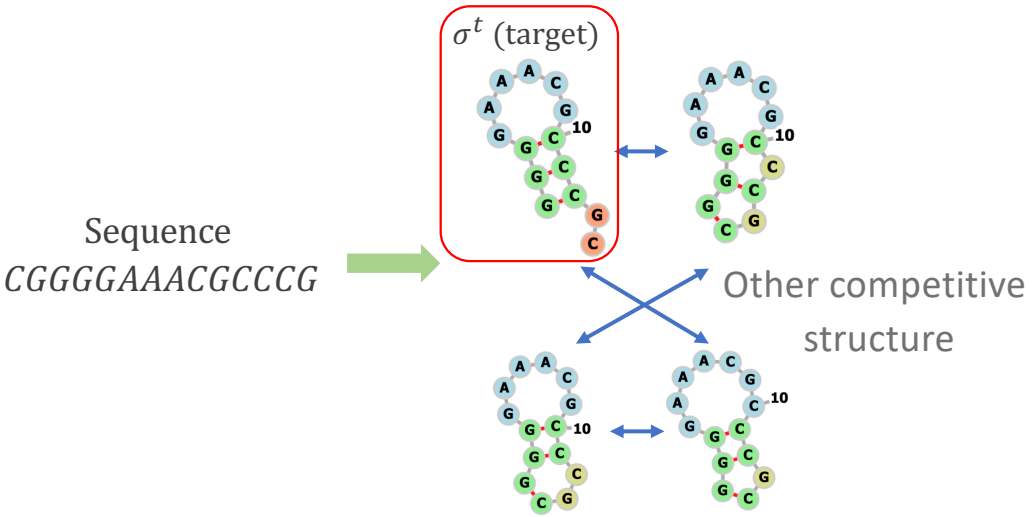
Why MFE agreement is not enough

RNA folds into an ensemble of competing structures,

not just a single MFE structure.

How strongly is the target supported in the ensemble?

→ **Folding Probability: $p(\sigma^t \mid s)$**

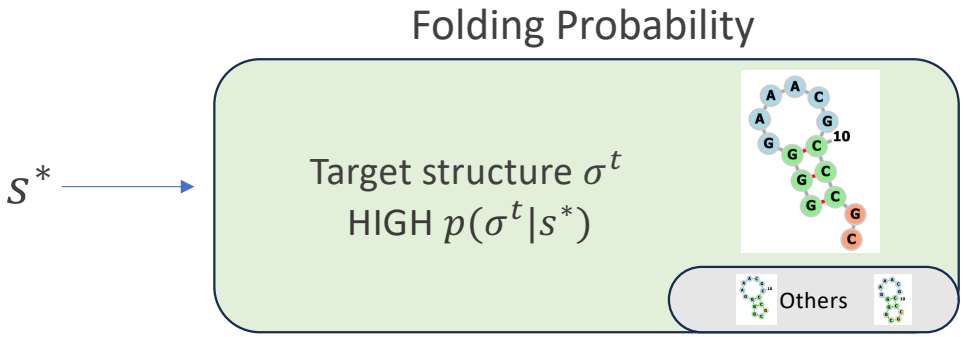


Probability-based inverse folding

- Find the **sequence that maximizes the target folding probability.**

$$s^* = \text{argmax}_s \, p(\sigma^t \mid s)$$

- The objective naturally extends to multiple target structures.
  - e.g., maximize $p(\sigma_1|s) + p(\sigma_2|s)$



The gap: probability-based design for pseudoknots

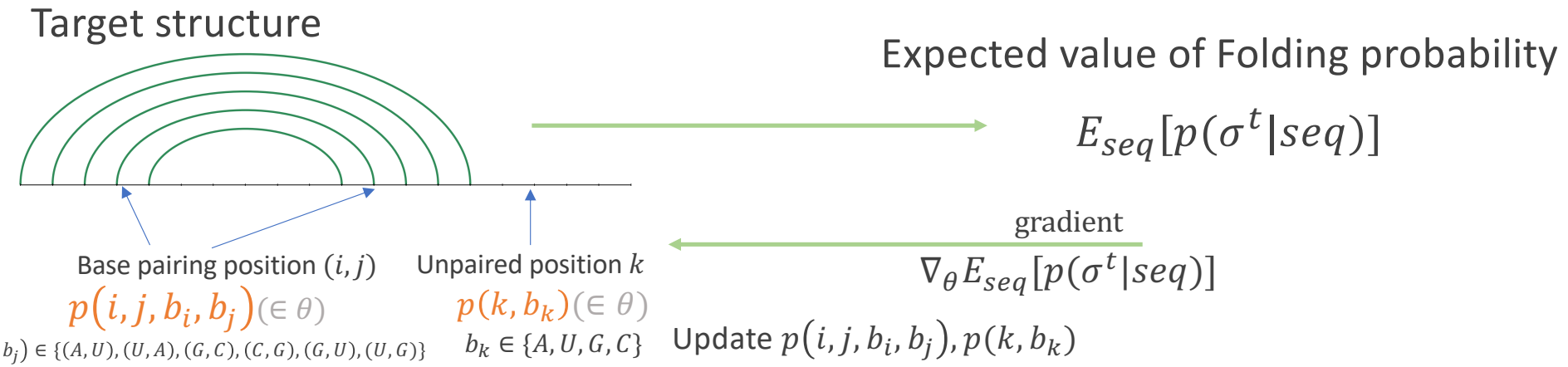
- Probability-based design requires a folding-probability evaluator.
- For pseudoknotted structures, integrating such an evaluator into a practical inverse-folding framework remained challenging.

→We propose PKProbDesign;

A pseudoknot-aware probability-based framework for RNA inverse folding.

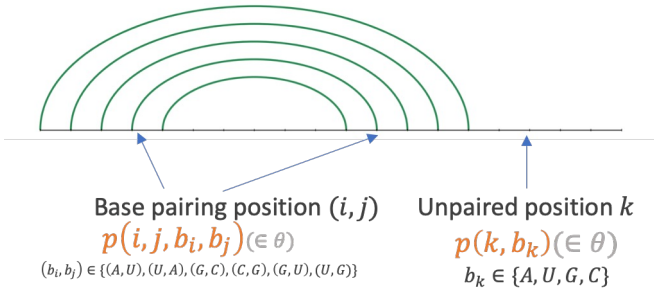
Previous work
SamplingDesign: probability-based inverse folding

- Sear 21the sequence which maximizes **the target folding probability** based on the gradient descent.
  - **Folding probability of the Pseudoknot-free structure is possible!**
- The gradient of folding probability is approximated by Monte Carlo Sampling.



Tang, Wei Yu, Ning Dai, Tianshuo Zhou, David H. Mathews, and Liang Huang. 2026. "SamplingDesign: RNA Design via Continuous Optimization with Coupled Variables and Monte-Carlo Sampling." *Nature Communications* 17 (1): 2950.

SamplingDesign formulation



$$J(\theta) = E_{S \sim p(\cdot|\,\theta)}\,[f(S)]$$

The gradient $\nabla_\theta J(\theta)$is; $\mathbb{E}_{S\sim p_\theta}\left[f(S)\nabla_\theta \log p_\theta(S)\right].$

We can approximate the gradient by sampling,

$$\nabla_\theta J(\theta) \approx \frac{1}{K}\sum_{k=1}^{K} f(S_k)\nabla_\theta \log p_\theta(S_k).$$

PKProbDesign replaces the standard pseudoknot-free evaluator with a pseudoknot-aware thermodynamic folding probability.

$f(S)$ can be black box score for sequence S.

Only sequence scores are required.

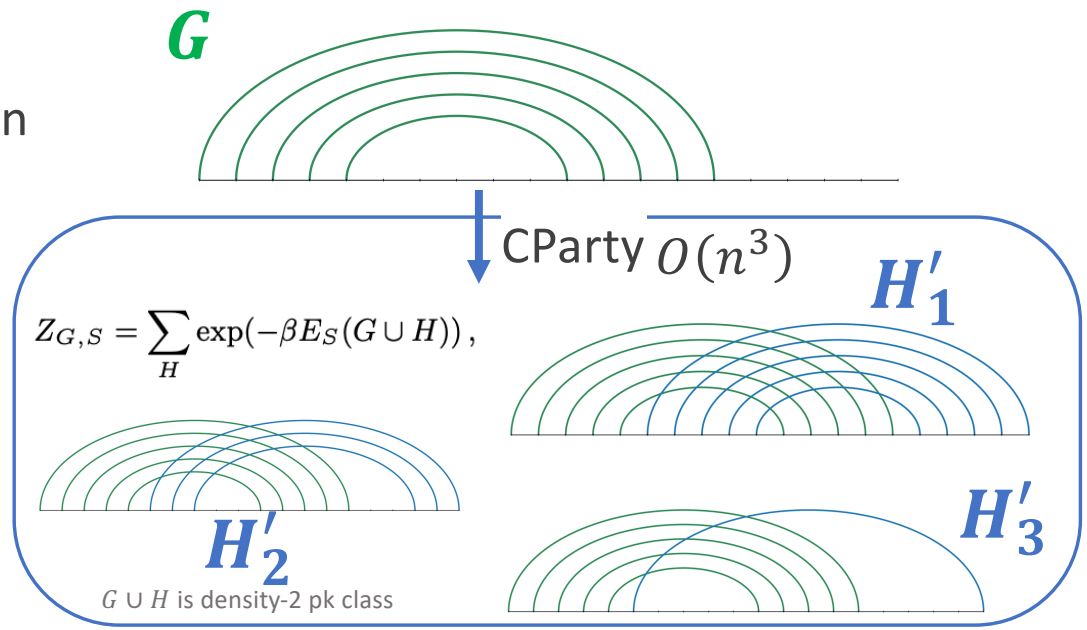
The evaluator f does not need to be differentiable.

CParty enables conditional probabilities for density-2 pseudoknots

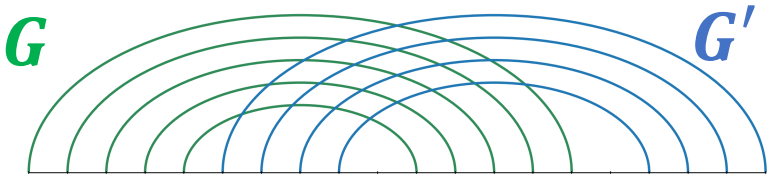
Given a pseudoknot-free scaffold G,
CParty computes a conditional partition function over extensions H s.t. $G \cup H \in$ density2 class.

It provides the conditional thermodynamic evaluator required by PKProbDesign.

Pseudoknot energy model (DP09)



$$P(G' \mid G, S) = \frac{\exp(-\beta E_S(G \cup G'))}{Z_{G,S}}.$$



Gray, Mateo, Luke Trinity, Ulrike Stege, Yann Ponty, Sebastian Will, and Hosna Jabbari. 2024.
"CParty: Hierarchically Constrained Partition Function of RNA Pseudoknots." *Bioinformatics (Oxford, England)* 41 (1): btae748.

Pseudo-joint folding probability for density-2 pseudoknotted structure

A density-2 pseudoknot struct R can be decomposed as.

$\rightarrow R = G \cup G', G \cap G' = \emptyset$, G, G' are pseudoknot-free.

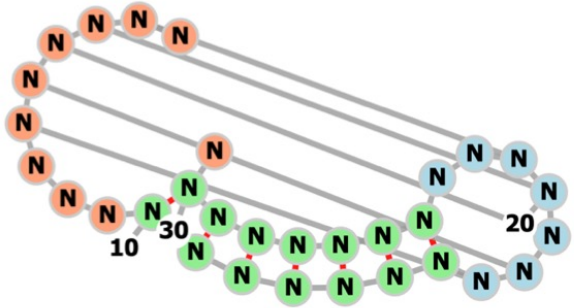
Under the hierarchical folding assumption,
we define the pseudo-joint folding probability as:

$$\tilde{p}(R \mid S) = p(G \mid \mathbf{S})\, \mathbf{p}(\mathbf{G}' \mid \mathbf{G}, \mathbf{S})$$

$p(G|S) \leftarrow$ LinearPartition$O(nb^2)$ (b: beam size)
$p(G'|G,S) \leftarrow$ CParty $O(n^3)$.

Exact $p(R|S)$ is $\sum_{(G,G') \in \mathcal{D}(R)} P(G \mid S)\, P(G' \mid G, S)$.

Target Structure R (density2)



R = .(((((...[[[[[[]))))).]]]]]].

Decomposition

G = .(((((.....))))).....
G' = ...[[[[[[[.....]]]]]]].

Greedy scaffold decomposition and its limitation

We decompose R in a greedy way.

Algorithm 1 Greedy scaffold-extension decomposition

Input: target base-pair set R

Output: scaffold G, extension component G'

1. **Sort** the pairs in R by increasing span $|j - i|$.
2. **Initialize** $G \leftarrow \emptyset$.
3. **For each** pair p in sorted order, add p to G if p does not cross any pair already in G.
4. **Set** $G' \leftarrow R \setminus G$.

Limitation

- Scaffold decomposition is heuristic (greedy).
- No guarantee that G' is pseudoknot-free.
- This issue did not occur in our benchmark dataset.

Dataset

Dataset: Pseudobase++ [Taufer et al., 2009]

- 357 pseudoknotted structures (≤100 nt)
- Removed 55 non-density-2 structures
- Removed 2 structures incompatible with the energy model.
- **300 benchmark targets**

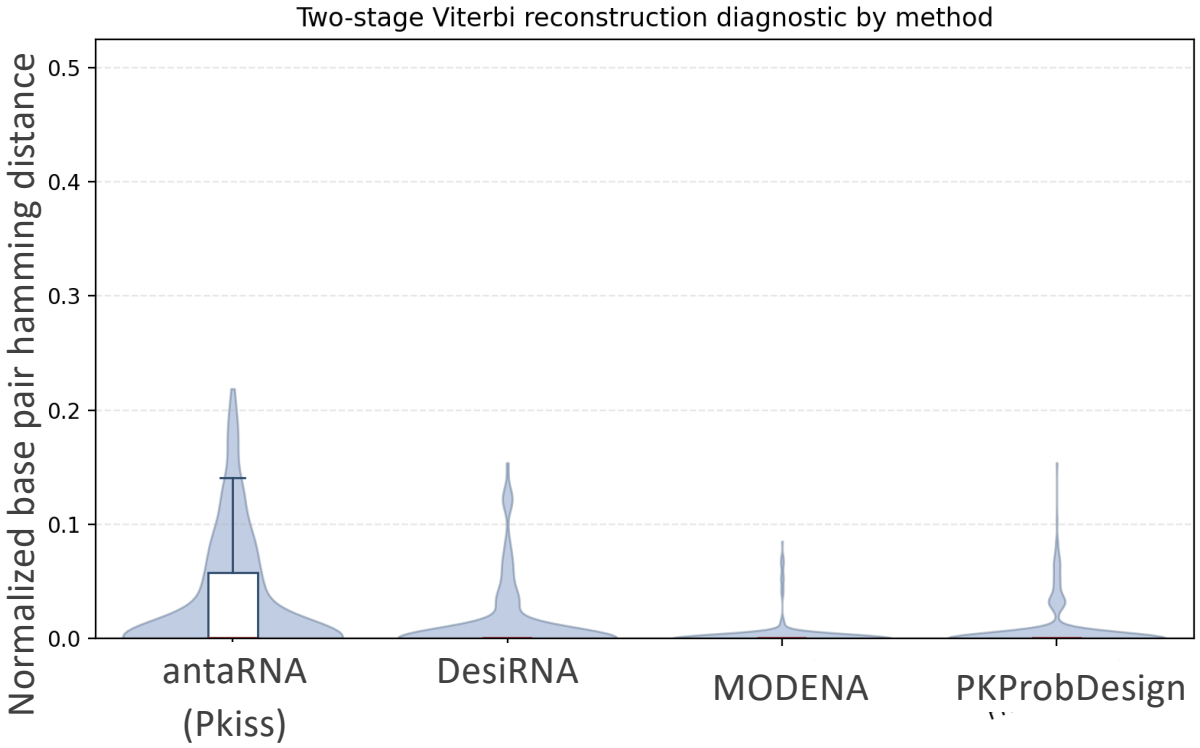
Compared methods:

- DesiRNA [Wirecki et al., 2025]
- MODENA [Taneda, 2011]
- antaRNA [Kleinkauf et al., 2015]

Structure-prediction based evaluation

1. 20 designed seqs for each methods and targets.
2. For each designed sequences;
   2.1. predict MFE G_1 excluding pk.
   2.2. predict MFE $G_2 \cup G_1$ on G_1.
3. Plot the min base_pair_distance$(G_1 \cup G_2, \sigma_t)$
   $\mathrm{Min}_{i=1\ldots20}\ d_{BP}^{norm}(G_1^i \cup G_2^i, \sigma^t)$

$$d_{\mathrm{BP}}^{\mathrm{norm}}(P_{\mathrm{pred}}, P_{\mathrm{tgt}}) = \frac{1}{|S|} \sum_{1 \le i < j \le |S|} \mathbf{1}[\, P_{\mathrm{pred}}[i,j] \neq P_{\mathrm{tgt}}[i,j]]$$

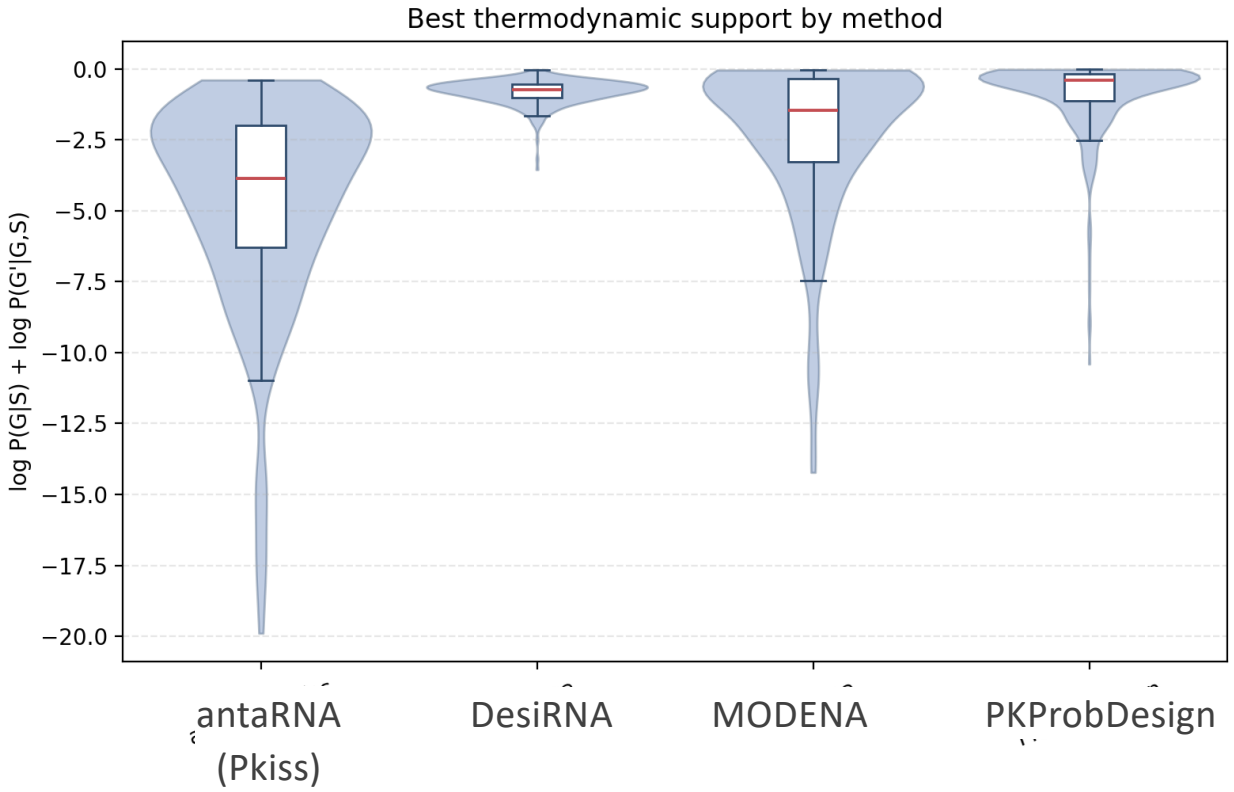


Pseudo-joint folding probability comparison

Higher better

1. 20 designed seqs for each methods, targets.
2. Plot the maximum $\log p(G|S)p(G'|G,S)$

PKProbDesign achieves high pseudo-joint folding probability comparable to DesiRNA



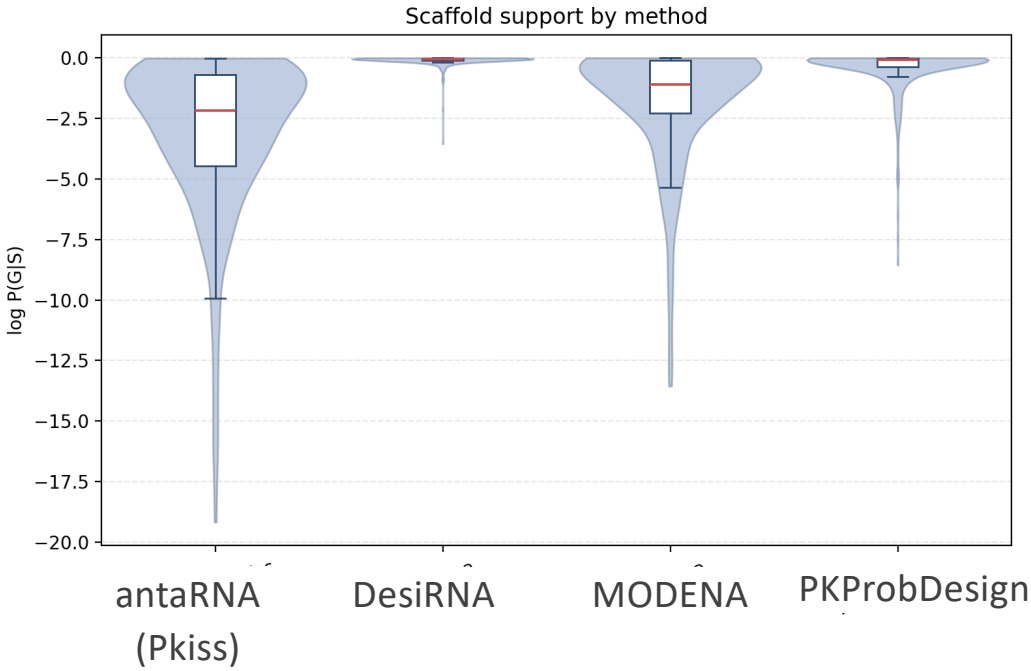
$p_{value} = 0.01$
(pkprobdesign v.s. desirna)

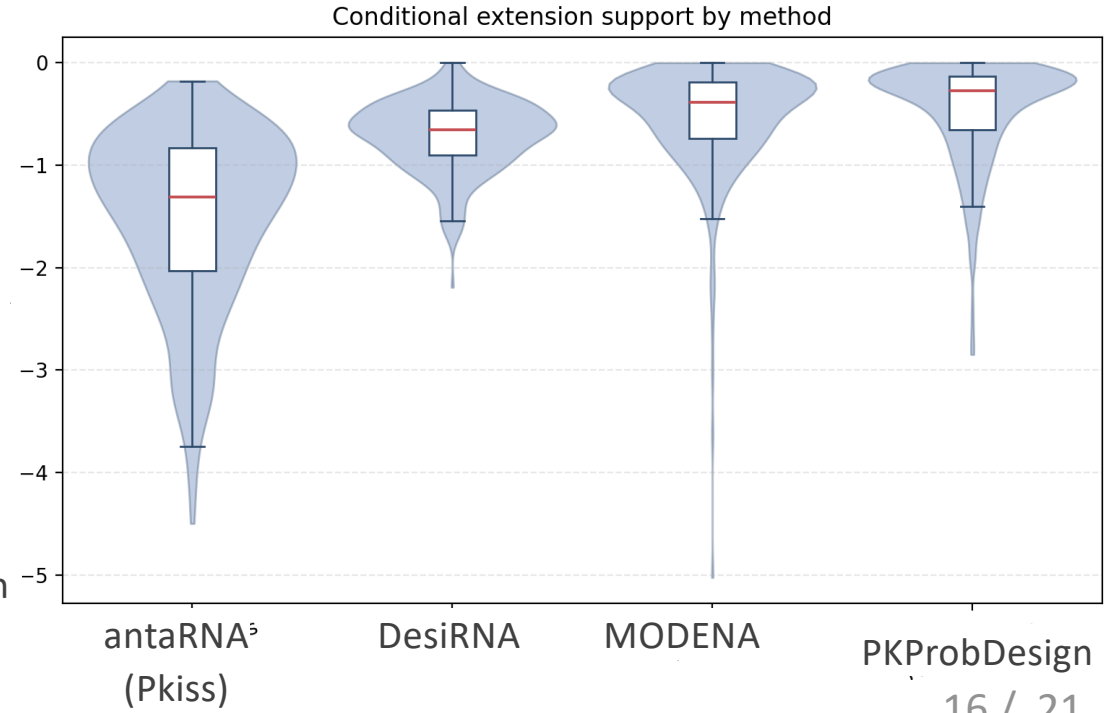
The same target decomposition was used for all methods.

Results

DesiRNA favors scaffold support; PKProbDesign favors conditional extension support

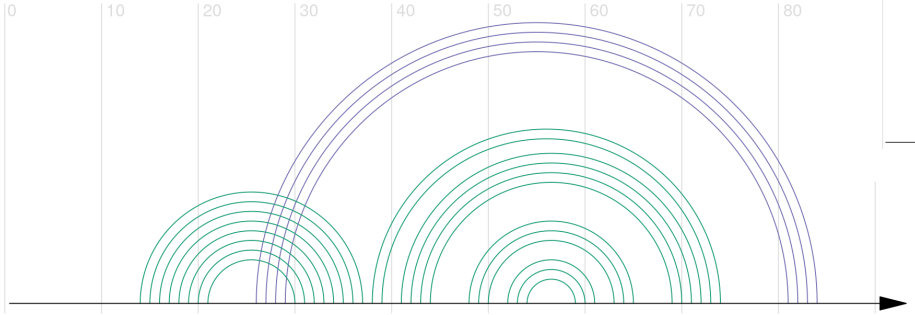
1. 20 designed seqs for each methods, targets.
2. For each target and method, select the sequence with the highest pseudo-joint probability.
3. Decompose its score into scaffold prob $\log p(G|S)$ (left) and extension prob $\log p(G'|G,S)$ (right).



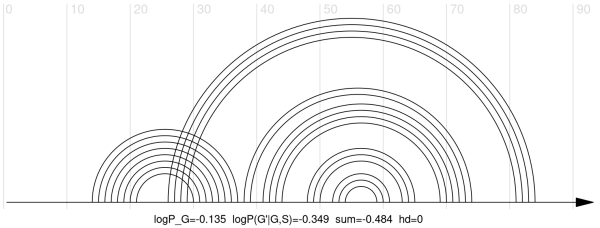


Case Study: PKB00362, viral 5'UTR
A target showing clear differences among methods.

Target σ^t



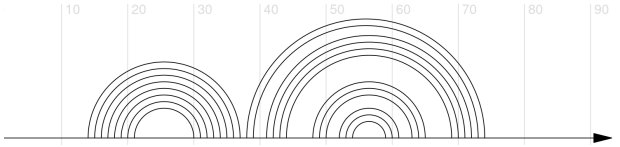
PKProbDesign



$\log p(G|S)p(G'|G,S) = -0.48$

HD=0

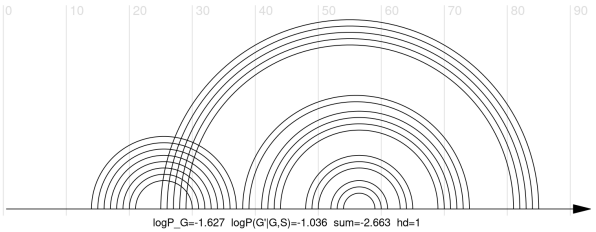
DesiRNA



$\log p(G|S)p(G'|G,S) = -0.82$

HD=4

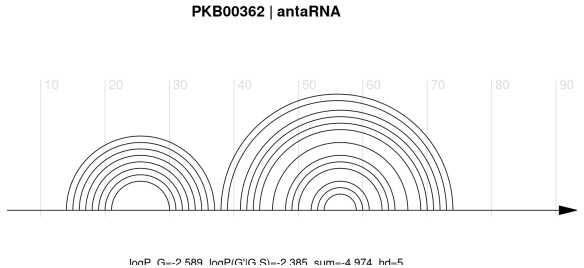
MODENA



$\log p(G|S)p(G'|G,S) = -2.66$

HD=1

antaRNA



$\log p(G|S)p(G'|G,S) = -5.00$

HD=5

1. 20 designed seqs for each methods, targets.
2. For each designed sequence;
   2.1. compute pkfree mfe G.
   2.2. compute mfe $G_2 \cup G_1$ on G_1

Interpretation of method-specific performance

- PKProbDesign showed strong conditional-extensionprobability, consistent with directly optimizing a pseudoknot-aware thermodynamic objective.
- MODENA performed well in MFE-based evaluation, consistent with its predictor-based optimization objective.
- DesiRNA excelled in scaffold probability, reflecting its design strategy (maximizing folding prob)
- antaRNA may have been affected by the fixed GC-content soft-constraint:GC ratio fixed at 0.5, which we could not disable.

Current limitations

- The objective is a decomposition-dependent pseudo-joint probability, not the exact probability of σ.
- The greedy decomposition is heuristic and may fail to produce a pseudoknot-free G'.
  - Future work:
    Use graph-coloring-based decomposition and maximizing the num of bps in G
- The current implementation is restricted to density-2 pseudoknots.
- The model relies on the hierarchical folding assumption.

Toward longer and more diverse pseudoknot-aware design

Algorithmic extensions

- Beam-pruned CParty for longer RNA targets
- Marginalization over, or optimization of, multiple decompositions to obtain the exact joint probability

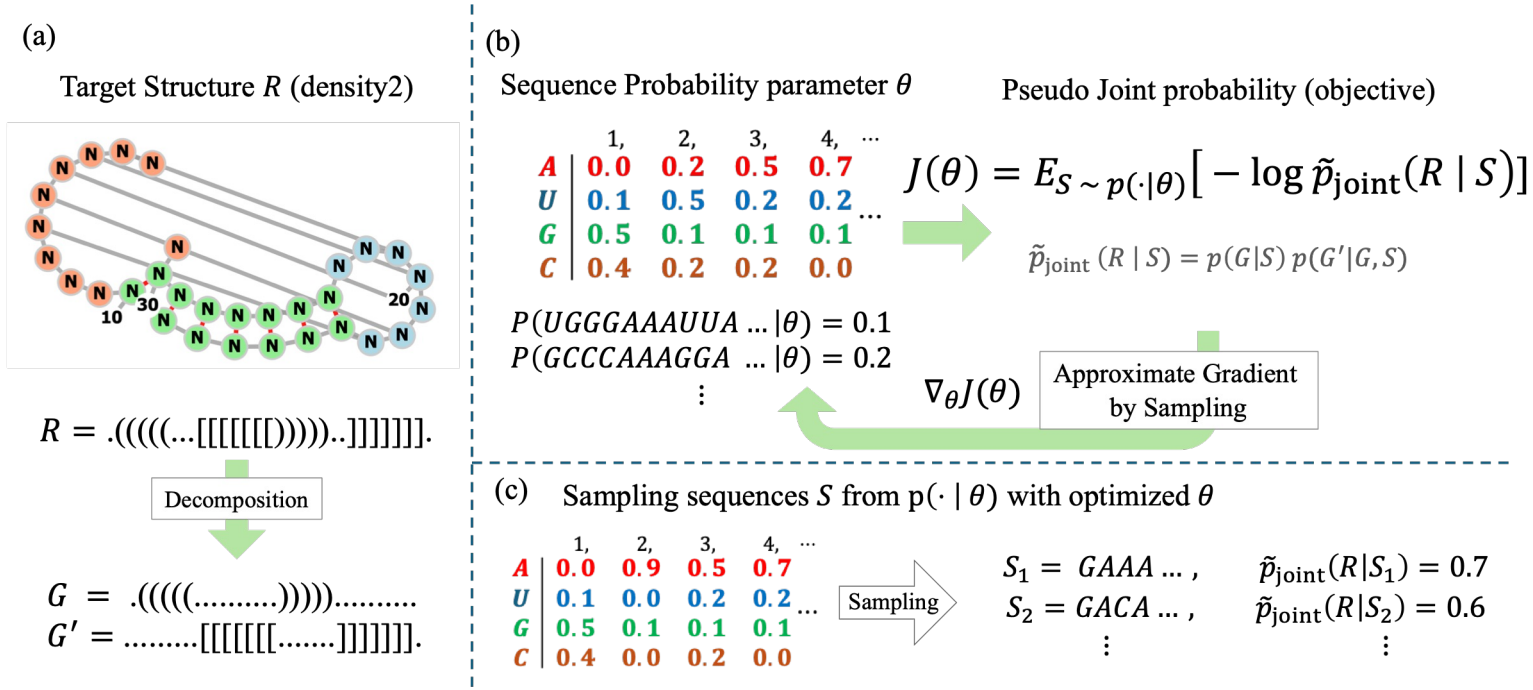
Biological applications

- IRES design
- Riboswitch design

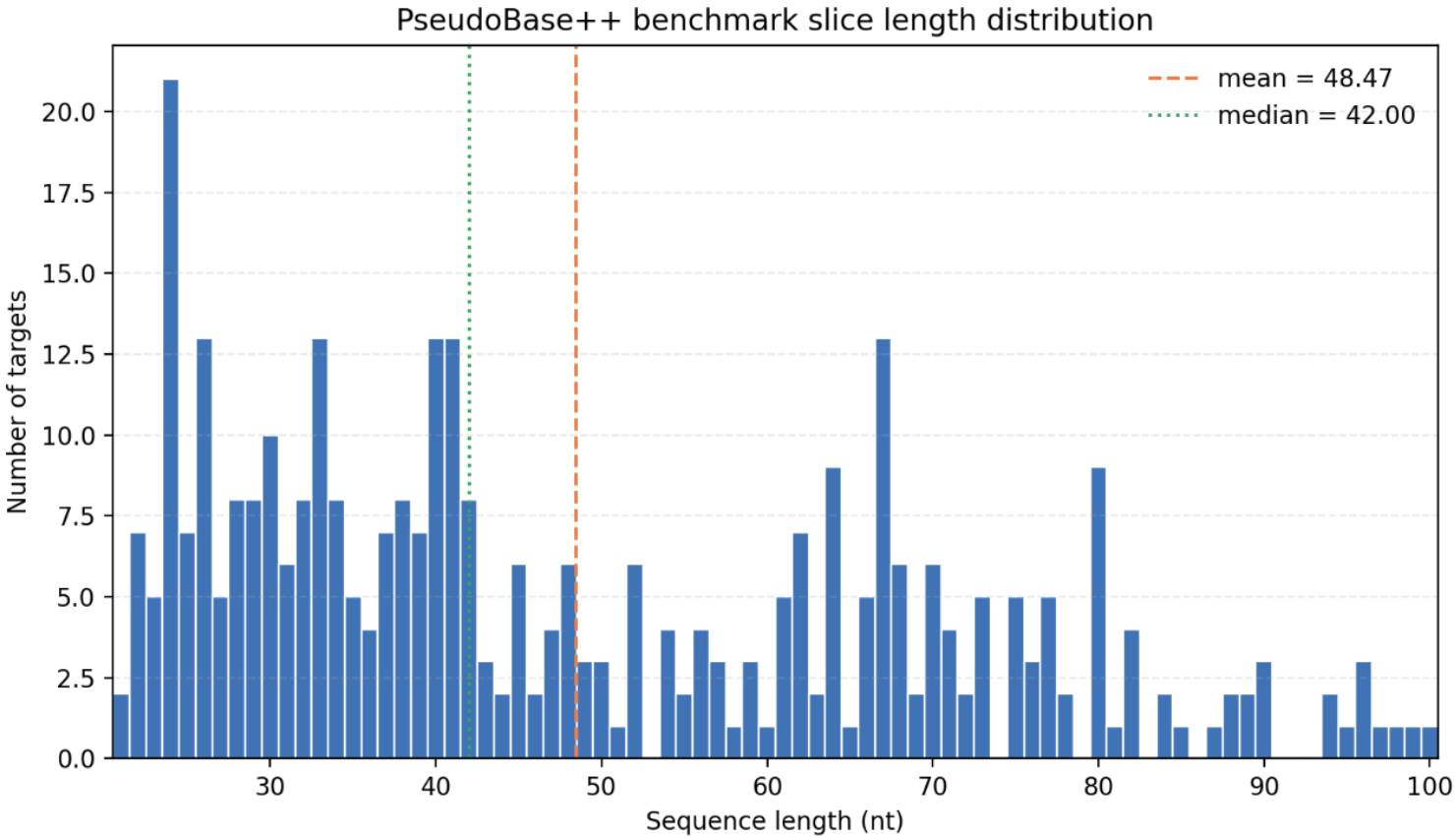
Summary

Thank you!

- Probability-based inverse folding for density-2 pseudoknots
- SamplingDesign optimization with LinearPartition and CParty evaluators
- Improved thermodynamic folding support on 300 PseudoBase++ targets

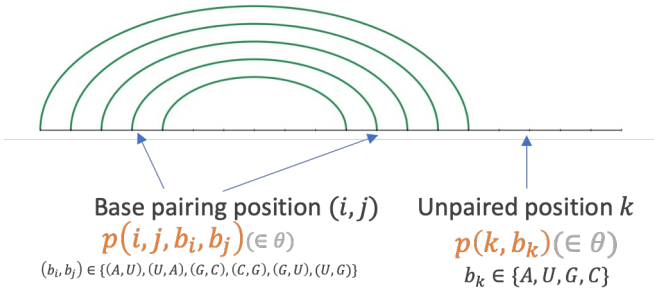


The sequence length distribution



N=300

SamplingDesign formulation



$$J(\theta) = E_{S \sim p(\cdot|\,\theta)}\left[f(S)\right]$$

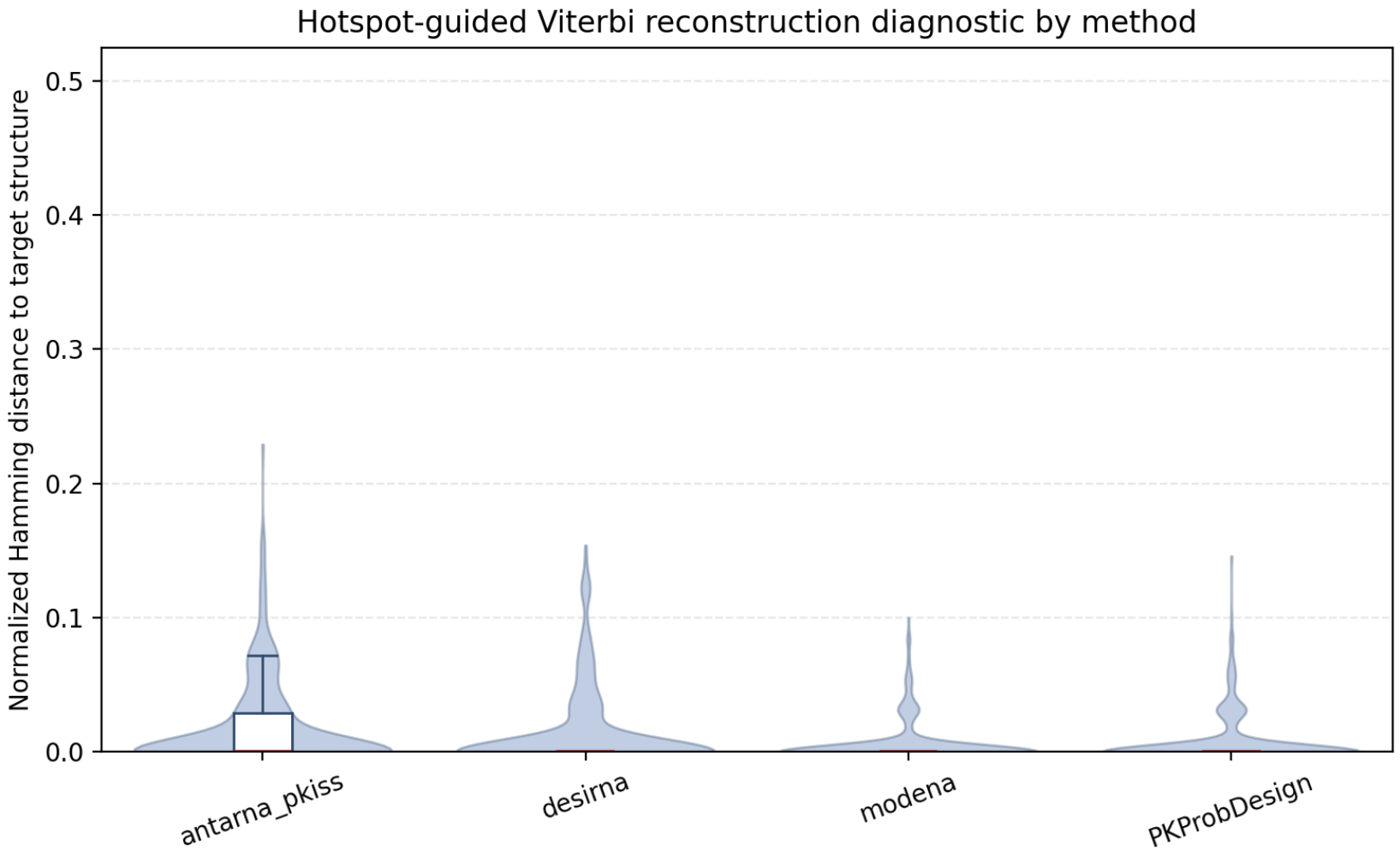
The gradient $\nabla_\theta J(\theta)$is $\nabla_\theta J(\theta) = \nabla_\theta \sum_S p_\theta(S) f(S)$

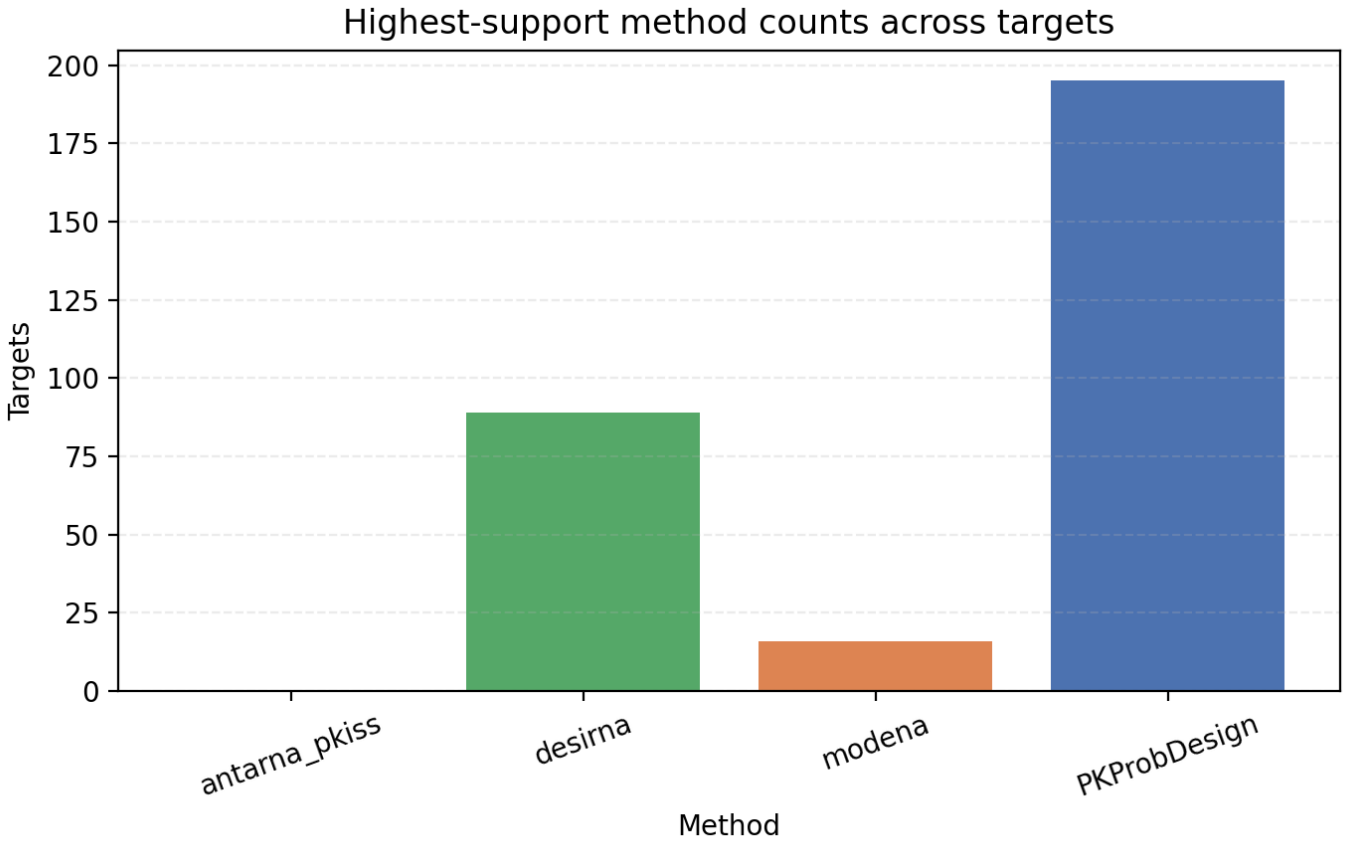
$$= \sum_S f(S)\, p_\theta(S)\, \nabla_\theta \log p_\theta(S)$$

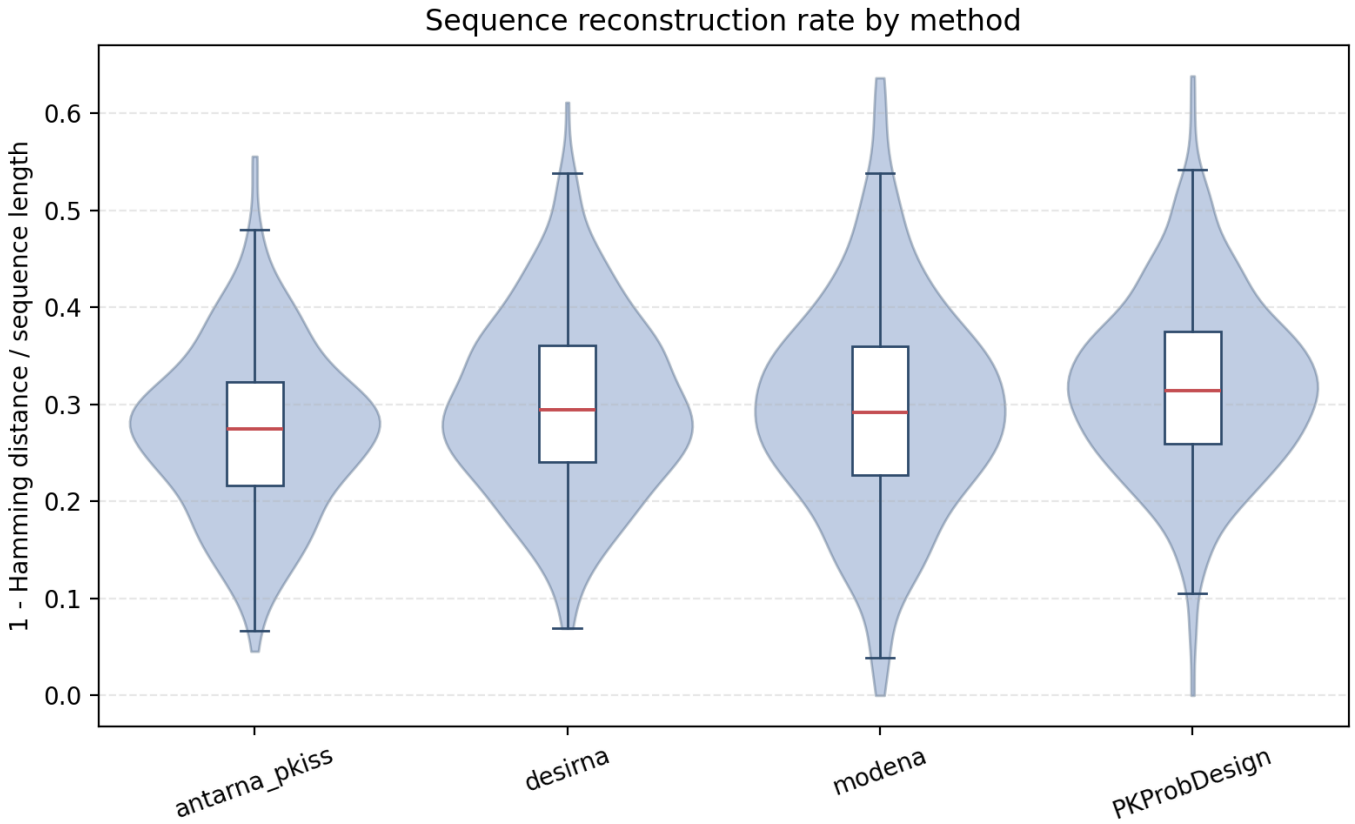
$$= \mathbb{E}_{S \sim p_\theta}\left[f(S) \nabla_\theta \log p_\theta(S)\right].$$

We can approximate the gradient by sampling,

$$\nabla_\theta J(\theta) \approx \frac{1}{K} \sum_{k=1}^{K} f(S_k) \nabla_\theta \log p_\theta(S_k).$$


Hotspot-guided Viterbi reconstruction diagnostic by method
Normalized Hamming distance to target structure
0.0
0.1
0.2
0.3
0.4
0.5
antarna_pkiss
desirna
modena
PKProbDesign


Highest-support method counts across targets
Targets
200
175
150
125
100
75
50
25
0
antarna_pkiss
desirna
modena
PKProbDesign
Method


Sequence reconstruction rate by method
1 - Hamming distance / sequence length
0.0
0.1
0.2
0.3
0.4
0.5
0.6
antarna_pkiss
desirna
modena
PKProbDesign