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Supervised and Unsupervised Classification of lncRNA Subtypes

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Abstract: Many small nucleolar RNAs and many of the hairpin precursors of miRNAs are processed from long non-protein-coding (lncRNA) host genes. In contrast to their highly conserved and heavily structured payload, the host genes feature poorly conserved sequences. Nevertheless there is mounting evidence that the host genes have biological functions. No obvious connections between the function of the host genes and the function of their payloads have been reported. Here we inverstigate whether there is an association of host gene function or mechanisms with the type of payload. To assess this hypothesis we test whether the miRNA host genes (MIRHGs), snoRNA host genes (SNHGs), and other lncRNAs host genes can be distinguished based on sequence and structure features. A positive answer would imply a correlation between host genes and their payload. While the three classes can be distinguished reliably when the classifier is allowed to extract features from the payloads, this is no longer the case when only sequence and structure of parts of the host gene distal from the snoRNAs or miRNA payload is used for classification. We conclude therefore, that MIRHGs and SNHGs do not form separate non-coding RNA classes that are distinct from each other or from lncRNAs without payloads.

Keywords: host gene; miRNA; snoRNA; *k*-mers; secondary structure; random forest

1. Introduction

A wide variety of molecular and biological functions have been reported for long non-coding RNAs (lncRNAs), recently reviewed in [1]. Specific lncRNAs regulate chromosome architecture and chromatin remodeling, modulate inter- and intrachromosomal interactions and recruit or prevent the recruitment of chromatin modifiers. LncRNAs regulate transcription by forming R-loop thus recruiting transcription factors and interfere with the Pol II machinery to inhibit transcription. Other lncRNAs are structural components on nuclear bodies. In the cytoplasm, lncRNAs regulate mRNA turnover, translation and post-translational modification. Many lncRNAs serve as host genes for the production of small RNAs [2], including microRNAs (miRNAs) and small nucleolar RNAs (snoRNAs), or act as sponges that modify the miRNA pool [3]. In contrast to protein-coding genes, where function is closely tied to protein families, sequence similarity is a poor predictor of functional similarity in lncRNAs. As a consequence, it has remained impossible to predict the biological function or molecular mechanism of lncRNAs for sequence data alone. This is in stark contrast also to the small, structured ncRNAs. These are readily recognized through their highly conserved sequences (such as ribosomal

or spliceosomal RNAs), or by class specific features (such as the cloverleaf shape of tRNAs or the ultra-stable hairpins of miRNAs) [4].

Unsupervised clustering using normalized *k*-mer abundances as similarity measure revealed an association of *k*-mer profiles with lncRNA function, in particular with protein binding and sub-cellular localization [5]. With a plethora of RNA-binding proteins typically recognizing a wide array of local binding motives that may be structured, modular, and gapped [6], the correlation of short *k*-mers and function is not surprising. Nevertheless, it remains unclear whether there are distinct, well-separated classes of lncRNAs or whether the universe of lncRNAs is organized as a continuum of functions and associated molecular features.

Here we consider two classes of lncRNAs that, in addition to any intrinsic function they might have, also act as host genes for small RNAs with well known and quite specific molecular functions: snoRNA hosts genes (SNHGs) and miRNA host genes (MIRHGs). Due to the differences in their payloads, they undergo distinctive processing. The processing of snoRNAs for SNHGs in human is linked to splicing, with snoRNAs exclusively located in introns, see e.g. [7].

The precursors of canonical miRNAs, whether intronic or exonic, are extracted from the primary transcript by the microprocessor complex centered around Drosha and DGCR8 [8]. A second smaller group of miRNAs, the mirtrons, are processed with the help of the splicing machinery [9]. Functionally, miRNAs orchestrate post-transcriptional gene silencing, affecting a large fraction of protein-coding transcriptome [10,11].

SNHGs have received rapidly increasing attention in particular in cancer research, see e.g. [12–17] and the references therein. SNHG15, for instance, is dysregulated in a wide variety of cancers and interacts with several distinct molecular pathways apparently using different molecular mechanisms, reviewed in [14]. Similar, there are several competing, not mutually exclusive, mechanistic explanations for the function of GAS5 [12]. There is strong indication that at least one of the modes of action of most SNHGs is to act as miRNA sponge [15,18]. The snoRNAs themselves are mostly involved in the maturation of rRNAs and snRNAs, where they direct chemical modifications, although an increasing number of secondary functions have been reported very recently [19]. However these do not seem to be coupled to functions of the host genes.

In contrast to SNHGs, very few MIRHGs have known functions independent of the miRNAs that they harbor. Two notable examples are MIR100HG and MIR31HG [20]. MIR100HG has been reported to interact with HuR/ELAVL1 [21] and to form RNA-DNA triplex structures with the p27 locus [22], and possibly it may also be a ceRNA [23].

In this contribution we ask a simple question related to the global map of the lncRNA universe proposed in [5]: are SNHGs and MIRHGs distinct classes of lncRNA? To answer this question we use supervised and unsupervised methods of machine learning and ask whether machines can be trained that reliably separate SNHGs and MIRHGs from each other and from a background set of lncRNAs that harbor neither snoRNAs nor miRNAs, which we will refer to as NoHGs.

2. Results

For a comprehensive investigation into the separability of SNHGs, MIRHGs and NoHGs we first had to curate data from available annotation and define sets of features than can be reliably used for training and prediction. We resort to a mixture of supervised and unsupervised machine learning techniques, random forests to help us understand the weight of selected features as well as unsupervised techniques which may pick up on features which escape our human perception.

To answer the underlying question of whether or not SNHGs and MIRHGs are indeed distinct classes of lncRNAs, we investigate the influence of the payload itself. Comparing an inclusive approach to one where we explicitly exclude the payload and flanking sequences that may harbor processing signals as those should relatively easy be picked up by ML methods and bias results. Details on the curated datasets can be derived from figure 1 and table 2.

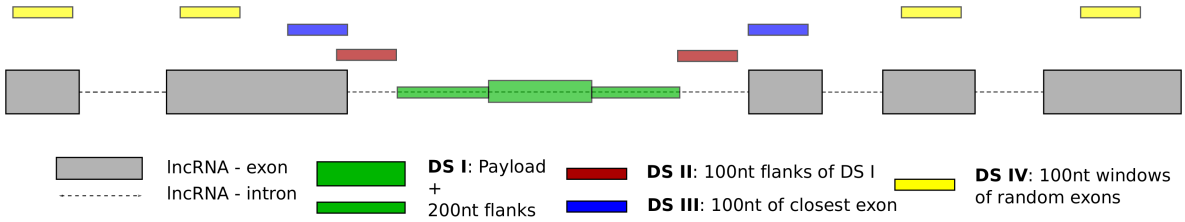

Figure 1. A schematic of the datasets curated for this study and their distribution over the gene body of a generic host-lncRNA. DS I (green) consists of the payload and 200nt flanking sequence. DS II (red) flanks DS-I by 100nts. DS III consists of the first 100nts of the exon closest to the annotated payload. DS IV consists of non-overlapping 100nt windows taken from random exons of the host-lncRNA. More details can be found in section 4.1

2.1. Supervised Machine Learning

In order to gain insight into the usefulness of selected features, we trained a set of random forest classifiers with combinations of distinct feature sets extracted from different genic regions of the three lncRNA classes. Details on this procedure can be found in 4.1, results of 10-fold cross-validation (10xCV) on all data and feature sets can be found in table 1, details to training, testing and CV can be found in section 4.4.

Payload-aware prediction.

MicroRNAs and snoRNAs are readily distinguished from transcribed background by means of machine learning approaches, see e.g. [24–27] and the references therein. We therefore consider this classification task as benchmark for our dataset/feature-selection strategy and expect that the three classes of lncRNAs can be distinguished with satisfactory reliability on *Dataset 1*, which makes the payload, i.e., miRNA and snoRNA precursors, resp., explicitly available to the classifier. We trained a random forest classifier, with feature sets ranging from sequence only features like weighted *k*-mer sets to structuredness and conservation (see section 4.3). Ficket score was added as extra feature to each comparison, to investigate the influence of coding potential. Table 1 summarizes the results for 10xCV on a training/test set split of 80/20. Accuracy of $> 84\%$ shows that random forests are indeed suitable for this kind of classification task. An interesting finding, however, is that the inclusion of information derived from structure/conservation features leads to a decrease in accuracy, probably due to the high structuredness of both miRNA and snoRNA payloads and similar conservation patterns. We note, finally, that a perfect separation of snoRNAs and miRNA precursors cannot be expected since there is a group of ncRNAs that appear to be in transition between the two functions [28–30].

Influence of the payload.

Next we tested whether the actual payload of the host genes is necessary for our machinery to classify the host-gene type. As the payload potentially, and in the case of MIRHGs even likely contains sequence or structure level signals needed for processing, we assume that machine learning techniques will be able to pick up on those signals, drastically influencing the prediction accuracy. To this end we restricted *Dataset 2* to 200 nucleotides of flanking sequence on either side of the payload without overlap and random lncRNA subsequence for NoHGs. As expected, the exclusion of actual payload sequences had a strong impact on prediction accuracy, dropping from $> 84\%$ to below 45%. Although integration of structure and conservation features led to an increase of accuracy to more than 51%, it becomes clear that the sequences in close vicinity to the actual payload does not give away the type of payload.Ranking of features according to their importance for classification shows that with the exclusion of the actual payload, sequence and conservation become important features for the classification task (supplementary table 2).

Exonic sequences only.

Since snoRNAs are located in introns of the host gene, *Dataset 2* is almost exclusively restricted to intronic regions. Functions of the matured host genes or other lncRNAs presumably reside in their exonic sequences. Furthermore, sequence and structure features involved in splicing as well as the initial processing of exonic miRNAs are like to be found in the matured transcript. *Dataset 3*, which is designed to focus on such effects, comprises the 200nt of exonic sequence flanking the payload-bearing intron in the case of an intronic payload or the miRNA precursor in case of exonic miRNAs. Although by far not as good as the accuracy for classification including payloads, we see accuracy rises again to above 66%, including structure and conservation features even $> 70\%$. Our interpretation of this finding is that the exonic flanking regions contain sufficient information related to the processing of payload to be recognizable. For miRNAs this can be explained in particular by sequence motifs associated with microprocessor activity.

Random exonic sequences.

From shown results, we deduct that exonic regions flanking the payload contain some amount of information about the type of the payload, we decided to test this approach focusing in *Dataset 4* on random portions of exonic regions around the payload. Although the accuracy after inclusion of structure and conservation is higher than for payload flanking sequences of *Dataset 2* ($> 62\%$ in comparison to $> 51\%$), it is still well below the accuracy obtainded from the flanking exonic regions with $> 70\%$.

Fickett score.

Invented as a measure for coding potential, we investigated whether the Fickett score [31] can be used as informative feature for our classification problem. While we observed a small increase in accuracy for flanking exons (*Dataset 3*) when only sequence features are used, every other prediction did not profit from its inclusion, thus we render the Fickett score a non-functional feature for the classification problem at hand.

Secondary Structure.

SnoRNAs are highly structured RNA species, depending on the correct fold for their biological function. MiRNAs on the other hand are usually loaded into the RISC complex as single strands, for prior processing however, they are also heavily structured. Thus, secondary structuredness, or the lack thereof, could indicate regions which are better suited for the integration of either payload. Using `RNAplFold` [32] of the ViennaRNA package [33,34], we included the probability of being unpaired as feature vectors into our predictions, as explained in detail in section 4.1. In contrast to the Fickett score, inclusion of these features always increased accuracy, although not by much in most cases. In combination with Fickett Score, prediction accuracy is even decreased, except for dataset 2.

Sequence Conservation.

Sequence conservation is a key indicator of biological function. It therefore seems sensible to include conservation features in our classifications. However, this limits application to genomic regions where reliable sequence alignments can be constructed, from which conservation scores can be deducted. We integrated the `PhastCons` score, i.e., the probability that a given position is part of a conserved region [35], as a convenient measure of position-wise conservation, see section 4.1. Similar to structure derived features, adding conservation increases accuracy except when focusing directly on the payload. This effect is strongest when random exonic regions (*Dataset 4*) are used and in any combination with Fickett Score.

Table 1. Overview of 10-fold cross validation accuracy for supervised machine learning on combinations of data and feature sets (*k*-mers only, *k*-mers plus secondary structure, or *k*-mers plus secondary structure and sequence conservation), both with and without the Fickett score as measure of coding potential.

Dataset	Fickett Score	*k*-mer	+structure	+conservation
Dataset 1	without	85.50%	85.51%	84.06%
sno: 345, mir: 400, lnc: 400	with	84.06%	80.19%	82.61%
Dataset 2	without	44.69%	47.58%	51.69%
sno: 690, mir: 800, lnc: 750	with	40.34%	43.96%	50.72%
Dataset 3	without	66.67%	67.03%	70.61%
sno: 1287, mir: 464, lnc: 2101	with	67.38%	65.59%	70.25%
Dataset 4	without	42.86%	50.00%	62.24%
sno: 162, mir: 168, lnc: 750	with	41.84%	35.71%	50.00%

2.2. Unsupervised machine learning

Visualizing data using PCA for the four data sets we could not identify a credible clustering or separation between NoHG, MIRHG, and SNHG, see supplemental figures S5-S8. Using *k*-means clustering we found the assignment of the three groups of lncRNAs to the clusters is effectively random: accuracies for all combinations of features and datasets are below 36% (see supplemental table S1 and supplemental figures S1-S4). The use of convolutional neural network (CNN) with an autoencoder also showed no credible separation of the lncRNA classes, which is to be expected, however, given the small size of training and test sets.

2.3. MicroRNA target sites as a feature

At least some SNHGs can act as miRNA sponges [15,18]. This suggests to use the distribution of miRNA binding sites as features for classification. To this end, we retrieved predicted and experimentally validated miRNA binding sites from `miRTarBase` [36], which in contrast to other sources contain not only 3'UTR located sites but any experimentally validated binding site regardless of its genic location. Intersection of this resource with our list of SNHGs and MIRHGs, however, revealed no significant overlap of miRNA binding sites and any of our host lncRNAs. To improve on this list of experimentally validated targets in regards to lncRNAs, which constitute less than .1% of the reported target genes, we used the *k*-mer feature to scan for miRNA seed region matches. A list of seed regions for each miRNA is readily available at `TargetScan` [37]. We restricted our search to regions covered in the analyzed datasets for which we had weighted *k*-mers available. No conclusive enrichment for any of the analyzed seeds could be detected comparing SNHGs and the other classes. This approach is of course limited due to the rather small regions we cover in our datasets, for a comprehensive analysis the whole gene body of all host genes has to be taken into consideration. Such an approach, however, is not feasible in context of our machine learning based classification task.

3. Discussion

In this contribution we asked whether there is a clear distinction between the host genes of snoRNAs and miRNAs on the one hand and lncRNAs without such highly conserved payloads on the other hand. Our answer is largely negative. While ML methods readily distinguish the three classes based on generic features of their payload, the classification task appears to become very difficult if the information about the payload and its immediate vicinity is not made available to the classifier. While the immediate vicinity – both exonic and intronic – appears to contain payload-specific information, the association between payload and features obtained from distant regions of the lncRNA is weak. Features derived from structuredness and conservation of these regions have in all cases a positive

effect on classification accuracy and are readily ranked among the most important features. They do, however, not compensate for the weak association.

Given that there is mounting evidence for biological function of not only lncRNAs but also specifically for snoRNA and miRNA host genes, we argue that this *lack of detectable association* is of biological interest. It suggests that the function of the host genes is not closely tied to the function of the payload. This is in stark contrast to the protein-coding host genes of snoRNAs, many of which encode ribosomal proteins [38] and thus also contribute to the maturation of the ribosome.

Although a function as miRNA sponge has been reported for many SNHGs, we could not detect features that that might connect the sequence or structure of the SNHGs with specific *k*-mers (namely those complementary to the seed regions of miRNAs) or to predicted miRNA target sites. Considering available, experimentally validated miRNA targets limits such a study for ncRNAs in general and even more so for lncRNAs, as they represent only a very small fraction of known target genes. A dedicated investigation into that matter is missing and would help to shed light onto the regulatory interplay between SNHGs, MIRHGs and their payload.

Applying *k*-means clustering to our datasets and features showed poor classification accuracy. Even though lncRNAs as such may harbor sequence motifs that give away conserved RNA binding protein target function among lncRNA groups, our investigation shows only poor classification potential for MIRHGs and SNHGs, even if the payload is considered. Although no distinctive clusters of MIRHGs and SNHGs were observed, our data are consistent with clustering of lncRNA classes observed in [5]. There, prominent SNHGs such as GAS5 have remained outside the identifiable clusters.

From an evolutionary point of view it may not be surprising that the host genes of miRNAs and snoRNAs do not exhibit recognizable class-specific features. Most likely, the molecular function of the payload, miRNAs or snoRNAs is much older and pre-dates functions of the non-coding host genes, which likely arose secondarily, maybe long after the transcripts have come under negative selection as host genes. The lack of common features of the host genes together with the usually very poor sequence conservation suggests they may even have acquired different functions in different lineages. A better understanding of the host genes thus will require a much more detailed investigation into the patterns of conservation than what is available at present. With rise of new sequencing technologies and advances in functional screening methods we can expect that more detailed data on host gene functions will be forthcoming. This may revise the current picture of distribution of lncRNA functions, which shows only a rather loose association of biological function and molecular mechanism with sequence and structure features of transcripts.

4. Materials and Methods

4.1. Sequence retrieval

MiRNA sequences were collected from miRBase [39], snoRNA sequences from the Human snoRNA Atlas [40] and snoDB [41], and lncRNA sequences were retrieved from GENCODE v29 and GENCODE v33 [42]. Where multiple lncRNA isoforms were annotated we used the longest one as representative. The coordinates of miRNA precursors from miRBase were used to identify MIRHGs and the exact position of the payload(s) within the MIRHG. All overlaps were computed with the bedtools suite [43] and customized Python scripts. Since all snoRNAs are intronic, no further processing was needed for SNHGs. In MIRHGs we defined both intronic and exonic miRNA precursors with 100 nt flanking sequence as the "payload". The intron/exon annotation provided by the GENCODE annotation was used to define the exonic part of the lncRNA.

4.2. Datasets

The four datasets differ in regions selected, e.g. including payload, i.e., miRNAs and snoRNAs, or not. The difference is illustrated in Fig. 1. All data used for classification were extracted following

Table 2. Distribution of sequences contained in each dataset.

	Dataset 1	Dataset 2	Dataset 3	Dataset 4
sno	345	690	1287	162
mir	400	800	464	168
lnc	400	750	2101	750

GENCODE annotation (version 33) using custom scripts and the `bedtools` suite [43]. For purpose of training and testing, datasets were always balanced based on the smallest number of sequences available for any given class, to prevent prediction artefacts.

Dataset 1.

For a set of 400 MIRHGs and 345 SNHGs, we extracted the payload sequence together with 100nt flanks. Flanks are considered to contain information necessary for the processing of the payload, *i. e.* for example processing of pre-miRNAs by the DROSHA/DICER machinery. As negative control set we collect 400 subsequences of random lncRNAs excluding overlaps with our positive sets on gene and sequence and level. Sequence length was set to 500nt as this was the mean length of sequences in our positive sets.

Dataset 2.

To investigate the influence of the information derived directly from payload on our classification approach, we next prepared a dataset consisting of 800 samples from MIRHGs, 690 samples from SNHGs and 750 samples from NoHGs. For the host genes we extracted 100nt windows flanking the regions used as dataset 1. Overlaps between extracted sequences were discarded. These can arise from closely spaces payloads. This makes sure we have no information on the actual payload while we preserve close-by context. Here we do not focus on the genic region of these flanks. As negative set we again derive random subsequences of lncRNAs without overlap with the positive set, this time of size 100nt to match the positive sequences.

Dataset 3.

Since the genic sequence information itself is of particular interest, we constructed a set that is similar to dataset 2, with the additional constraint that 100nt sequences are chosen from the closest exon up- and downstream of the payload. Dataset comprises 464 miRNA and 1287 snoRNA host gene sequences and differs from dataset 1 by exclude payload as well as the intronic sequence surrounding the payload. As negative set we selected at random 2101 exonic regions of length 100 from randomly picked lncRNAs without overlap to the positive set.

Dataset 4.

To avoid any localized effect associated with the payload, we constructed dataset 4 by selecting multiple, non-overlapping regions of length 100nt from the exonic parts of the host genes. This data set contains 168 sequences extracted from miRNA precursors and 162 extracted sequences from snoRNA precursors. For the negative set we chose multiple, non-overlapping, 100nt windows of random exons of random lncRNA genes without overlap with the positive set, amounting to 750 sequences.

4.3. Features

The following features were computed for each sequences in the four datasets described above:

1. k-mer counts for $2 \leq k \leq 7$ were calculated using jellyfish [44]. From these data, a triple $(key, kmer_norm, freq)$ was computed for each k-mer and each transcript, where key is a

catagorical variable describing presence or absence of the *k*-mer is a given sequence, $freq$ is its relative frequency and *kmer_norm* is the normalized count of the *k*-mer. The entries were converted into features using a MultiLabelBinarizer class from `scikit-learn` [45]) as binarizer.
2. The Fickett score [31], a measure for the coding potential of sequences, was computed with a custom script inspired by [46].
3. Sequence conservation scores were obtained from the UCSC Table Browser (PhastCons30way) and the scores for individual exons were extracted [47,48]. The PhastCons score was used in two ways; once as mean of all the scores across all the nucleotides and as normalized scores after being divided into 5% bins.
4. RNA secondary structure is often better conserved than the sequence. We therefore utilized structural attributes of the sequences, pairing probabilities of the nucleotides were calculated using `RNAplfold` [32]. Three different windows of lengths 60, 80, 120 were used for the calculation. Each window was divided into 20 bins, binary encoding each position that falls into the bin with 1 and the rest of the positions with 0.

Since at least a fraction of the annotated lncRNAs is annotated from incomplete transcript models [49], we did not use parameters such as the number of exons, the transcript length, or polyadenylation in any of the classification tasks.

4.4. Machine Learning and evaluation framework

`Scikit-learn` [45,50] was used to perform training, testing and cross-validation of results.

Supervised Machine Learning.

Random forests are decision tree based classifiers, which work on the basis of an aggregator, training on different subspaces of the training data in parallel. A major advantage of random forest approaches is that they enable us to rank features based an their importance for classification. This allows us to investigate the latter in detail or discard less useful ones to increase training accuracy and time. We used 100 trees for the initial classification, at default settings. 10-fold and 5-fold cross validation was performed with tree sizes $[100, 300, 500, 1000]$, both impurity criteria (gini and entropy), bootstrapping, and taking out of bag samples. A random forest classifier has been trained considering all possible parameter combinations to achieve hyperparameter optimization using grid search. For each combination of feature and data set, we chose the best perfoming hyperparameter setting (details can be found in supl.tab.3). Only little differences in prediction accuracies were observed between training runs on the available parameter space. This is likely related to the rather small size of available datasets, should, however, not diminish gained insights.

Unsupervised Machine Learning.

As an alternative to the supervised classification strategies discussed above we pursued unsupervised clustering. To this end we used the same four datasets and the same features. To obtain a visual overview of the data we used Principal Component Analysis on the *k*-mer vectors as implemented in `R`. We then used *k*-means clustering in attempt to distinguish the sequences into three distinct clusters [51]. We further evaluated a convolutional neural network (CNN) based autoencoder instead of a classical clustering approach. For this purpose the *Keras* package (`https://keras.io/`) was used within the *TensorFlow* framework [52]. We used accuracy, F-measure, and rand index to evaluate the quality of the clusters w.r.t. to distinguishing the three classes of lncRNAs.

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Abbreviations

The following abbreviations are used in this manuscript:

miRNA	microRNA
snoRNA	small nucleolar RNAs
lnRNA	long non-coding RNA
MIRHG	miRNA host gene
SNHG	snoRNA host gene
NoHG	lncRNAs that harbor neither snoRNAs nor miRNAs

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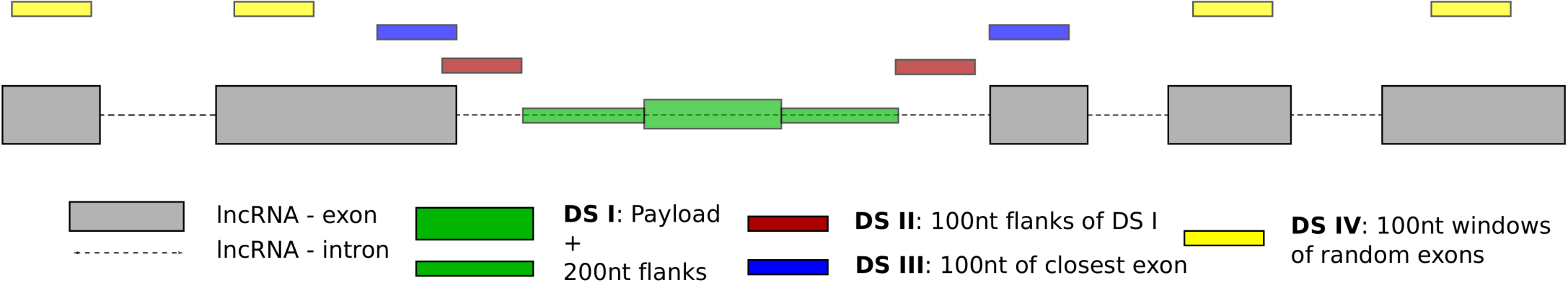
lncRNA - exon
lncRNA - intron
DS I: Payload
+
200nt flanks
DS II: 100nt flanks of DS I
DS III: 100nt of closest exon
DS IV: 100nt windows
of random exons