2L-piRNA: A Two-Layer Ensemble Classifier for Identifying Piwi-Interacting RNAs and Their Function

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Involved with important cellular or gene functions and implicated with many kinds of cancers, piRNAs, or piwi-interacting RNAs, are of small non-coding RNA with around 19–33 nt in length. Given a small non-coding RNA molecule, can we predict whether it is of piRNA according to its sequence information alone? Furthermore, there are two types of piRNA: one has the function of instructing target mRNA deadenylation, and the other does not. Can we discriminate one from the other? With the avalanche of RNA sequences emerging in the postgenomic age, it is urgent to address the two problems for both basic research and drug development. Unfortunately, to the best of our knowledge, so far no computational methods whatsoever could be used to deal with the second problem, let alone deal with the two problems together. Here, by incorporating the physicochemical properties of nucleotides into the pseudo K-tuple nucleotide composition (PseKNC), we proposed a powerful predictor called 2L-piRNA. It is a two-layer ensemble classifier, in which the first layer is for identifying whether a query RNA molecule is piRNA or non-piRNA, and the second layer for identifying whether a piRNA is with or without the function of instructing target mRNA deadenylation. Rigorous cross-validations have indicated that the success rates achieved by the proposed predictor are quite high. For the convenience of most biologists and drug development scientists, the web server for 2L-piRNA has been established at http://bioinformatics.hitsz.edu.cn/2L-piRNA/, by which users can easily get their desired results without the need to go through the mathematical details.

INTRODUCTION

With a length of around 19–33 nt, piRNAs (piwi-interacting RNAs) distinctly belong to the largest class of small non-coding RNA molecules in animal cells.1–4 They are involved with many cellular or gene functions including the transposon silencing, specific protein translation, gene expression regulation, and the formation and maintenance of germ cells.5–7 Moreover, many studies (see, e.g., Mei et al.,8 Cheng et al.,9 Moyano and Stefani,10 and Hashim et al.11) have shown that piRNAs have been implicated with many kinds of cancers. Therefore, knowledge about piRNAs and their functions is very important for drug development, as well as for RNA biology and many other relevant areas.

Given an RNA molecule, can we identify whether it belongs to piRNA? Lee et al.12 and Nishibu et al.13 had developed some experimental methods to address this problem, greatly stimulating the development of this area. But purely using experimental methods alone to do the sequence analyses is not only inefficient and expensive, but also insensitive for many cases (e.g., it is difficult to get sufficient quantity of samples for observation). Facing the explosive growth of RNA sequences in the postgenomic age, to make the piRNA analysis in a more efficient way, as well as in a faster pace and at a deeper level, we could not help but resort to the computational approach.

Actually, several computational methods have been proposed for classifying piRNA from non-piRNA sequences. For instance, by combining the k-mer scheme and support vector machine (SVM), Zhang et al.14 proposed a model called piRNApredictor. Three years later, Wang et al.15 proposed a different model for predicting piRNAs by using the transposon interaction and SVM. Recently, two more papers were published for identifying piRNAs. One was authored by Luo et al.,16 who considered the physicochemical properties of RNA sequences, and the other was authored by Li et al.,17 who used the powerful ensemble approach. Both methods were quite powerful, reaching the state-of-the-art performance.
corresponding results obtained by the powerful existing state-of-the-art methods simply did not have the function to yield any results for the second-layer prediction. The new method presented in this paper.

The present study was initiated in an attempt to fill in this empty area by developing a new predictor that not only can be used to identify piRNAs, but also can be used to identify their functional types.

The concretes procedures to construct the benchmark dataset of Equation 1 are as follows: (1) The piRNA sequences were taken from piRNAbase; (2) collected for \( S_{\text{inst}}^{+} \) are only those samples that were annotated with piRNA having the function of instructing target mRNA deadenylation; (3) collected for \( S_{\text{non-inst}}^{+} \) are only those samples that were annotated with piRNA without the function of instructing target mRNA deadenylation; (4) the corresponding non-piRNA sequences for the negative subset \( S^{-} \) were taken from Bu et al.; (5) the CD-HIT software with the cutoff threshold 0.8 was used to remove the redundancy for each of the aforementioned subsets; and (6) to minimize the negative effect caused by the skewed benchmark datasets, the random sampling method was applied to balance out each of the sub-datasets with its counterpart. The benchmark datasets \( S \) for the current study can be formulated as

\[
\begin{align*}
S^{+} &= S_{\text{inst}}^{+} \cup S_{\text{non-inst}}^{+} \\
S^{-} &= S_{\text{inst}}^{-} \cup S_{\text{non-inst}}^{-}
\end{align*}
\]

where \( S^{-} \) is the negative subset that contains the non-piRNA samples only, \( U \) is the symbol for union in the set theory, \( S_{\text{inst}}^{+} \) is the subset that contains the piRNA samples only, \( S_{\text{inst}}^{-} \) is the sub-subset that contains piRNA samples having the function of instructing target mRNA deadenylation, whereas \( S_{\text{non-inst}}^{+} \) is the sub-subset without such function.

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Conclusions

It is anticipated that the 2L-piRNA will become a very useful high-throughput tool in genome analysis and drug development, particularly in those areas involved with non-coding RNAs.

MATERIALS AND METHODS

Benchmark Dataset

According to Chou’s five-step rule for developing a really useful statistical predictor, the first important thing is to construct or select a reliable benchmark dataset. In literature the benchmark dataset usually consists of a training dataset and a testing dataset: the former is for the usage of training a model, whereas the latter is for testing the model. But as elucidated in a comprehensive review, there is no need to artificially separate a benchmark dataset into the aforementioned two parts if the prediction model is examined by the jackknife test or subsampling (K-fold) cross-validation because the outcome thus obtained is actually from a combination of many different independent dataset tests. Thus, the benchmark datasets \( S \) for the current study can be formulated as

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Shown in Figure 2 is the sequence length distribution of the samples in the benchmark dataset; their detailed sequences and the relevant codes are given in the Supplemental Information.

**Pseudo K-Tuple Nucleotide Composition**

With a good benchmark dataset, the next thing we need to consider is how to formulate the samples therein. Actually, this is one of the most challenging problems in computational biology. This is because all the existing machine learning algorithms were designed to handle the discrete models or vectors only. But a biological sequence expressed by a vector may completely miss its sequence order or pattern, so as to limit the prediction quality. The pseudo amino acid composition (PseAAC) was proposed to deal with such a dilemma. Ever since the concept of PseAAC was introduced, it has been rapidly and widely applied in nearly all the areas of computational proteomics (see, e.g., Du et al., Lin and Lapointe, Chou, Khan et al., and Meher et al. and a long list of references cited in these papers). Inspired by the great successes of using PseAAC to represent protein-peptide sequences, the PseKNC (pseudo K-tuple nucleotide composition) was introduced to represent DNA/RNA sequences. Likewise, since its introduction, PseKNC has also been increasingly applied in many areas of genome analysis.37,38

For an RNA sample with \( L \) nucleotide, its sequence expression is generally given by

\[
\mathbf{R} = N_1 N_2 N_3 \cdots N_j \cdots N_{L},
\]

where

\[
N_i \in \{ \text{A (adenine), C (cytosine), G (guanine), U (uracil)} \}
\]

denotes the nucleotide at the \( i \)-th sequence position, and \( \in \) is the a symbol in the set theory meaning “member of.” According to a recent review paper, the general form of PseKNC for \( \mathbf{R} \) of Equation 2 can be formulated as

\[
\mathbf{R} = [\phi_1 \ \phi_2 \ \cdots \ \phi_u \ \cdots \ \phi_Z]^T,
\]

where the components \( \phi_u (u = 1, 2, \cdots) \) and \( Z \) is an integer; their values will depend on how the desired features are extracted from the RNA sample; and \( T \) is the transposing operator to a matrix or vector.

In this study, we take

\[
\phi_u = \begin{cases} 
\sum_{i=1}^{4^u} f^u + \sum_{j=1}^{3} \theta_j, & (1 \leq u \leq 4^K) \\
\sum_{i=1}^{4^u} f^u + \sum_{j=1}^{3} \theta_j, & (4^K + 1 \leq u \leq 4^K + \lambda)
\end{cases}
\]

where \( f^u_j \) is the \( u \)-th component of the K-tuple nucleotide composition for the RNA sample sequence, and

\[
\theta_j = \frac{1}{L - \lambda} \sum_{i=1}^{L-\lambda} C_{i+j} (j = 1, 2, \cdots, \lambda; \lambda < L - K).
\]

In Equation 6, the correlation function or coupling factor is given by

\[
C_{i+j} = \frac{1}{\mu} \sum_{\xi=1}^{\mu} \left[ H_i (N_{i+1} \cdots N_{i+4K-1}) - H_i (N_{i+j} N_{i+j+1} \cdots N_{i+j+4K-1}) \right]^2,
\]

where \( \mu \) is the number of physicochemical properties considered, whereas \( H_i (N_{i+1} \cdots N_{i+4K-1}) \) is the numerical value of the \( i \)-th physicochemical property for the K-mer \( N_{i+1} \cdots N_{i+4K-1} \) in the RNA sequence, and so forth.

In this study, we consider pseudo dinucleotide composition. Thus, we can substitute \( K = 2 \) into Equations 5, 6, and 7. Also, we used the values of the following six RNA dimer’s physicochemical properties: rise, roll, shift, slide, tilt, and twist (Table 2). Thus, we can substitute \( \mu = 6 \) as well as \( \text{Rise (N}_{i+1}\text{), Slide (N}_{i+1}\text{), Shift (N}_{i+1}\text{), Twist} \)
values in Table 2 were subjected to a standard conversion, as described by the following equations:

\[
\text{Rise}(N_{i+1}) = \frac{\text{Rise}(N_{i+1}) - \langle \text{Rise} \rangle}{\text{SD}(\text{Rise})}, \\
\text{Slide}(N_{i+1}) = \frac{\text{Slide}(N_{i+1}) - \langle \text{Slide} \rangle}{\text{SD}(\text{Slide})}, \\
\text{Shift}(N_{i+1}) = \frac{\text{Shift}(N_{i+1}) - \langle \text{Shift} \rangle}{\text{SD}(\text{Shift})}, \\
\text{Twist}(N_{i+1}) = \frac{\text{Twist}(N_{i+1}) - \langle \text{Twist} \rangle}{\text{SD}(\text{Twist})}, \\
\text{Roll}(N_{i+1}) = \frac{\text{Roll}(N_{i+1}) - \langle \text{Roll} \rangle}{\text{SD}(\text{Roll})}, \\
\text{Tilt}(N_{i+1}) = \frac{\text{Tilt}(N_{i+1}) - \langle \text{Tilt} \rangle}{\text{SD}(\text{Tilt})}, \\
\]

where the symbol “< >” means taking the average of the quantity therein over 16 different dinucleotides. The converted values obtained by Equation 8 will have a zero mean value over the 16 different dinucleotides and will remain unchanged if going through the same conversion procedure again. Listed in Table 3 are the corresponding values obtained via the standard conversion of Equation 8 from those of Table 2.

**Operation Engine**

Below, let us consider the third step of the five-step rule, i.e., what kind of algorithms should be used to operate the training and predicting.

**Support Vector Machine**

Being widely used in many different areas of computational biology (see, e.g., Feng et al., Han et al., Liu et al., Qumar et al., Kiu et al., Liu et al., Rahimi et al., and Chen et al.), SVM is a powerful algorithm in cluster analysis. Its basic idea has been elaborated in the aforementioned papers, and hence there is no need to repeat it here. For those who are interested in knowing more about SVM, refer to the previous papers or a monograph.

In this study, we used the Scikit-learn as the implementation of the LIBSVM with the radial basis function (RBF) kernel.

**Two-Layer Classification Framework**

Inspired by the recent study, we constructed a two-layer classification framework as done in Chou and Shen, Wang et al., Xiao et al., and Shen and Chou. The SVM model in the first-layer classifier was trained with \( S \) of Equation 1, serving to predict a query RNA sample as of piRNA or non-piRNA; the SVM model in the second layer was trained with \( S^* \) of Equation 1 to further identify whether the predicted piRNA sample is with the function of instructing target mRNA deadenylation. Shown in Figure 3 is a flowchart to illustrate how the two-layer classifier is working.

**Ensemble Learning**

As we can see from Equations 5 and 6, the RNA sample defined by the PseKNC approach in this study contains three uncertain parameters: \( K, \lambda, \) and \( w \). In this study, the ranges considered for these parameters are

\[
\begin{align*}
1 \leq K & \leq 6, \quad \text{with step gap } \Delta K = 1, \\
1 \leq \lambda & \leq 17, \quad \text{with step gap } \Delta \lambda = 4, \\
0.1 \leq w & \leq 0.9, \quad \text{with step gap } \Delta w = 0.2
\end{align*}
\]

![Figure 2. Length Distribution of the Sequences in the Benchmark Dataset](image)

**Table 2. The Original Values of Rise, Roll, Shift, Slide, Tilt, and Twist for the 16 Dinucleotides in RNA**

| Physicochemical Property | Rise | Roll | Shift | Slide | Tilt | Twist |
|--------------------------|------|------|-------|-------|------|-------|
| AA                       | 3.18 | 7.0  | -0.08 | -1.27 | -0.8 | 31    |
| AC                       | 3.24 | 4.8  | 0.23  | -1.43 | 0.8  | 32    |
| AG                       | 3.30 | 8.5  | -0.04 | -1.50 | 0.5  | 30    |
| AU                       | 3.24 | 7.1  | -0.06 | -1.36 | 1.1  | 33    |
| CA                       | 3.09 | 9.9  | 0.11  | -1.46 | 1    | 31    |
| CC                       | 3.32 | 8.7  | -0.01 | -1.78 | 0.3  | 32    |
| CG                       | 3.30 | 12.1 | -0.30 | -1.89 | -0.1 | 27    |
| CU                       | 3.30 | 8.5  | -0.04 | -1.50 | 0.5  | 30    |
| GA                       | 3.38 | 9.4  | 0.07  | -1.70 | 1.3  | 32    |
| GC                       | 3.22 | 6.1  | 0.07  | -1.39 | 0.0  | 35    |
| GG                       | 3.32 | 12.1 | -0.01 | -1.78 | 0.3  | 32    |
| GU                       | 3.24 | 4.8  | 0.23  | -1.43 | 0.8  | 32    |
| UA                       | 3.26 | 10.7 | -0.02 | -1.45 | -0.2 | 32    |
| UC                       | 3.38 | 9.4  | 0.07  | -1.70 | 1.3  | 32    |
| UG                       | 3.09 | 9.9  | 0.11  | -1.46 | 1.0  | 31    |
| UU                       | 3.18 | 7.0  | -0.08 | -1.27 | -0.8 | 31    |

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In other words, \( K \) may be 1, 2, 3, 4, 5, and 6; \( \lambda \) may be 1, 5, 9, 13, 17, and 19; \( w \) may be 0.1, 0.3, 0.5, 0.7, and 0.9. Accordingly, there are a total of \( 5 \times 6 \times 5 = 150 \) individual classifiers for each layer.

Suppose each of these individual classifiers is expressed by \( C(i) \) \((i = 1, 2, \cdots, 150)\), their ensemble classifier \( C^E \) can be formulated as

\[
C^E = C(1) \lor C(2) \lor \cdots \lor C(150) = \lor_{i=1}^{150} C(i), \tag{10}
\]

where the symbol \( \lor \) denotes the fusing operator.\(^{32}\) The ensemble predictor formed by fusing an array of individual predictors via a voting system can yield much better prediction quality, as demonstrated by a series of previous studies including signal peptide prediction,\(^ {86,92}\) membrane protein type classification,\(^ {64,89}\) protein subcellular location prediction,\(^ {95,96}\) protein fold pattern recognition,\(^ {97}\) enzyme functional classification,\(^ {98}\) protein-proteins interaction prediction,\(^ {99}\) protein-protein binding site identification,\(^ {100}\) and DNA recombination spot identification.\(^ {68}\)

Unfortunately, if all of the 150 classifiers in Equation 10 were directly used to form an ensemble predictor by the voting approach, it would be not only computationally inefficient, but also might reduce the success rate because of too much noise. One of the effective approaches is to select some key classifiers from them. To realize this, let us introduce the concept of “complementing degree” between two individual classifiers, \( C(i) \) and \( C(j) \), or their “mutually strengthening degree,” \( D(i,j) \), as defined below:

\[
D(i,j) = 1 - \frac{1}{2m} \sum_{t=1}^{m} G_t(i,j) \quad (0 \leq D(i,j) \leq 1), \tag{11}
\]

where \( m \) represents the number of training samples, and

\[
G_t(i,j) = \begin{cases} p_t(i) + p_t(j), & \text{if both fail} \\ 0, & \text{otherwise} \end{cases} \tag{12}
\]

In Equation 12, \( p_t(i) \) denotes the probability or output when applying the classifier \( C(i) \) on the \( t \)-th sample, \( p_t(j) \) the corresponding output for \( C(j) \), and “both fail” means that both predicted results are incorrect.

By means of Equations 11 and 12, all of the 150 classifiers in each layer were clustered with the AP (affinity propagation) clustering algorithm\(^ {101}\) using the default parameters. Four clusters were thus obtained for each of the two layers. Subsequently, the classifiers in the four cluster centers were selected as the representative classifiers, respectively, that have the highest complementing/strengthening degrees, as illustrated by the flowchart in Figure 4. Suppose the four
representative classifiers thus selected for the first and second layers are denoted by
\[
\left\{ \begin{array}{l}
C''(1st, 1), C''(1st, 2), C''(1st, 3), C''(1st, 4) \\
C''(2nd, 1), C''(2nd, 2), C''(2nd, 3), C''(2nd, 4)
\end{array} \right.
\] \tag{13}

Listed in Table 4 are the detailed values of their parameters for the first and second layers, respectively. Thus, instead of Equation 10, the final ensemble classifier should be formulated as
\[
C_E = \begin{cases} 
V_i^{1}C'(1st, i), & \text{for the 1st layer} \\
V_i^{2}C'(2nd, i), & \text{for the 2nd layer}
\end{cases}
\] \tag{14}

Note that different from the ensemble classifiers formed in Chou and Shen\textsuperscript{102-105} and Qiu et al.,\textsuperscript{106,107} the voting weighted factors \( V_w \) were included during the fusion process for each layer, and their optimal values can be easily derived by optimizing success rates during the validation process as shown in Table 4 (Voting Weighted Factor \( V_w \) column).

The predictor developed via the above procedures is called 2L-piRNA, where 2L represents the two-layer ensemble classifier and piRNA represents the piwi-interacting RNA and its function.

Prediction Quality Measurement

How to measure the prediction quality is one of the five indispensable steps\textsuperscript{31} in developing a new prediction method for a biological system. It consists of two issues: What scales should be used to measure the predictor’s quality? And what test method should be adopted to score them? Below, let us address the two problems one by one.

Formulation of Measurement Scales

The following metrics were widely used in the literature to measure the prediction quality from four different aspects: (1) \( \text{Acc} \) that was used for checking the overall accuracy of a predictor, (2) \( \text{MCC} \) for its stability, (3) \( \text{Sn} \) for its sensitivity, and (4) \( \text{Sp} \) for its specificity.\textsuperscript{108}

Unfortunately, the four metrics’ original formulations copied directly from mathematical books are difficult to understand for most biologists due to lack of intuitiveness. Fortunately, by using the scales defined by Chou\textsuperscript{109} in studying signal peptides, Xu et al.\textsuperscript{110} and Chen et al.\textsuperscript{55} had successfully converted them into a set of intuitive equations that are much easier for most biologists to understand, as given below:

\[
\begin{align*}
\text{Sn} &= 1 - \frac{N^+}{N^+} \\
\text{Sp} &= 1 - \frac{N^-}{N^-} \\
\text{Acc} &= 1 - \frac{N^+ + N^-}{N^+ + N^-} \\
\text{MCC} &= \frac{1 - \frac{N^+ + N^-}{N^+ + N^-}}{\left(1 + \frac{N^+}{N^-} + \frac{N^-}{N^+}\right)\left(1 + \frac{N^+}{N^-} + \frac{N^-}{N^+}\right)}
\end{align*}
\] \tag{15}

where \( N^+ \) represents the total number of the positive samples investigated, whereas \( N^+ \) is the number of the positive samples incorrectly predicted to be negative, and \( N^- \) is the total number of the negative samples investigated, whereas \( N^- \) is the number of the negative samples incorrectly predicted to be positive.
**Table 4. List of the Four Individual Representative Base Classifiers Selected by Using the Affinity Propagation Clustering Algorithm**

| Base Classifier | Feature | Dimension | Voting Weighted Factor \(Y_w\) | Acc (%) |
|-----------------|---------|-----------|-------------------------------|---------|
| **First Layer** |
| \(C'(1st, 1)\) | PseKNC\(^a\) | 17 | 0.200 | 84.1 |
| \(C'(1st, 2)\) | PseKNC\(^b\) | 21 | 0.100 | 84.0 |
| \(C'(1st, 3)\) | PseKNC\(^c\) | 69 | 0.300 | 84.6 |
| \(C'(1st, 4)\) | PseKNC\(^d\) | 257 | 0.400 | 82.1 |

**Second Layer**

| Base Classifier | Feature | Dimension | Voting Weighted Factor \(Y_w\) | Acc (%) |
|-----------------|---------|-----------|-------------------------------|---------|
| \(C'(2nd, 1)\) | PseKNC\(^e\) | 17 | 0.100 | 73.8 |
| \(C'(2nd, 2)\) | PseKNC\(^f\) | 69 | 0.800 | 77.0 |
| \(C'(2nd, 3)\) | PseKNC\(^g\) | 4,101 | 0.000 | 71.4 |
| \(C'(2nd, 4)\) | PseKNC\(^h\) | 4,113 | 0.100 | 70.1 |

\(^a\) The optimal parameters were \(K = 2, \lambda = 1, w = 0.1, C = 2^7, y = 2\).
\(^b\) The optimal parameters were \(K = 2, \lambda = 5, w = 0.3, C = 2^{15}, y = 2^{-1}\).
\(^c\) The optimal parameters were \(K = 3, \lambda = 5, w = 0.1, C = 2^3, y = 2^{-1}\).
\(^d\) The optimal parameters were \(K = 4, \lambda = 1, w = 0.3, C = 2^3, y = 2^{-1}\).
\(^e\) The optimal parameters were \(K = 2, \lambda = 1, w = 0.9, C = 2^{13}, y = 2\).
\(^f\) The optimal parameters were \(K = 2, \lambda = 5, w = 0.1, C = 2^3, y = 2\).
\(^g\) The optimal parameters were \(K = 6, \lambda = 17, w = 0.9, C = 2^{11}, y = 2\).

Based on the definition of Equation 15, the meanings of \(S_n\), Sp, Acc, and MCC have become much more intuitive and easier to understand, as discussed and used in a series of recent studies in various biological areas (see, e.g., Jia et al., Liu et al., Xiao et al., Lin et al., Chen et al., Qiu et al., Xu et al., and Ding et al.).

It should be pointed out, however, that for the multi-label systems (see, e.g., Xiao et al., Qiu et al., Xiao et al., Chou et al., Lin et al., and Cheng et al.), a much more sophisticated set of scales is needed as elaborated by Chou.

**Cross-Validation**

There are three different cross-validation methods that are widely used in literature: (1) jackknife test, (2) subsampling (or K-fold cross-validation) test, and (3) independent dataset test. Of these three, however, the jackknife is the least arbitrary that can always yield a unique outcome for a given benchmark dataset, as elaborated by Chou and widely recognized and increasingly adopted by researchers to analyze the quality of various predictors (see, e.g., Kabir and Hayat, Kumar et al., Chen et al., Ali and Hayat, Khan et al., Mondal and Pai, Dehzangi et al., Ahmad et al., Ju et al., and Behbahani et al.). In this study, however, to reduce the computational time, we adopted the 5-fold cross-validation method for each layer in 2L-piRNA, as done by many investigators with SVM as the prediction engine. For each layer, the benchmark dataset was divided into five subsets; for each run, four subsets were used as the training set, and the remaining one was used as the test set to evaluate the performance. This process was repeated five times until each subset was used as a test set once. To do this, we first randomly divided the benchmark datasets in Equation 1 into five subsets with approximately the same size. For instance, for the first benchmark dataset in Equation 1, we have

\[
\begin{align*}
S_1 &= S_1^1 \cup S_2^1 \cup S_3^1 \cup S_4^1 \cup S_5^1 \cup \bigcup_{i=1}^{5} S_i^1, \\
\mathcal{O} &= S_1 \cap S_2 \cap S_3 \cap S_4 \cap S_5 = \bigcap_{i=1}^{5} S_i,
\end{align*}
\]

where \(\cup\), \(\cap\), and \(\bigcup\) represent the symbols for union, intersection, and empty set in the set theory, respectively, and

\[
S_i = S_i^1 \cup S_i^- \quad (i = 1, 2, \ldots, 5)
\]

with

\[
\begin{align*}
|S_1| &\leq |S_2| \leq |S_3| \leq |S_4| \leq |S_5|,
\end{align*}
\]

where \(|S_i|\) denotes the number of samples (or cardinalities) in \(S_i^1\), and so forth.

Then, each of the five sub-benchmark datasets was singled out one by one and tested by the model trained with the remaining four sub-benchmark datasets. The cross-validation process was repeated five times, with their average as the final outcome. In other words, during the process of 5-fold cross-validation, both the training dataset and testing dataset were actually opened, and each sub-benchmark dataset was in turn moved between the two. The 5-fold cross-validation test can exclude the “memory” effect, just like conducting five different independent dataset tests.

**Web Server and User Guide**

In Chou’s five-step rule for developing a useful predictor, the last one is to establish a user-friendly web server. This not only represents the future direction for developing any computational methods, but is also particularly important for most experimental scientists working in drug development. Accordingly, as done in a series of recent studies, the web server for 2L-piRNA has been established as well. Moreover, to maximize users’ convenience, a step-by-step guide is provided below.

Step 1. Open the web server at http://bioinformatics.hitsz.edu.cn/2L-piRNA/ and you will see its top page as shown in Figure 5. Click on the Read Me button to see a brief introduction about the server and the caveat when using it.

Step 2. You can either type or copy/paste the query RNA sequence into the input box. You can also directly upload your input data via the Browse button. The input sequence should be in the FASTA format for the examples of sequences in the FASTA format, click the Example button right above the input box.

Step 3. Click on the Submit button to see the predicted results. For example, if you use the four query RNA sequences in the Example
window as the input, you will see on your computer screen that the first and second query sequences are of non-piRNA. The third one is of piRNA with the function for instructing target mRNAs deamination. The fourth one is of piRNA, but without that function. All these predicted results are fully consistent with the experimental observations as reported in Gou et al.18

SUPPLEMENTAL INFORMATION
Supplemental Information includes one data file and can be found with this article online at http://dx.doi.org/10.1016/j.omtn.2017.04.008.

AUTHOR CONTRIBUTIONS
B.L. conceived of the study and designed the experiments, participated in drafting the manuscript and performing the statistical analysis. F.Y. participated in coding the experiments and drafting the manuscript. K.-C.C. participated in revising the manuscript. All authors read and approved the final manuscript.

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